THE ROLE OF INTEGRINS IN MECHANO-TRANSDUCTION LEADING TO ENHANCED COLLAGEN SYNTHESIS IN HUMAN CARDIAC FIBROBLASTS

by

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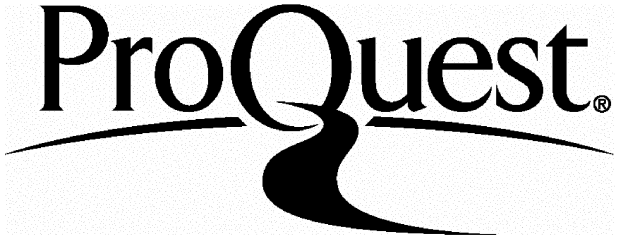

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ABSTRACT

The mechanisms that regulate the development of cardiac hypertrophy and fibrosis are poorly understood. Cardiac hypertrophy results from changes in the mechanical environment and is accompanied by increased extracellular matrix (ECM) synthesis and remodelling. Collagen types I and III the major constituents of the ECM, have previously been shown to be increased during cardiac hypertrophy. In this study the importance of fibroblast:ECM interactions and subsequent activation of intracellular signalling pathways in the load-induced stimulation of collagen synthesis were investigated. More specifically the role of integrins as mechanotransducers was investigated. The data suggest that an interaction between human cardiac fibroblasts and a fibronectin matrix (rather than collagen or elastin) results in the most effective stimulation of procollagen synthesis when subjected to a cyclical mechanical load. Further, the addition of blocking antibodies to specific fibronectin integrins inhibited collagen synthesis induced by mechanical load, whereas blocking antibodies to a collagen integrin did not. These results suggest that fibronectin integrins act as mechanotransducers, allowing the mechanical stimulus to be transduced into a biochemical event. Conversion of the mechanical stimulus into a biochemical event appears to involve the formation of focal adhesion complexes. More specifically the focal adhesion proteins focal adhesion kinase (FAK), $p130^{Cas}$, and the cytoskeleton associated protein paxillin, all showed increased tyrosine phosphorylation in response to mechanical load. Inhibitors of protein tyrosine phosphorylation not only prevented mechanical load induced tyrosine phosphorylation but also inhibited collagen synthesis. Inhibition of paxillin phosphorylation also suggests a role for the cytoskeleton in mechanical load activation of procollagen synthesis. Finally, experiments investigating the

intracellular signalling pathways activated downstream of the focal adhesion complex showed that the MAP kinase and the phospholipid pathway may play a role in mediating the effects of mechanical load on procollagen synthesis. In conclusion, this thesis has shown for the first time that integrins act as mechanotransducers in human cardiac fibroblasts and that phosphorylation of FAK, as well as paxillin and $p130^{Cas}$ is required for mechanical load to be converted into signalling pathways leading to procollagen synthesis.

DEDICATION

This thesis is dedicated to my grandparents and my parents who have supported me throughout this PhD. Thank you for all the special memories you have given me and for all your love and support.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my supervisor Dr Jill Bishop for helping and encouraging me throughout my 3 years and for being so enthusiastic about my research. I will miss discussing football with you during lunch. I would also like to thank Professor Geoff Laurent for his support and enthusiasm. Thanks to everyone at work who has made my time working in the lab great fun, especially David Howell. I would especially like to thank Jenny P, Helen, Olivier and Cecelia for being such good friends and for great nights out! Gisela, for being there when I needed advice. Thanks also to Carmel, Ian and Maddy who have gone through doing a PhD with me. I would like to say a big thank you to my flatmates, Astrid and Beth, who I have lived with for over 3 years, and who have been great friends and the best flat mates ever. I would like to thank Simon, Matt and Dave for being great friends and for their great parties. Last, but not least I would like to give special thanks to all my family who have been so supportive during my PhD. I couldn't wish for a better family.

Finally I would like to thank Aj, who has been with me, put up with me and looked after me for the last 3 years. I've enjoyed every moment of being with you.

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ABBREVIATIONS

4-NBDCl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
AII	angiotensin II
α-SMA	α–smooth muscle actin
BAEC	bovine aortic endothelial cells
bFGF	basic Fibroblast Growth Factor
BSA	bovine serum albumin
cas	crk associated substrate
cm	centimetres
CO_2	carbon dioxide
c-src	C-terminal src
DAG	diacylglycerol
DCM	dilated cardiomyopathy
ddH_20	deionised distilled water
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetracetic acid
ERK 1/2	extracellular-related kinase 1/2
ET-1	endothelin-1
EtOH	ethanol

FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluorescein-5-isothiocyanate
Fn	fibronectin
FRNK	FAK related non kinase
g	unit of gravity
g	gram
GST	glutathione-s-transferase
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
hGF	human growth factor
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
h	hour(s)
HUVEC	human umbilical vein endothelial cell
Hz	Hertz
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
Il-1B	interleukin-1B
IP_3	inositol trisphosphate
JNK1	Jun-related kinase
kDa	kiloDalton
L	litre
LPA	lysophosphatidic acid

M	molar
MAP kinase	mitogen activated protein kinase
mg	milligram
MI	myocardial infarction
min	minute(s)
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
Mw	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
NIH 3T3	mouse Swiss 3T3 fibroblasts
nM	nanomolar
nmoles	nanomoles
NP-40	nonident P40
NBDCl	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
OHpro	hydroxyproline
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PI 3-kinase	phosphatidyl inositol 3 kinase
PIP	phosphatidylinositol monophosphate
PKC	protein kinase C
PMSF	phenylmethylsulfonylfluoride

rER	rough endoplasmic reticulum
RGD	Arginine-Glycine-Aspartic acid
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SH2/3	src homology 2/3
SMA	smooth muscle actin
SMC	smooth muscle cells
t0	time zero
TBS-T	tris buffered saline-Tween 20
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	transforming growth factor beta
Tris	tris(hydroxymethyl)methylamine
TRITC	tetramethylrhodamine isothiocyanate
Tween 20	polyoxyethylenesorbitan monolaurate
μg	micrograms
μl	microlitre
μm	micrometer
υM	micromolar
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

vWF	von Willebrand factor
w/v	weight/volume

CHAPTER 1: INTRODUCTION

1.1 FOREWARD

In the cardiovascular system the mechanical environment is a key determinant of cellular activity. It regulates protein turnover, cell growth and cell division. Since mechanical forces continually change during development and under disease conditions, these forces play an important role in tissue remodelling during growth and disease. Collagen is a major protein component of the extracellular matrix of the heart and vasculature - it contributes to the functional integrity of the myocardium and blood vessel walls. Collagen is continually synthesised and degraded throughout life. Stimuli such as increased mechanical stress stimulate collagen turnover resulting in changes in the amount, distribution or types of collagen which may affect the cardiovascular function of the heart. The regulation of collagen synthesis by fibroblasts, the major collagen producing cells in the heart, is poorly understood. There is also little known as to how cells of the cardiovascular system detect changes in the mechanical environment - termed mechano-signal transduction - and how such signalling leads to changes in collagen synthesis and deposition. This thesis addresses the role of ECM composition in mechanical load induced procollagen synthesis and the importance of fibronectin integrins in mechano-signal transduction. Signalling pathways activated by mechanical load, and leading to procollagen synthesis are also examined.

1.2 STRUCTURE AND FUNCTION OF THE CARDIOVASCULAR EXTRACELLULAR MATRIX

The cardiac interstitium is populated by cardiac fibroblasts and cardiac myocytes. The fibroblasts occupy the interstitial space between the cardiac myocytes and are the source of many components of the cardiac extracellular matrix (ECM). Regulation of ECM proteins

secreted by these cells plays an important role in normal heart function and in diseased states.

The cardiac extracellular matrix is composed mainly of collagen and plays a vital role in determining the structure and function of the cardiovascular system. The formation of collagen fibrils into weaves and struts provides a network of physical support that maintains myocardial structure and myocyte alignment throughout the cardiac cycle (Borg & Cauldfield, 1981) (Figure 1.1). The network of collagen fibrils has multiple functions including the preservation of tissue architecture and chamber geometry, by formation of lateral connections between cells and muscle bundles. Given its tensile strength, alignment, location and configuration, relative to cardiac myocytes, the collagen matrix also represents a major determinant of myocardial stiffness (Horowitz *et al*, 1988, Ohayon & Chadwick, 1988). Because of its tensile strength and close association with the myocytes, the collagen network plays a vital role in the transmission of an even distribution of force generated by myocytes throughout the myocardium (Borg *et al*, 1982). This is important in both contraction (systole) and relaxation (diastole) phases in the cardiac cycle (Robinson *et al*, 1983). It also protects the sarcomeres from overstretch (Robinson *et al*, 1988). The composition of the ECM also influences how cells in the cardiovascular system perceive forces applied to the cardiac tissue. The 3D structure of the matrix and the resulting stiffness can influence the extent to which cells within the matrix can be physically deformed e.g. cells within a collagenous vessel may be less susceptible to deformation than those in a more elastic vessel. The matrix may also influence the alignment of cells within the matrix and thus determine which aspect of the cell is exposed to force, which will bias the extent and direction of force to which the cells are exposed.

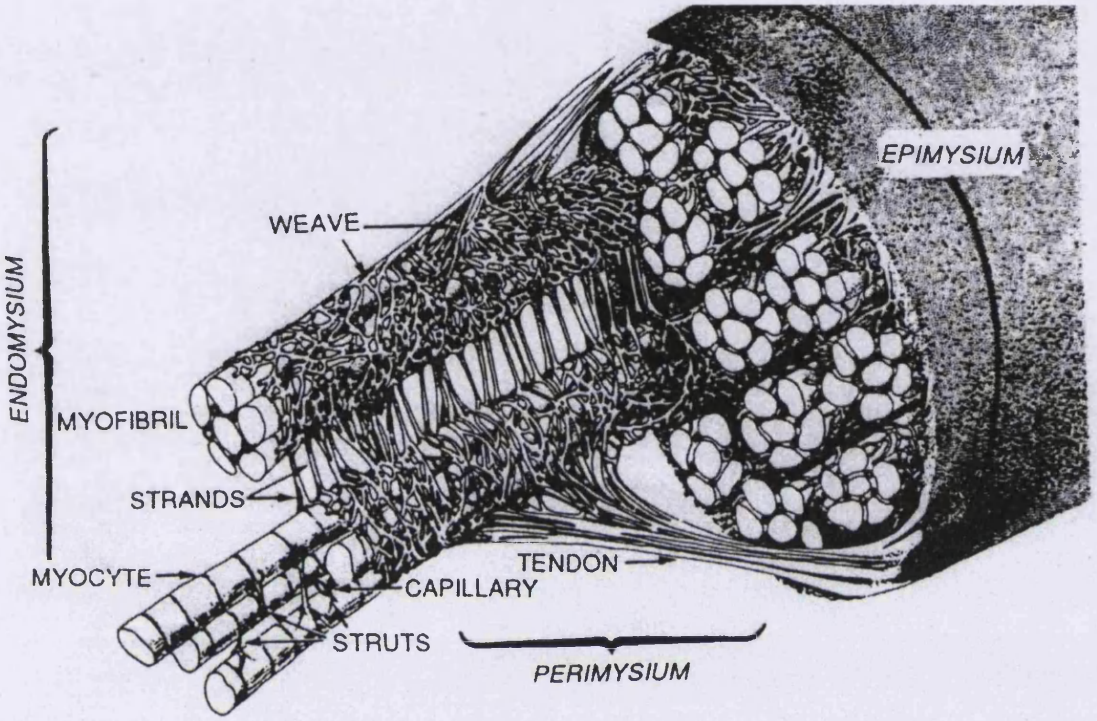


Figure 1.1. A schematic diagram of the heart's collagen matrix and its various components. Collagen weaves surround and group myocytes together. Struts of collagen join neighbouring myocytes and myocytes with capillaries. Adapted from Weber *et al,* 1988.

The cardiac ECM, in addition to its structural role, plays a critical role in regulating cell phenotype. Both *in vivo* and *in vitro*, ECM proteins markedly influence the growth, migration and differentiation of cells (Mosher, 1989, Hynes, 1990). Cardiac fibroblasts adhere to collagen matrices through integrins. Interactions of the individual components of extracellular matrix with specific integrin receptors, and proteoglycans initiates a cascade of signal transduction leading to varied short-term or persistent cellular responses. *In vitro*, it has been observed that adult rat cardiac myocytes require the ECM components laminin and collagen type IV for cell survival and spreading, suggesting the interaction of cell surface components with the ECM influences cell function (Lundgren *et al*, 1985).

There are eight known cardiovascular collagens. Types I, III and V are the classic fibril-forming interstitial collagens which act to confer tensile strength and contribute to the mechanical properties and functional integrity of the myocardium. The non-fibril forming collagen type IV forms an open-meshed network in association with other ECM proteins including laminin, heparan sulphate, nidogen and type V collagen. Type VI and the short-chain collagen type VIII are present in the interstitium and pulmonary vasculature (Amenta *et al*, 1986). The FACIT (fibril-associated collagens with interrupted triple helices) collagens, type XII and XIV, do not themselves form fibrils but act as molecular bridges between different ECM components, are also present in the cardiovascular system (Walchil *et al*, 1994, Lethias *et al*, 1996, Shaw & Olsen, 1991 (review)). These collagen types will be discussed in more detail below.

Collagen type I is the major collagenous product of cardiac fibroblasts (Egbahli *et al*, 1989) and accounts for ~ 80% of the total cardiac collagen content, as measured by pepsin digest

of tissue and gel electrophoresis (Bashey *et al*, 1989, Weber, 1989) and cyanogen-bromide extraction (Bishop *et al*, 1990). This observation is in agreement with the results of a study of collagen isoforms synthesised by cultured cardiac fibroblasts (Bashey *et al*, 1992). Collagen type III, produced principally by fibroblasts (Egbahli, 1989) is relatively abundant in the myocardium, accounting for ~ 12% of myocardial content. Together, collagen types I and III represent more than 90% of all myocardial collagen. They form the major structural collagens in the heart and are arranged as weaves and struts around individual and groups of myocytes (Borg & Cauldfield, 1981). Immunohistochemical studies have suggested that type I collagen forms thick fibres which confer high tensile strength and therefore structural support (Boerrigter *et al*, 1998). Type III collagen forms a fine network of fibrils and is associated with tissues in which a high degree of elasticity is required, such as the lung, blood vessels and skin (Boerrigter *et al*, 1998).

The other collagen types (VI, V, IV, VIII), make up the remaining 10% of the total cardiovascular collagen content. Type IV collagen is a basement membrane protein, produced by most cardiovascular cells, particularly myocytes (Eghbali *et al*, 1988) and endothelial cells. It influences cell adhesion, spreading and proliferation and acts as a filter of nutrients and growth factors to the underlying mesenchymal cells of the vessel wall and of the myocardial interstitium.

Type V collagen is associated with type IV in the basement membrane and in the interstitium is associated with types I and III, where it modulates fibril diameter (Birk *et al*, 1990). Type VI collagen, associated with other fibrillar collagen, may also regulate fibril diameter (Amenta *et al*, 1986). Type VIII is a short chain collagen, thought to play a role

in angiogenesis (Rooney *et al*, 1993) and cardiac morphogenesis, being present in the fetal heart, with only low levels detected in the neonate and adult heart (Iruela-Arispe & Sage, 1991). In the adult it is associated with blood vessels, forming a component of the intimal basement membrane and co-localising with elastic components of the ECM (Sawada & Konomi, 1991). Type VIII collagen is produced by SMCs and endothelial cells (Stiemer *et al*, 1993).

Fibronectin is another important component of the cardiac ECM, providing a link between collagen fibres and myocytes and fibroblasts (Bashey *et al*, 1992). This link has been shown by ultrastructural analysis, whereby fibronectin was found to be inserted along the surfaces of cardiac myocytes, connecting these cells to perimyocytic collagen (Ahumada & Saffitz, 1984). These ultrastructural relationships imply that fibronectin may affect myocardial compliance and control the motion of myocytes during the contraction and relaxation of the heart.

During cardiac organogenesis there is an accumulation of fibronectin mRNA. At the onset of development of the heart and during the fetal stage a progressive decrease in fibronectin levels occurs and continues through senescence. However, levels have been shown to change during hypertension. Fibronectin is known to be chemotactic for both fibroblasts and macrophages and may act directly as a growth factor, possibly through its role in cell attachment via specific integrin ECM receptors (Mosher, 1989, Ruoslahti, 1988). In the wound healing setting much research has been carried out on the possible role of fibronectin acting as a "scaffold" for newly synthesised collagen. Kurkinen *et al* (1980) showed *in vivo* that fibronectin appears early along with invading fibroblasts into granulation tissue. This

suggests that fibronectin functions as a primary matrix for organization of the collagenous connective tissue during the tissue repair process.

Relatively little information is available about the structure and function of cardiac elastin. About 18% of cardiac elastin is arranged in helical form (Debelle *et al*, 1992). Endomysial connections between myocytes are comprised of elastin fibres as well as collagen fibres (Robinson *et al*, 1985). Elastin like collagen, is a structural protein and confers resiliency to the heart. Its mechanical stiffness is lower than that of collagen (Vesely, 1998) but is thought to act in valve function, returning the valve back to a resting configuration between loading cycles (Vesely, 1998).

Laminin is an extracellular glycoprotein with adhesive properties that is secreted by cardiac fibroblasts, myocytes and endothelial cells. It is a basement membrane protein and forms a fine fibrillar network along the basement membrane in association with transverse tubules of myocardial cells (Kim *et al*, 1999). Laminin promotes and regulates morphogenesis of the myocardial cells in the mammalian heart (Kim *et al*, 1999). It also functions in cell adhesion and cell migration in the embryonic heart (Loeber *et al*, 1990, Davis *et al*, 1989).

The role of proteoglycans in the heart is still unclear. Proteoglycans are cell surface receptors and therefore may be important in cell:cell or cell:ECM adhesion in the heart. Synthesis of collagen types I and III by bovine synovial fibroblasts is accompanied by synthesis of proteoglycans, it has been suggested by the authors that proteogylcans may play a structural as well as adhesive role (Simionescu & Kefalides, 1991).

In summary, both structural and adhesive proteins which make up the cardiovascular ECM are essential for the normal function of the heart. Structural ECM proteins function to support and align myocytes, blood vessels and lymphatic vessels to one another, thereby preserving myocardial architecture. They prevent muscle fibre and myocyte slippage, allowing the transduction of myocyte-generated force and provide the basis for diastolic myocardial stiffness. Adhesive ECM proteins including fibronectin and laminin, play important roles in regulating cell phenotype within the heart. The cardiac ECM is also essential in modulating the cell phenotype during ontogenic development and hypertrophy.

1.3 CARDIOVASCULAR REMODELLING

ECM remodelling occurs in the heart in response to increases in mechanical forces, such as raised blood pressure. The increase in collagen synthesis that occurs during the development of cardiac hypertrophy may be "compensatory", i.e. it matches the growing myocardium,or it may exceed that required for the maintenance of normal heart growth and fibrosis develops. These two processes are discussed below.

1.3.1 Cardiac hypertrophy

Cardiovascular cells are exposed to continual mechanical stresses. During the development of cardiovascular diseases including hypertrophy, the mechanical environment changes, and in an attempt to normalise these forces, the heart and vasculature remodel at both the cellular and extracellular level (Glagov, 1994).

Initially, heart muscle responds to changes in the mechanical environment by inducing myocyte growth, with myocytes growing in proportion to the increase in cardiac mass (Zak,

1973). An increase in collagen synthesis is also observed but this is matched by the myocyte hypertrophy, resulting in an enlarged ventricle with a normal collagen composition (Brilla & Maisch, 1994). This is known as compensatory growth, whereby hypertrophy occurs and the matrix remodels to accommodate the increase in muscle mass. Cardiac hypertrophy is defined as an increase in cardiac mass which is related to an increase in myocyte size rather than number and is caused by chronic pressure overload, volume overload, or a combination of the two. Although myocytes are considered to be terminally differentiated (Takahashi, 1992) it is thought that myocyte hyperplasia is possible in a variety of pathological conditions, including cardiac hypertrophy (Anversa *et al*, 1980, Anversa *et al*, 1990, Olivetti *et al*, 1987, Olivetti *et al*, 1990).

During the onset of cardiac hypertrophy changes in the cardiovascular ECM take place. In animal models an increase in collagen type III is observed early on (Weber *et al*, 1988), followed by a large and sustained increase in type I (Chapman *et al*, 1990). Pressure overload has been shown to increase total protein and procollagen fractional synthesis rates (Bishop *et al*, 1994, Eleftheriades *et al*, 1992, Chapman *et al*, 1990). The increase in procollagen synthesis rates is due to increased collagen gene expression by fibroblasts (Bishop *et al*, 1994, Villareal *et al*, 1992). The sustained pressure overload that accompanies hypertension leads to an increase in wall thickness without chamber enlargement. Thus, pressure overload results in the development of concentric hypertrophy, i.e. an increase in ventricular wall thickness without increase of the inner ventricular radius (Mirsky, 1979). Parallel sarcomere replication resulting in increased myocyte dimension produces the increased wall thickness. In chronic or more severe pressure overload, collagen deposition exceeds the hypertrophic response leading to

excessive collagen deposition and fibrosis (see section 1.3.2 below).

In volume overload hypertrophy sustained overload leads to increased ventricular wall tension, resulting from increased volume. However, unlike pressure overload hypertrophy (although similar in early phases), this is matched by myocyte hypertrophy resulting in an enlarged ventricle with a normal collagen composition. Myocyte elongation occurs but does not result in an increase in wall thickness (Anversa *et al*, 1983, Kramer *et al*, 1998). This is defined as eccentric hypertrophy. To determine if volume overload caused interstitial collagen accumulation as observed in pressure overload, an aortocaval (AC) shunt was created in rats, to induce eccentric LV hypertrophy (Namba *et al*, 1997). AC shunt lead to an increase in type I and III collagen mRNA levels compared to control. In contrast, the collagen protein concentration was comparable between control and AC shunt rats. Immunohistochemical staining of both collagen types showed no changes in distribution or density of fibrillar collagen between control and AC shunt rats. This suggests the upregulation of collagen gene expression and protein accumulation may be different in pressure overloaded and volume overloaded hypertrophy (Namba *et al,* 1997).

1.3.2 Cardiac fibrosis

Cardiac fibrosis occurs when severe pressure overload causes an increase in collagen deposition which exceeds the hypertrophic response, leading to excessive collagen deposition and hence an increase in myocardial collagen concentration, resulting in fibrosis. In various models of left ventricular pressure overload it has been observed that collagen fibres change with respect to their physical dimension, their alignment with cardiac muscle fibres and the relative proportions of type I and III collagens (Medugorac, 1976, Willems *et*

al, 1994). Structurally distinct arrangements of these collagen fibres with respect to muscle have been identified leading to the proposition of the occurrence of different patterns of fibrosis (Weber, 1989 (Review)).

Cardiac fibrosis presents as an accumulation, and increase in dimensions, of fibrillar collagen, within the cardiac interstitium (Abrahams *et al*, 1987, Doering *et al*, 1988, Pick *et al*, 1989). The existing collagenous matrix increases in dimension leading to an increased number of intermuscular spaces being occupied by collagen fibres, creating an interstitial fibrosis. This type of fibrosis is termed reactive fibrosis because it is due to direct stimulation of fibroblasts and is not secondary due to myocyte necrosis (Okoshi *et al*, 1997). After an initial period of hypertension, an accumulation of fibrillar collagen is seen within the adventitia of intramyocardial cornoary arteries (Jalil *et al*, 1989, Thiedemann *et al*, 1983). From their perivascular locations, these collagen fibres radiate outwards into intermuscular spaces (Figure 1.2).

Although the precise mechanisms involved in the transition from normal expansion of collagen mass to fibrosis during the development of hypertrophy are not fully understood, growing evidence suggests a role for locally produced growth factors including transforming growth factor-beta (TGF-β) and angiotensin II (AII) (Crawford *et al*, 1994, Campbell & Katwa, 1997). In addition, insufficient perfusion and oxygen supply during severe pressure overload leads to regions of myocyte necrosis (Anderson, 1979, Thiedemann *et al*, 1983, Jalil *et al*, 1989, Pick *et al*, 1989). Following necrosis, collagen is deposited at the site during scar formation. This is known as reparative fibrosis (Figure

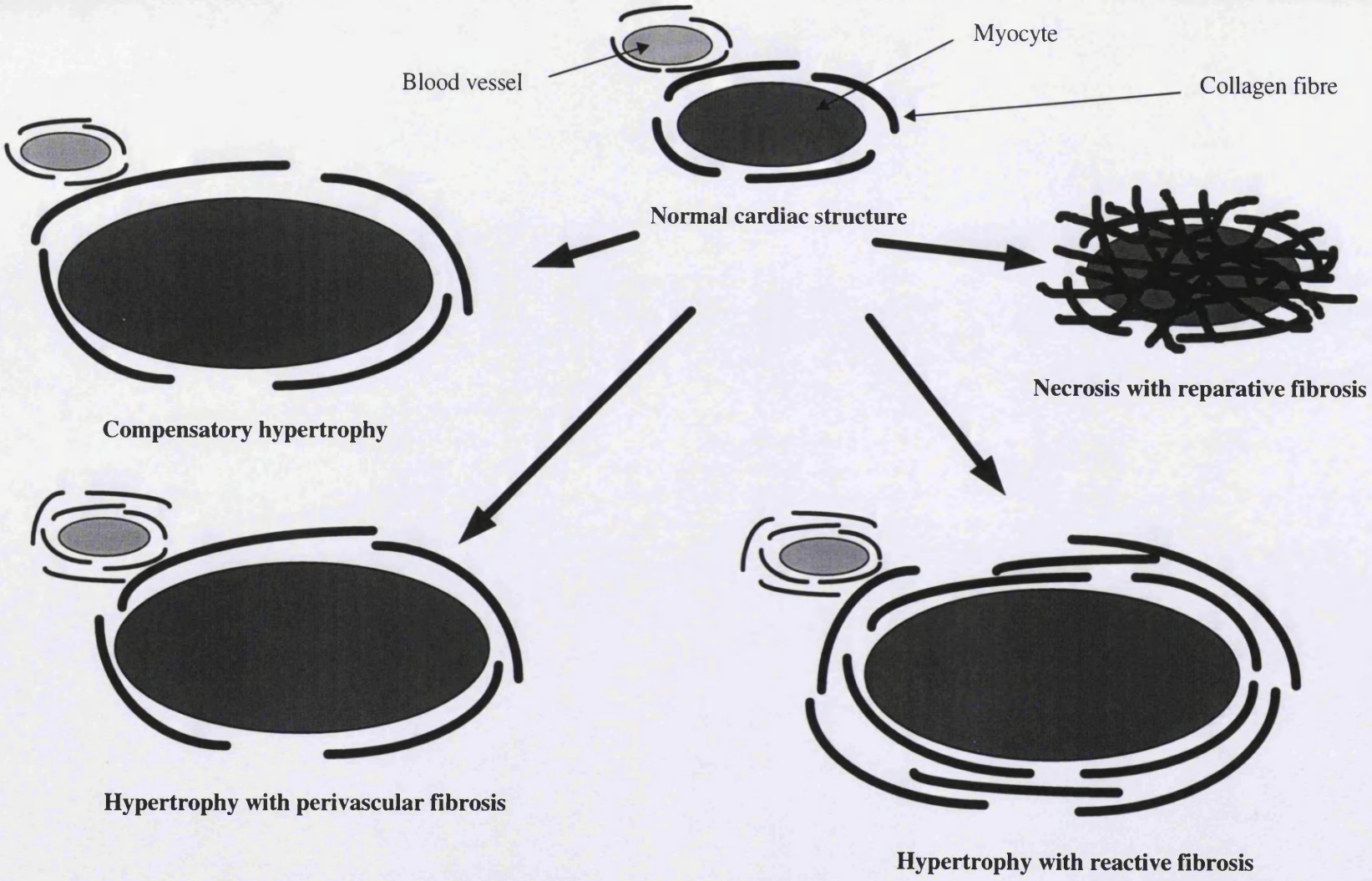


Figure 1.2. Schematic diagram showing the types of cardiac fibrosis.
Adapted from Bishop, J.E. 1998.

1.2) since collagen is deposited as a result of a wound healing response to tissue injury or cell death. During this process thick collagen fibres bridge gaps created by necrotic cells. These fibres are directed parallel to the long axis of muscle that previously occupied the space (Carroll *et al*, 1989). Thin collagen fibres, running parallel to the long axis of muscle, become entwined in the thick fibres. Together these form a dense patch that preserves the structural integrity of the myocardium.

In addition to changes in the amounts of collagen deposited, changes may also occur in the relative proportions of the collagen types in the development of cardiac hypertrophy. Type III collagen appears to increase in the early stages (Weber *et al*, 1988b) but the later stages show an increase in type I (Turner and Laurent, 1986, Chapman *et al*, 1990), a sustained increase in type III (Medugorac and Jacob, 1983, Mukherjee *et al*, 1983, Morioka *et al*, 1992) or a return to normal levels (Weber *et al*, 1988b) in both human disease and animal models. These differences are dependent on the species, age and time at which the measurements are made in the development of hypertrophy.

Following an insult such as myocardial infarction (MI) or ischaemia in addition to reparative fibrosis, there is enhanced collagen deposition distal to the infarct. This is termed reactive fibrosis. Infarction leads to the production of TGF-β (Hao *et al*, 1999) and AII (Silvestre *et al*, 1999, Thai *et al*, 1999) in the surrounding tissue which may stimulate myocyte hypertrophy and matrix deposition. The fibrosis remote from the infarct site may also be the result of a direct stimulation of cell function by growth factors and/or increased mechanical load. The infarct and subsequent scar contracts, partly due to the

presence of myofibroblasts pulling at the surrounding tissue.

Early in established hypertrophy, when collagen concentration is increased, there is predominantly diastolic dysfunction of the left ventricle, while systolic function is still preserved. Later, with myocyte necrosis and atrophy and continued collagen accumulation, systolic dysfunction with a reduction in ejection fraction occurs accompanied by a marked diastolic dysfunction (Weber & Brilla, 1991). As a result of the apparent relationship between impaired stiffness and pump dysfunction and the accumulation of, or shift in, collagen types, it is suggested that this remodelling of matrix components represents a determinant of pathological hypertrophy in hypertension. This pathological LVH is a major risk factor associated with myocardial failure (Levy *et al*, 1990).

1.4 COLLAGEN METABOLISM

The collagens are a family of fibrous proteins found in all multicellular animals. Common to all members is a triple-helical domain composed of three polypeptide α chains which together form the triple helix. To date, 33 distinct collagen α-chains have been identified. The 33 α-chains interact to form at least 19 distinct collagen isotypes, the most predominant ones being collagen type I and III which are produced principally by fibroblasts.

1.4.1 Procollagen Synthesis

The interstitial collagens (types I and III), composed of 3 polypeptide α-chains, are initially

synthesized as procollagens. Each pro α-chain consists of a central left-handed helical region (Ramachandran *et al*, 1954) containing the repeating triple unit of amino acids, Gly-X-Y (X is often proline and Y the imino acid 4-hydroxyproline) (Miller, 1985) as well as short non-helical regions at the N-and C-terminal ends. The Gly-X-Y sequence is essential for the folding of the molecule into a right-handed triple helix, stabilized by inter-chain hydrogen bonds. Because the glycines have no side chains, they can be positioned in the centre of the triple helix thus allowing the peptide bonds to become buried within the interior of the molecule (Traub & Piez, 1971). This renders the triple helical region highly resistant to proteolysis. Secreted type I and III procollagens have amino- and carboxy terminal extension peptides which are cleaved off before fibrils are formed. However, type III collagen chains may retain the amino terminal. After their spontaneous assembly into fibrils, collagen molecules are cross-linked by pyridinium and deoxy-pyridinium.

Each procollagen α-chain is encoded by a separate gene (van der Rest & Garrone, 1991). Procollagen genes are widely dispersed throughout the genome and are characterised by high concentrations of guanine (G) and cytosine (C) which account for the high concentrations of proline and hydroxyproline in the recurring Gly-X-Y triplet. Collagen types I, II and III share a highly conserved gene structure consisting of 51 or 52 exons interspersed with introns. The major triple helical regions of the fibrillar collagens are encoded by 42-44 exons, most of which have a conserved 54-base pair structure (Kimura *et al*, 1989). The N-propeptide and the signal propeptide are typically encoded by the first six exons, and the C-propeptide by the last 4 exons.

Collagen is synthesised predominantly by fibroblasts. The process of collagen biosynthesis is well understood. Procollagen DNA is transcribed to pre-mRNA in the nucleus. Pre-mRNA is then spliced to form functional mRNAs (4-6 kB) before translation on the membrane-bound polysomes in the rough endoplasmic reticulum (rER). The translated product, termed preprocollagen α-chain, contains an N-terminal hydrophobic signal peptide. This peptide is thought to facilitate passage across the rER. The peptide is proteolytically cleaved during translation or shortly after polypeptide synthesis to form the procollagen α-chain (Nimni & Harkness, 1967). This undergoes a series of co- and post translational modifications, including hydroxylation of specific proline and lysine residues in the Y position of the Gly-X-Y triplet to form 4-hydroxyproline and hydroxylysine (Kivirikko *et al*, 1989). The enzymes required to catalyse this reaction are prolyl 4-hydroxylase and lysyl hydroxylase and involve the cofactors ferrous iron and ascorbate. Most of these reactions occur while the nascent polypeptide α chains are growing on the ribosomes, and proceed until virtually all Y-position prolines are hydroxylated. This process is essential for the stability of the triple helix. Procollagen that is underhydroxylated is unable to form stable triple helices and so is susceptible to rapid degradation (Steinmann *et al*, 1981).

Triple helix formation of the fibrillar collagens occurs through a nucleolation centre in the C-propeptide as a result of the alignment of the three procollagen α-chains in a way that juxtaposes appropriate cysteine residues in the C-terminal propeptides (Engel & Prockop, 1991). This results in the formation of intra- and interchain disulphide bonds between opposing lysine and hydroxylysine residues. Triple-helix formation then proceeds in a

zipper like fashion from the C terminus to the N-terminal domain. Procollagen molecules are eventually transported from the rER to the Golgi apparatus. Here they are packaged into secretory vesicles. During or immediately after secretion, the N and C terminal propeptides of fibrillar collagens are cleaved by procollagen N and C proteinase (PCP), respectively.

Fibre formation

Upon cleavage of the propeptides, spontaneous assembly of fibrils occurs at the cell membrane (Birk & Trelstad, 1986). The molecules align precisely in quarter-stagger arrays, first forming microfibrillar units, which are then packed together to form larger fibres (Birk & Trelstad, 1986). The fibres are initially held together by electrostatic interactions and thus lack tensile strength. Crosslinking of these fibres results in an increase in their tensile strength. Crosslinking formation is due to the action of lysly oxidase which oxidises susceptible lysine and hydroxylysine residues to lysine and hydroxylysine aldehydes.

1.4.2 Collagen Degradation

Two principle pathways exist by which collagen may be degraded; an intracellular pathway that rapidly degrades procollagen molecules and an extracellular pathway involving the degradation of fibrillar collagen.

Intracellular degradation

In vitro studies have shown that a proportion of newly synthesised collagen is rapidly degraded. Studies have suggested that this degradation takes place intracellularly within minutes of procollagen synthesis. This has been confirmed by *in vivo* studies which have

shown that degradation occurs within 15 minutes (Bishop *et al*, 1995). Intracellular degradation is thought to occur mainly in lysosomes, although a small amount has also been shown to be non-lysosomal (Bienkowski, 1984). The regulation of intracellular degradation is poorly understood, but several non-specific proteases with other functions in the cell may be involved.

The functions of intracellular degradation may be two-fold: to prevent secretion of defective molecules and secondly, to provide a rapid means of regulating collagen output in response to extracellular stimuli. Evidence in support of the former hypothesis comes from studies in which proline analogues are incorporated into the collagen chain, preventing them from forming a stable triple helix. These unstable collagen chains are rapidly degraded (Berg *et al*, 1980). Also, fibroblasts under-hydroxylate collagen during the log phase of growth and this stage is associated with the degradation of three times the proportion of newly synthesised collagen compared with cells which are confluent (Tolstoshev *et al*, 1981). The role of this degradative pathway as a regulator of the extent of collagen deposition has been demonstrated in studies of fibrotic disorders where enhanced collagen deposition is associated with a fall in the proportion of collagen degraded rapidly (Laurent & McAnulty, 1983, Bishop *et al*, 1994).

Extracellular degradation

The collagen fibrils are very robust and highly resistant to proteolytic degradation. However, they can be degraded by specific proteinases, the matrix metalloproteinases (MMPs), including collagenases, gelatinases (type IV collagenase) and stromelysin. Collagenolysis by collagenase may be regulated at several sites (Krane, 1985) including (a)

biosynthesis and secretion of the latent enzyme procollagenase, predominantly by fibroblasts; (b) activation of the latent enzyme by proteases for example, activation of latent (pro)collagenase probably occurs through the action of plasmin (via the plasminogen activator, Krane, 1985); (c) interaction of the active enzymes with the collagen substrate and (d) modulation of the effects of the active enzyme by proteinase inhibitors, such as tissue inhibitor of metalloproteinase (TIMP) and α_2-macroglobulin. Interstitial collagenases cleave the interstitial collagens at a single locus within the helical structure to produce a large fragment, TC_A (75% of the molecule length) and a smaller TC_B fragment. The cleaved fragments lose their triple-helical conformation and are susceptible to further proteolysis by the gelatinases.

This degradative pathway is thought to play a role in the remodelling of the collagen matrix during rapid tissue growth, since this process involves the breakdown of existing cross-linked collagen fibres. MMPs are activated in dilated cardiomyopathic hearts (DCM) (Tyagi *et al*, 1996), one of the hallmarks of DCM being left ventricular remodelling, with an increase in expression levels of MMP 1 compared to the normal heart (Tyagi *et al*, 1996, Thomas *et al*, 1998). In response to this increase in MMP activity and expression, TIMP expression is decreased in the DCM hearts compared to normal suggesting that a disruption in the balance of proteinase and anti-proteinase expression occurs in cardiac remodelling.

1.5 COLLAGEN METABOLISM IN THE NORMAL HEART AND IN RESPONSE TO CARDIAC HYPERTROPHY

In the cardiovascular system, collagen is continually being synthesised and degraded.

Collagen turnover in the normal heart has not been extensively studied. In the rabbit right and left ventricles, fractional collagen synthesis rates (the percent of total ventricular collagen synthesised in one day) of 3%/day and 6%/day respectively were found, of which one third is rapidly degraded in both ventricles (Turner *et al*, 1986). By comparison, non-collagen protein synthesis was 18 %/day in both ventricles. Fractional collagen synthesis rates in the rat were 9 %/day at 6 months of age, in comparison with values of 4 %/day and <1 %/day in the lung and skin, respectively (Mays *et al*, 1991). However, at this age in the heart over 90% of this collagen is degraded rapidly, compared to 70 % in the lung and only 14 % in the skin. Rates of collagen synthesis can be measured by injection of ^{3}[H]-proline, administered with a large dose of unlabelled proline, followed by measurement of the specific radioactivity of proline and hydroxyproline in tissues up to 3 hrs later.

Little information is available concerning the relative rates of synthesis for the different collagen types. In the skin, however, it appears that collagen synthesis is more rapid for type I and III. The half-life for processing type I collagen in rabbit skin has been estimated at 26 min compared to 3.9 hrs for type III (Robins, 1979).

During the development of cardiac hypertrophy, collagen synthesis rates are dramatically increased. A six-fold increase in the rate of synthesis in the right ventricle was observed 2 days after banding the pulmonary artery (Bishop *et al*, 1994), associated with a fall in the proportion degraded rapidly (although degradation in absolute terms was increased).

In the bleomycin model (which causes an increase in pulmonary artery pressure, leading to right ventricular hypertrophy), RV collagen synthesis rates increased by three-fold after 14

days. A fall in RV collagen concentration of 40 % was observed 6 days after the bleomycin administration. This was due to a fall in the total collagen content of the ventricle, indicating a breakdown of part of the existing collagen matrix. An increase in the free hydroxyproline in these tissues served to support the idea that collagen degradation increases (Turner *et al*, 1986). Electron microscope studies also give support for the degradation of the existing network to accommodate the increase in muscle mass (Doering *et al*, 1988). The observed increase in degradation levels of collagen is due to the activation of matrix metalloproteinases (MMPs) in the heart. In the normal myocardium MMPs are present in the latent form, but have been shown to contribute to extracellular remodelling in several disease states. A comparison of MMP activity between normal human cardiac tissue, infarcted, noninfarcted and dilated cardiomyopathic human cardiac tissue, showed that there was an increase in MMP 1 activity in the infarcted tissue compared to normal tissue (Cleutjens *et al*, 1995, Sato *et al*, 1983). These data suggest that activation of collagenase throughout the myocardium may contribute to its remodelling (Tyagi *et al*, 1996, Thomas *et al*, 1998).

With respect to pro-collagen α_1 (I) mRNA levels, a four-fold increase was observed (Bishop *et al*, 1994) in a rabbit model of RV hypertrophy (induced by pulmonary artery banding). Such an increase has also been seen with collagen types I and III mRNA in the hypertrophied rat myocardium in response to pressure overload (Chapman *et al*, 1990, Villarreal & Dillmann, 1992). These results suggest that the regulation of collagen deposition occurs at both the transcriptional level and post-translational level.

The regulation of collagen synthesis by fibroblasts, the major collagen producing cells in the heart, is poorly understood. Since pressure overload is frequently associated with increased collagen deposition, this thesis investigates the hypothesis that mechanical forces directly regulate cardiac fibroblast function and attempts to understand the process of mechano-signal transduction.

1.6 THE EFFECT OF MECHANICAL LOAD IN THE DEVELOPMENT OF CARDIAC HYPERTROPHY

1.6.1 Effect of mechanical load on tissue growth *in vivo*

All tissues in the body are exposed to mechanical forces. Studies have shown that biochemical and chemical signals (traditionally thought to be the major factors that govern cell function), work together with mechanical factors to regulate cell activity, growth and development. In skeletal muscle, neuronal and hormonal stimuli, although important in determining the final size of a muscle, are not sufficient to augment muscle growth without mechanical load. It had been shown that protein synthesis in rat skeletal muscle is promoted by stretch even in the absence of neural supply (Goldspink, 1978). Similarly, inflation of the uterus to mimic the pregnant state causes increased protein synthesis in the absence of pregnant hormonal influences (Douglas *et al*, 1988). Also, shrinkage of the uterus occurs after delivery once the stretch is removed.

Mechanical load is the driving force for muscle protein synthesis and growth (Goldberg, 1967). In bones and tendons, compression and strain are stimuli for growth and remodelling (Jones & Bingmann, 1991), with the application of mechanical load a classic approach for the treatment of orthopaedic disorders. The response of bone cells to load is

aimed at maintaining the bone structure and architecture. Studies in dogs, where the ulna was resected and as a result the strain on the radius increased, have shown a two-fold increase in the diameter of the radius after 3 months and a change in the orientation of trabeculae in the direction of maximum load (Goodship *et al*, 1979). In orthopaedics, application of mechanical load is used in the treatment of fractures. Absence of load on the contrary, leads to loss of bone thickness.

The lungs are subjected to passive mechanical forces during development and normal breathing processes (Riley *et al*, 1990b). After pneumonectomy in infants, mechanical load imposed on the remaining lung is thought to be the major stimulus for the regrowth of the tissue that occurs since filling the cavity after pneumonectomy abolishes the increase in lung size (Rannels, 1989, Brody *et al*, 1978).

Mechanical forces are extremely important in the cardiovascular system, playing a very important role in tissue growth. The cells of the cardiovascular system are continually subjected to mechanical forces due to changes in blood volume and pressure. During normal cardiovascular function, cells respond to these changes until they and the surrounding ECM they produce reach a state of mechanical and metabolic equilibrium. Changes in the normal levels of these forces have a greater effect on cardiovascular structures and function as described above. Increased mechanical load leads to cardiac hypertrophy which involves the enlargement of myocytes, in an effort to respond to the increased load imposed on the heart, an increase in the number of cardiac fibroblasts and increased collagen deposition. Studies have shown that cardiac myocyte hypertrophy, fibroblast replication and collagen synthesis by cardiac fibroblasts are directly stimulated by

mechanical load (Bishop *et al*, 1993, Butt *et al*, 1995, Carver *et al*, 1991). Thus, abnormal forces imposed on the heart, such as in pressure-overload due to hypertension, may be responsible for the development of the observed ventricular hypertrophy and ensuing fibrosis (Turner *et al*, 1986). Thus the mechanical environment is a key modulator of cell function.

1.6.2 Effect of mechanical load on cell growth *in vitro*

Mechanical load and its effects on cell phenotype and function have been well documented in the literature. To examine the effect of mechanical load on cell function, different *in vitro* techniques have been employed. This has allowed the study of the direct effects of mechanical forces on cell function. Cells are exposed to a number of different types of mechanical force, including stress, shear stress, compression and osmotic tension. Cells can be grown on flexible membranes (synthetic or natural). These membranes can be deformed in a uni-or bi-axial manner by the use of solid "prongs"; by the application of air pressure causing the membrane to balloon up; or by the application of a vacuum beneath the membranes, causing them to dome down. Cells may be subjected to a single static stretch, slow continuous stretch or cyclic repetitive stretching. Cells can also be grown in a three-dimensional matrix. By comparing cells maintained in bound lattices (under tension) compared to cells in free retracting lattices, the effect of mechanical forces in this environment can be compared (Vandenburgh, 1992)

It has been well documented that mechanical load stimulates cell proliferation and protein synthesis in many cell types *in vitro*. Cyclic load stimulates endothelial cell (Sumpio *et al*, 1987) and vascular smooth muscle cell (VSMC) proliferation (Wilson *et al*, 1993, Predel

et al, 1992). Although mechanical load has been shown to have no effect on thoracic aorta smooth muscle cell replication (Leung *et al*, 1976).

Mechanical forces stimulate cell replication *in vitro*, although this response appears to be dependent on the cell type examined. Cyclical mechanical strain profoundly increases cultured VSM cell growth (Wilson *et al*, 1995). Intervertebral disc cells-nucleus pulposus cells which *in vivo* are exposed to a multitude of physical forces, have been shown *in vitro* to increase DNA synthesis rates (Matsumoto, 1999). Enhanced replication also occurs for endothelial cells (Sumpio *et al*, 1987, Banes *et al*, 1993), smooth muscle cells (Wilson *et al*, 1993) and mixed foetal lung cells (Smith *et al*, 1994).

Total protein synthesis increases in response to mechanical load *in vitro*. *In vitro* in the pulmonary artery, stretch increased the rate of collagen and elastin synthesis, and caused hypertrophy in smooth muscle cells and hyperplasia in fibroblasts (Kolpakov *et al*, 1995). Cyclic load enhances procollagen synthesis in cardiovascular and pulmonary fibroblasts (Butt *et al*, 1997, Bishop *et al*, 1998) and mesangial cells (Yasuda *et al*, 1996). However, collagen synthesis is decreased in response to mechanical stain in endothelial cells (Sumpio *et al*, 1990). Elastin synthesis is increased in smooth muscle cell cultures in response to mechanical load (Sutclife & Davidson, 1990). Cyclic strain is associated with an increase in actin and myosin in airway smooth muscle cells (Smith *et al*, 1993), fibroblasts (Pender & McCulloch, 1991) and tendon cells (Harris *et al*, 1992).

Total protein synthesis increases in response to mechanical load by smooth muscle cells (Kollros *et al*, 1987) and skeletal myoblasts (Vandenburgh *et al*, 1989). This protein

accumulation is associated with cellular hypertrophy. Mesangial cells undergoing cyclic stretching also show an increase in their synthesis of protein, caused in part by cell growth and hypertrophy (Riser *et al*, 1992). Hypertrophy of myocytes exposed to cyclic stretch has been shown *in vitro* (Cadre *et al*, 1998, Terracio *et al*, 1988), resulting in an increase in cell size. Contrary to this, stretching of ventricular myocytes in another study did not induce a hypertrophic response alone, but when cultured with cardiac fibroblasts a hypertrophic response occurred (Harada *et al*, 1997), suggesting that the cardiac fibroblasts mediate the hypertrophic effect of mechanical stress on myocytes, by increasing endothelin production. AII may also be involved in the crosstalk.

From the literature presented above, it can be concluded that mechanical load is important in regulating both cell replication and protein synthesis, in many cell types. However, it is now thought that the matrix to which the cells attach plays an additional role in determining how cells respond to mechanical load, as demonstrated by Wilson and co-workers (1997). Vascular smooth muscle cell replication in response to mechanical load was found to be dependent on the nature of the matrix on which the cells are grown. Cells grown on a fibronectin or collagen matrix were found to be more responsive to mechanical load than those cells grown on elastin or laminin. This suggests that the cell:matrix interaction is important in determining how cells perceive mechanical forces, implicating that receptors expressed on the cell surface, which bind to the matrix, are involved.

Little is known regarding the effects of mechanical load on cardiac fibroblasts or the mechanisms involved in increased DNA synthesis and collagen production. This thesis examines the biochemical effects of mechanical load on human cardiac fibroblast

procollagen synthesis.

1.6.3 Synergy between mechanical load and growth factors

It has been suggested that growth factors may act synergistically with mechanical load to stimulate the changes in fibroblast activity that occur during cardiac hypertrophy and that load alone is unable to stimulate procollagen synthesis (Butt, R, Ph.D. Thesis 1997, Butt *et al*, 1997). Rat cardiac fibroblasts require the presence of high levels of serum or pure growth factors in order to increase production of collagen in response to mechanical load (Butt *et al*, 1997). However, this response may be specific to cardiovascular fibroblasts since Wilson *et al* (1995) showed that vascular smooth muscle cell (VSMC) replication is enhanced by load in the absence of any growth factors. However these cells produce growth factors such as platelet derived growth factor (PDGF) in response to load. PDGF has been shown to stimulate both fibroblast replication (Peacock *et al*, 1992, Butt *et al*, 1995) and cardiac fibroblast procollagen synthesis. Cyclic mechanical deformation of human lung fibroblasts results in increased replication, the effect of which may be mediated by the release of autocrine growth factors (Bishop *et al*, 1993). Conditioned media taken from stretched fibroblasts increased mitogenesis of non-stretched lung fibroblasts (Bishop *et al*, 1993). VSMCs, in response to mechanical strain release not only PDGF, but also transforming growth factor -β (TGF-β1), fibroblast growth factor and VEGF which act in autocrine or paracrine loops to influence VSMC function and cell growth (Wilson *et al*, 1998). TGF-β is a potent stimulator of cardiac fibroblast collagen synthesis (Rizzino, 1981, Butt & Bishop, 1997) and is seen in the early stages of cardiac hypertrophy induced by pressure overload. It has also been shown to stimulate myocyte hypertrophy (Parker *et al*,

1990, Schneider *et al*, 1991). Depending on the cell type, it appears that autocrine factors are produced in response to load or that they are required from a paracrine source to act in conjunction with mechanical load to enhance cellular activity. The nature of this synergistic interaction - whether through production of an intermediate protein, for example or through interaction of intracellular signalling pathways - is, as yet, unknown.

1.7 MECHANO-SIGNAL TRANSDUCTION: ROLE OF INTEGRINS

Little is known as to how cells sense mechanical load and transduce it into intracellular signals of gene regulation. This process involves detection of the load at the cell surface, transduction of this stimulus through intracellular signalling and the response at the level of gene expression. This particular section focuses on mechanisms of mechano-signal transduction at the level of the receptor.

Three possible mechanisms of mechano-signal transduction have so far been proposed, involving the cytoskeleton, stretch-activated ion channels (SACs) and integrins. Of these, the integrins appear to be the most likely candidates as they directly link cells to the extracellular matrix allowing the transmission of the mechanical signal of cellular distortion to the nucleus. Such evidence has evolved from work carried out in Ingber's laboratory where it has been demonstrated, using ligand-coated magnetic beads to disrupt the cell surface integrins, that there is a rearrangement of the cytoskeletal filaments leading to an increase in cytoskeletal stiffness, and a distortion of the nucleus along the axis of the applied tension field (Maniotis *et al*, 1997, Wang & Ingber, 1995). Thus there is a continuum of "mechanical" information from the cell surface through to the nucleus - the integrins providing the link between the extracellular mechanical signal and the intracellular response.

From this and studies using integrin blocking antibodies (see below), the integrins appear to be important in mechano-signal transduction. This section focuses on integrin structure and recent evidence implicating integrins as mechanotransducers in different cell types.

General structure

Integrins are the primary cell surface receptors for the extracellular matrix. Most cells express more than one integrin. Integrins are involved in a range of signalling, and cytoskeletal modulating functions. They accomplish these tasks by a process of outside-in signalling, whereby ligand-occupied and clustered integrins control cell shape and the organisation of the cytoskeleton and generate a variety of biochemical signals. Integrins play an important role in tissue homeostasis, cell migration, embryogenesis, wound healing, tumour invasion and extracellular matrix assembly.

At present, 22 integrin receptors have been described. All are composed of one of 16 different α subunits noncovalently associated with one of 8 β subunits (Hynes, 1992). Integrins are functionally expressed at the cell surface and are responsible for binding to the ECM and other cells by binding to other integrins or to other cell membrane adhesion molecules. These may include the immunoglobulin superfamily members ICAM-1,-2,-3, and VCAM-1 (Marlin *et al*, 1987, Webb *et al*, 1993, Arroyo *et al*, 1994, Ferrini *et al*, 1994, Ross *et al*, 1994).

By binding to the ECM, integrins form a conduit between the extracellular environment and the cellular cytoskeleton, to which they are attached.

Integrin α subunits vary in size between 120 and 180kDa, and each non-covalently associates with a slightly smaller β subunit. Specific α–β subunit combinations determine the ligand specificity of the integrin, for example α1β1 binds to collagen and laminin and α5β1 binds to fibronectin, although these interactions are not exclusive (Table 1.1).

Integrin subunit structure is characterised by a large cytosolic glycoprotein domain, a single hydrophobic transmembrane segment, and in most cases a short cytoplasmic tail (Figure 1.3). Each subunit is thought to form tight, compact domains due to extensive disulphide bridges, which render intact integrins resistant to proteolysis. Dimerisation is mediated through the extracellular domains, to form a cleft at the top of the heterodimer in which is located the α subunit conserved sequence Asp-X-Asp-X-Asp-Gly-X-X-Asp. This is thought to be involved in the divalent cation binding (Michishita *et al*, 1993, Mould *et al*, 1995) which is essential for integrin function, both in terms of affinity and specificity.

The integrin complex exists in a series of conformational states that correlate with differences in both ligand-binding ability and signaling activity, thus depending on the underlying matrix, an integrin complex will either be activated or redundant. If an integrin becomes activated this will lead to the activation of proteins, collectively known as focal adhesion proteins (Jewell *et al*, 1995, Burridge *et al*, 1988).

	β1	β2	β3	β4	β5	β6	β7	β8
α1	✔*							
α2	✔*							
α3	✔*							
α4	✔						✔	
α5	✔*							
α6	✔			✔				
α7	✔							
α8	✔							
α9	✔							
α_L		✔						
α_M		✔						
α_X		✔						
α_D								
α_V	✔		✔*		✔	✔		
αiib			✔					
αiel							✔	

Table 1.1. Integrin-ligand binding combinations. Those showing * indicate integrins that are expressed in cardiac tissue.

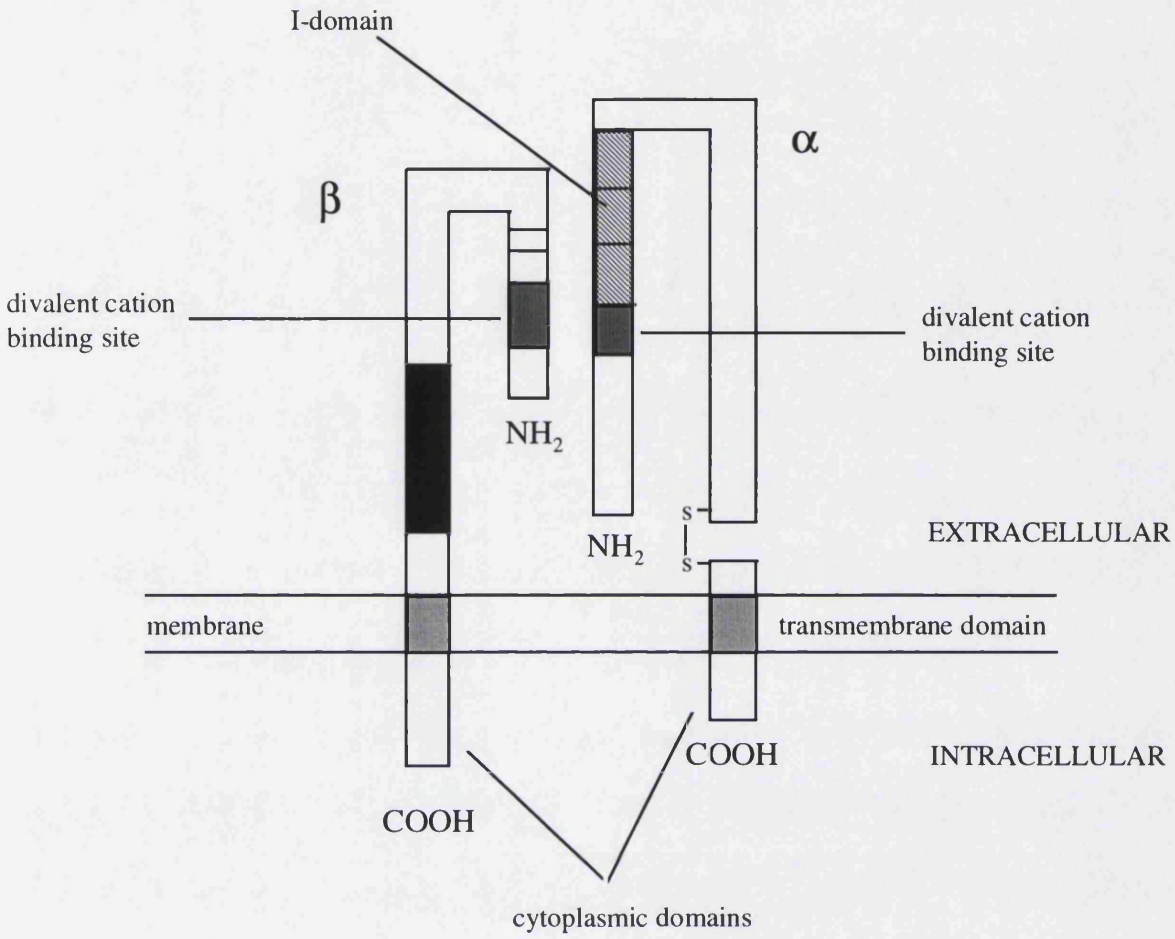


Figure 1.3. Schematic diagram of the integrin structure. Integrins comprise 2 subunits, one α and one β subunit. Both span the cell membrane. Specific motifs on the intracellular cytoplasmic domain of the β subunit act as binding sites for focal adhesion molecules, including FAK and paxillin. The I domain is involved in ligand-binding function.

Integrins function in concert with growth factors, cytokines and hormone receptors to regulate cell behaviour. The I-domain present on the α subunit is critically involved in ligand-binding function (Dickeson & Santoro, 1998, review). Ligand binding results in conformational changes in the integrins. After association with appropriate ligands, the integrins transmigrate along the plasma membrane giving rise to the association of a number of integrins. Activation of the integrins by ligand binding is associated with a structural change in the cytoplasmic domain of both subunits (Lee Jie-O *et al*, 1995). This allows the recruitment of cytoskeletal and signalling molecules to the focal adhesion complex. These include the cytoskeletal proteins talin, tensin and paxillin, and the protein kinases FAK (focal adhesion kinase), c-src and protein kinase C (PKC) which then interacts with signalling complexes which in turn can activate signal transduction cascades.

1.7.1 Integrins as mechanotransducers

Fibroblasts adhere to the cardiac ECM via matrix-specific receptors principally the integrins. Integrins form structural links between the cardiac ECM and the cytoskeleton allowing attachment, cell spreading and matrix assembly. Cell adhesion to the ECM triggers the release of lipid second messengers (McNamee *et al*, 1993), activation of protein kinases and changes in intracellular pH and Ca^{2+}, and it is this that has provided a mechanistic link between the ECM and the regulation of cell behaviour. Because integrins are the primary receptors for the ECM, they may act as mechanotransducers in the cardiovascular system. It is hypothesised that cardiac fibroblasts respond to mechanical load through integrins. There has been much speculation as to the role played by integrins in the cells "sensing" changes in mechanical load (Banes *et al*, 1995). Evidence has begun

to emerge suggesting that integrins act as mechanosignal transducers, converting physical stimuli into biochemical events. Wilson *et al* (1995) showed that addition of the antibodies to αvβ3 and αvβ5 fibronectin integrins could block the mitogenic response of neonatal rat vascular smooth muscle cells to mechanical load *in vitro*. This suggests that cells sense mechanical load through interaction of integrin receptors with a particular substrate.

Using a magnetic drag force device to apply forces on integrin receptors in an osteoblastic cell line, Schmidt and co-workers (1998) were able to induce enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. Mechanical stress was applied to the integrin subunits β1 and α2 to induce the phosphorylation. They were also able to show that MAP kinase phosphorylation was increased in response to mechanical integrin stressing. They concluded that integrins sense physical forces that control gene expression by activation of the MAP kinase pathway. Recently the integrins α4β1 and a non-α5β1 integrin have been shown to activate ERK 2 and JNK 1 in response to mechanical load in rat cardiac fibroblasts (MacKenna *et al*, 1998). Mechanical stimulation of human osteosarcoma cells produced a stimulation of expression of the β1 integrin as well as expression of integrin mRNA (Carvalho *et al*, 1995), again suggesting a role for integrins in mechanosignal transduction.

1.7.2 Integrins and cardiovascular disease

Multiple integrins are expressed in the heart and participate in a wide variety of biological processes. They are important regulators in early cardiac development *in vivo*, providing positional information for migrating and differentiating cells. In the heart, interaction of the

ECM components with the surface of cells is regulated in part by integrins. The α1- and α2- integrin subunits have been demonstrated to have an affinity for interstitial collagens, collagen type IV and laminin (Kramer *et al*, 1989, Dedhar *et al*, 1990, Languino *et al*, 1989). The α3- subunit has been shown to interact with fibronectin, laminin and collagen type IV and the interstitial collagens (Gehlsen *et al*, 1989). The α5- subunit has a specific interaction with fibronectin (Takada *et al*, 1987). The fibronectin integrins are particularly important in the assembly and development of the heart and vasculature, demonstrated using knockout mutations of fibronectin or the α5β1 and αv integrins which prevented angiogenesis and normal development of the heart (Hynes *et al*, 1997). α1β1 has been identified on cardiac fibroblasts and is important for angiotensin II-mediated contraction in collagen gels (Carver *et al*, 1995). Other integrins identified on cardiac fibroblasts include α2β1 and α3β1. Terracio *et al* (1990) showed that during cardiac development and hypertrophy there is a correlation of expression of the β1- subunit along with its associated α-chains in response to changes in pressure and volume overload.

There has been very little research on the role of integrins in disease states, with the exception of atherosclerosis. The integrin αvβ3 appears to be a critical molecule in several processes involved in atherosclerotic progression, including VSM cell migration (Brown *et al*, 1994). Immunohistochemical analysis showed an increase in expression of αvβ3 in atherosclerotic arteries (atherosclerotic plaques) versus normal arteries (nonatherosclerotic diffuse intimal thickening) (Hoshiga *et al*, 1995). More specifically, the β3 subunit is present in arteries with atherosclerosis (Veinot *et al*, 1999). It is thought this integrin may influence cell migration and adhesion.

Another role of integrins in the heart may be during cardiac remodelling. Cardiac fibroblasts have been shown to respond to angiotensin II (AII) by increasing expression of integrins (AII being implicated as a growth promoter of cardiac myocytes and fibroblasts in cardiac remodelling that occurs with cardiac hypertension, Booz & Baker, 1996). Increasing evidence suggests that integrins play an important role in cardiovascular modeling during development. In addition, the $\alpha_{11b}\beta3$ antagonists, which inhibit platelet aggregation, also inhibit the $\alpha v\beta3$ integrins. This effect is thought to play a role in the ability of $\alpha_{11b}\beta3$ antagonists to inhibit restenosis after vascular injury, thus implying a role for $\alpha v\beta3$ in vascular remodeling.

Terracio *et al* (1995) observed increased expression of collagen binding integrins during cardiac development and hypertrophy. c-Src has been implicated in playing a critical role in hypertrophic growth regulation in right ventricular pressure overloading, in association with cytoskeletal structures, possibly via load activation of integrin-mediated signalling (Kuppaswamy *et al*, 1997).

Integrins regulate gene expression *in vitro*. Signalling in monocytes via the integrin $\alpha4\beta1$ is known to regulate the production of inflammatory mediators such as IL-1b (Yurochko *et al*, 1992). While ECM proteins can influence integrin activity, integrin activation can in turn regulate components of the ECM. For example, collagen and fibronectin, through their binding to integrins, can induce the expression of metalloproteinases, gelatinase, and other enzymes that regulate the degradation of specific proteins in the ECM. Cell contact with collagen type I, mediated through the $\alpha2\beta1$ integrin, stimulates MMP-1 expression

(Piltcher *et al*, 199) in wound healing. In tumour cell progression, inhibition of the integrin α2β1 resulted in a down-regulation of MMP-9 activity and thus tumour cell invasion (Vo *et al*, 1998). α5β1 and α4β1 regulate metalloproteinase expression in synovial fibroblasts (Huhtala *et al*, 1995).

However, there is currently no evidence to suggest a link between integrins and collagen gene expression. In fact there is little information currently available at all on the signalling pathways involved in the upregulation of collagen gene expression.

1.8 INTEGRIN SIGNALLING

1.8.1 Formation and structure of the Focal Adhesion Complex

Cell attachment to the extracellular matrix leads to integrin clustering and activation (Jewell *et al*, 1995). This in turn results in the formation of molecular structures called focal adhesions (Burridge *et al* 1988). Focal adhesions are points of close apposition between the cell membrane and the ECM. The structure of focal adhesions is complex, involving integrins which physically link the ECM to the actin cytoskeleton network allowing them to transmit signals from the ECM to the cytoplasm. It is the cytoplasmic domain of integrins which link to actin bundles or stress fibres, mediated by an intricate structure comprised of focal adhesion-associated proteins. Talin and α-actinin have been shown to interact directly with the cytoplasmic domains of β integrin subunits (Tapley, 1989, Otey 1990) and this association is thought to contribute to the formation of focal adhesions and actin stress fibre organisation.

Formation of focal adhesion complexes, and the associated cytoskelatal reorganisation appears to be primarily controlled by signalling pathways in which the Rho family of small GTPases are essential (Miyamoto *et al*, 1995, Renshaw *et al*, 1996, Braga *et al*, 1997, Takaishi *et al*, 1997, Hall 1998). This family of G-protein related molecules include Rho, Rac and cdc42. Rho has been shown, in response to extracellular ligands, through a number of signalling events (Ridley *et al*, 1992a, Ridley *et al*, 1992b), to assemble actinomysin contractile filaments in 3T3 fibroblasts (Ridley *et al*, 1992a). This led to the conclusion that Rho acted as a key participant in the signalling cascade which linked the membrane receptors to the cytoskeleton. Specific inhibitors of Rho prevent the formation of integrin containing focal adhesion complexes and reorganisation of the cytoskeleton into stress fibres, as dominant-negative mutants, or microinjection of inhibitors prevents cytoskeletal re-organisation (Hall, 1994, Norman *et al*, 1994, Nobes *et al*, 1995, Lim *et al*, 1996, Machesky *et al*, 1997, Hall, 1998). Also, activation of these molecules is essential for continued maintenance of focal adhesion complexes and cytoskeletal association, inactivation leads to rapid loss of the focal adhesion complex (Ridley *et al*, 1992).

Evidence also suggests that tyrosine phosphorylation is important in the formation and organisation of focal adhesions (Kornberg *et al*, 1992). In cells transformed by the tyrosine kinase oncogene $pp60^{src}$, two focal adhesion-associated proteins, tensin and paxillin, became highly phosphorylated on tyrosine (Turner *et al*, 1990, Davies *et al*, 1991, Burridge *et al*, 1992). In normal cells, immunofluorescence analysis with antibodies to phosphotyrosine reveals prominent staining of focal adhesions indicating the presence of significant levels of tyrosine phosphorylation (Burridge *et al*, 1988).

Focal adhesions are multi-molecular superstructures, the components of which have still not been fully identified. They may be composed of FAK, Src and C-terminal Src kinase, the serine/threonine kinases, PKC, the small G proteins Ras, Rho, and Rac, protein tyrosine phosphatase 1D, PLCγ, PI 3-kinase and adaptor proteins such as paxillin and Grb2. They are essential for proper signal transduction by integrins, since integrins themselves have no endogenous catalytic activity. The integrin cytoplasmic domains must interact with catalytic molecules present in the focal adhesion complex so that they can propagate signals to the inside of the cell. Research has shown that actin is the most abundant protein in the focal adhesion complex and is associated with bridging molecules such as vinculin, tensin, and talin which are subsequently linked to the β subunit of the integrins via paxillin (Jockusch *et al*, 1995). Paxillin appears to be a further essential structural component of the focal adhesion complex, it interacts both with cytoskeletal linked proteins, focal adhesion kinase (FAK) and the integrin β subunits (Bellis *et al*, 1995, Hildebrand *et al*, Schaller *et al*, 1995) (Figure 1.4).

1.8.2 Focal Adhesion Kinase (FAK)

Autophosphorylation of FAK was one of the first integrin mediated signalling events identified (Schlaepfer *et al*, 1998). This is important in recruiting other molecules to the focal adhesion allowing further signalling events to occur. As discussed above, cell adhesion to the ECM stimulates protein tyrosine phosphorylation. Since integrins do not possess intrinsic enzymatic activities on their own, they are unable to participate in

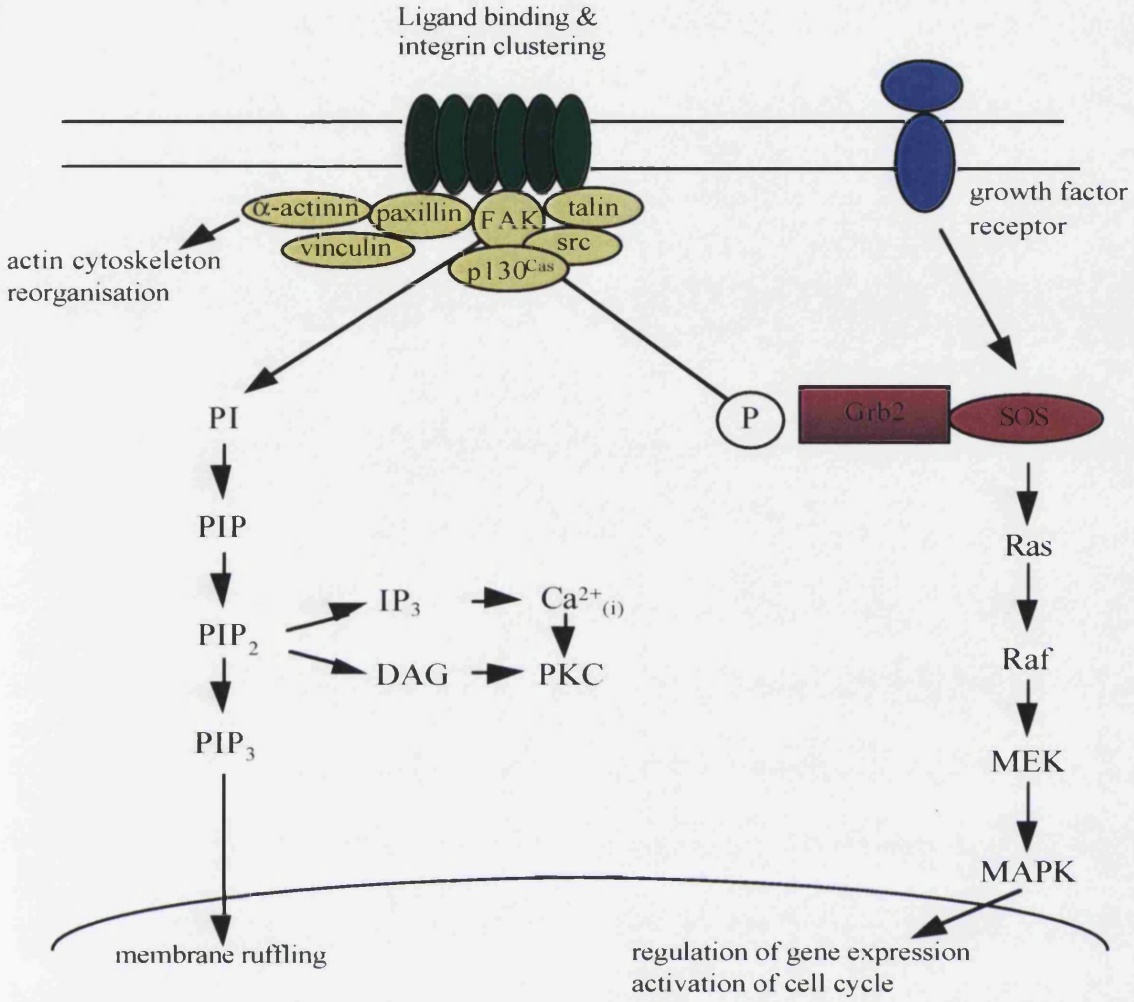


Figure 1.4. Schematic diagram showing some of the molecular events involved in integrin-mediated "outside-in" signal transduction. Integrin occupation and clustering results in the spatial organisation of a focal adhesion complex, which is composed of cytoskeletal and signalling molecules.

phosphorylation of signalling proteins. Therefore, protein tyrosine kinases such as FAK and PKC are responsible for tyrosine phosphorylation of intracellular signalling molecules.

Structure

FAK is a 125kDa nonreceptor cytoplasmic tyrosine kinase localised to focal contacts (Burridge *et al*, 1992). It can be divided into 3 domains (N-terminal, kinase, C-terminal domains), each consisting of ~ 400 amino acids (Schaller *et al*, 1992) (figure 1.5). Unlike other nonreceptor tyrosine kinases, FAK lacks the src homology 2 or 3 domains (SH2 or SH3), thus making it unique among the families of protein tyrosine kinases. However, it does contain a consensus phosphorylation site for protein kinase-C, two proline-rich stretches, a motif (YEAI) for the binding of the Src SH2 domain, a motif (YMXM) for the binding of the SH2 domain of the p85 subunit of PI 3-kinase, and a motif (YENV) for binding the Grb2 SH2 domain. Chen & Guan (1994) reported a stable association of FAK with phosphatidylinositol 3-kinase which is dependent on cell adhesion and FAK phosphorylation. They also suggest that PI 3-kinase may be a substrate for FAK. FAK is the most prominent of the cytoplasmic proteins to become tyrosine phosphorylated in response to integrin binding to ligands (Danker *et al*, 1998). Phosphorylation of FAK may occur via integrin clustering. Integrin oligomerization may lead to clustering of their cytoplasmic domains, promoting transduction of a signal from the ECM into the cell. Integrin clustering may result in clustering of FAK and subsequently autophosphorylation of FAK.

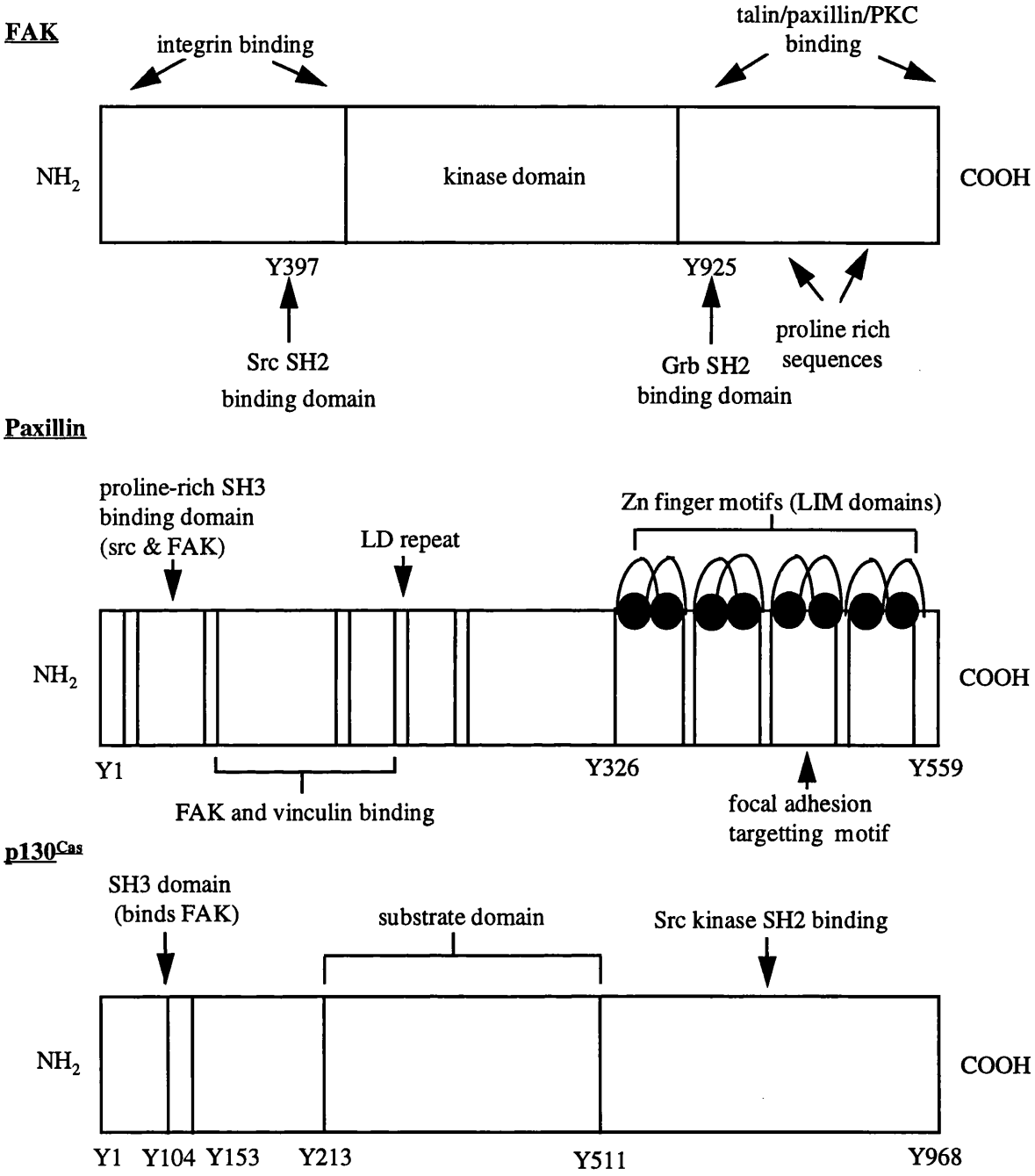


Figure 1.5. Schematic diagram of FAK, paxillin and p130^{Cas}, showing main structural domains and binding sites involved in focal adhesion complex formation and signalling.

This in turn leads to activation of signalling molecules that can bind to pp125FAK. Experimentally, integrin clustering can be mimicked by antibody-mediated cross-linking of integrins, which induces autophosphorylation of FAK (Kornberg *et al*, 1992, Kornberg *et al*, 1991). Although the mechanism for FAK activation by integrins is not well understood at the present time, it appears to involve the aggregation of FAK with integrins and other cytoskeletal proteins at focal contacts. Treatment of cells with cytochalasin D, which selectively disrupts F-actin filaments, can block FAK activation and phosphorylation induced by integrins binding to ligands (Burridge *et al*, 1992).

It is thought that tyrosine phosphorylation of FAK and other proteins may result in the creation of SH2 binding sites for other focal adhesion components, allowing their recruitment and leading to the generation of multiprotein complexes, important both structurally and for signalling from the focal adhesion (Schaller *et al*, 1994).

Mutational analysis and the use of fusion proteins has led to the identification of the functional roles of the N-and C-terminal domains of FAK. The C-terminal domain plays a role in the recruitment of FAK to the focal adhesions. Hildebrand *et al* (1993) identified a region in the C-terminus of FAK (residues 856-1012), that is essential for efficient localisation of FAK to adhesion sites. Variants of FAK that contain mutations in this region fail to localise properly in cultured fibroblasts. Further experiments determining the importance of this sequence in localisation, whereby the sequence is inserted into a cytosolic protein unrelated to FAK, allowed the protein to localise to the focal adhesions. These results are further supported by the finding that certain cells express a truncated form of FAK, called FRNK (**F**AK **r**elated **n**on-**k**inase). This protein is identical to the C-

terminus of FAK (Schaller *et al*, 1993, Richardson *et al*, 1997) but lacks a kinase domain and is therefore catalytically unreactive. However, FRNK can localise to focal adhesions. The C-terminus has also been shown to bind to two other focal adhesion proteins, paxillin and talin.

The N-terminal domain of FAK is important for the binding of FAK to integrins. Experiments have shown that FAK binds directly, through its N-terminal domain, to the cytoplasmic tail of the β1 integrin, and not via an intermediary protein or second messenger (Schaller *et al*, 1995). Other evidence suggests however that it is unlikely that integrin binding alone is sufficient to activate FAK, since the membrane-distal portion of the β1 integrin cytoplasmic tail is also required in order to upregulate FAK phosphorylation (Guan *et al*, 1991, Akiyama *et al*, 1994). It may be that the membrane-proximal region of the β1 tail is used as a docking site for FAK, but that conformational changes that occur upon integrin clustering are communicated through the membrane-distal β-integrin tail in order to activate kinase activity of FAK. Further support for a role of FAK in integrin signalling comes from its unique subcellular localisation in the focal contacts. A stretch of 159 amino acids (853-1012) in the C-terminal domain of FAK, called the FAT sequence (**F**ocal **A**dhesion **T**argetting) is responsible for its focal contact localisation (Hildebrand *et al*, 1993).

After association of FAK with the integrin β subunit a series of phosphorylation events occurs which leads to the recruitment of signalling molecules that are essential for activation of downstream signalling events. Two of these signalling molecules will be

discussed in detail below, paxillin and $p130^{Cas}$. FAK autophosphorylation is the first step required in recruiting other molecules to the focal adhesion complex. Autophosphorylation occurs on the tyrosine residue 397 (Schaller *et al*, 1994) and is essential for Src binding, through its SH2 domains, and the function of FAK in integrin signalling. This site also binds PI3-kinase. After binding to FAK, Src family kinases phosphorylate $p130^{Cas}$ leading to the association of $p130^{Cas}$ with Crk (Cas-related kinase). Furthermore, Src can phosphorylate FAK on tyrosine residues 925, creating a binding site for the Grb2/Sos complex. Sos can then function as a guanine exchange factor for Ras, which is involved in the MAP kinase pathway (Figure 1.4). Grb2 acts as an adaptor protein and the SH3 domains of Grb2 directly associates with the proline rich region in the Sos molecule. Integrins can stimulate MAPK without involvement of FAK (Lin *et al*, 1997). Firstly, a β1 integrin subunit deletion mutant affecting the FAK binding site supports activation of MAP kinase in adhering fibroblasts but not tyrosine phosphorylation of FAK. Expression of FRNK, the non-catalytic COOH terminal domain of FAK, completely blocks FAK tyrosine phosphorylation without affecting integrin-mediated activation of MAP kinase.

FAK is important in normal cell function, including cell migration, proliferation (Gilmore *et al*, 1996) and differentiation. Inhibition of focal adhesion kinase signalling in focal adhesions leads to a decrease in both motility and proliferation. Because these phenomena are all of critical importance during morphogenesis, and because FAK is expressed in embryonic cells, evidence has been accumulating to indicate that FAK may be an important modulator of developmental processes. Indeed, FAK deficient mice die early in embryogenesis (Furuta *et al*, 1995, Ilic *et al*, 1995).

1.8.3 FAK activation by non-integrin receptors

Activation of FAK can occur in the absence of integrins. FAK has recently been shown to be activated by mitogenic peptides and growth factors and a number of agents that act independently of integrins. Mitogenic peptides and growth factors such as PDGF (Abedi *et al*, 1995), lysophosphatidic acid (LPA) (Chrzanowska-Wodnicka & Burridge, 1994, Seufferlin & Rozengurt, 1994, Rodriguez-Fernandez & Rozengurt, 1998), bombesin (Zachary *et al*, 1993, Sinnett-Smith *et al*, 1993), VEGF (Abedi & Zachary, 1997) stimulate the tyrosine phosphorylation of FAK. However, since these agents also require an organised cytoskeleton in order to activate FAK, it is likely that their effects also depend on integrin occupancy. But, it is also possible that these growth factors and neuropeptides might activate FAK independently of integrins, which would imply that FAK could be a point of convergence of several distinct signalling pathways (Zachary & Rozengurt, 1992).

Integrin-mediated signalling can also occur in the absence of FAK, suggesting it may not be important in focal adhesion formation by integrins. In FAK-/- cells integrin-mediated signal transduction still occurs due to the expression of a substitute for FAK in these cells, CAKβ (cell adhesion kinase β) (Ueki *et al*, 1998)

<u>Evidence for FAK phosphorylation by mechanical load</u>

Due to their ability to form structural links between the ECM and the cytoskeleton, integrins are likely to be able to detect significant changes in the mechanical environment. Thus integrins allow the mechanical stimuli to be converted into a biochemical response within the cell. However, because they possess no catalytic activity, for the mechanical

stimulus to become converted into a biochemical signal, integrins require binding of other molecules which themselves can become phosphorylated allowing the activation of downstream signalling. FAK may provide this role, and it may therefore be involved in mechanical load induced signalling.

In vitro, FAK phosphorylation has been shown to occur in response to mechanical load. Rat mesangial cells, isolated from rat renal glomeruli, responded to a 10 min 10% membrane stretch with an increase in FAK phosphorylation (Hamasaki *et al*, 1995). In a similar study, canine airway smooth muscle cells were cultured on collagen type I coated silicone rubber culture dishes and subjected to 30 min of cyclic deformation strain (2 s of 25% deformation of the substratum, 2 s relaxation) (Smith *et al*, 1998). Strain caused a rapid increase in tyrosine phosphorylation of pp125 FAK and paxillin compared to identical non-stretched cells. Phosphorylation levels then decreased by 4 hr. Fluid shear stress ($12 dyn/cm^2$ for 5 min) applied to bovine aortic endothelial cells increased tyrosine phosphorylation and the kinase activity of FAK, compared to static controls (Li *et al*, 1997).

In tracheal smooth muscle, isometrically contracted with acetylcholine, higher levels of tyrosine phosphorylation of FAK and paxillin were observed at the optimal muscle length, than at a shorter length (Tang *et al*, 1999). FAK is also rapidly phosphorylated when rat cardiac myocytes are subjected to a pulsatile stretch (Seko *et al*, 1999). These data suggest that mechanical load increases tyrosine phosphorylation of FAK, and since FAK is a major constituent of focal adhesions, it suggests that the focal adhesion may be a site where mechanical forces are translated into biochemical events, and that FAK may play an

important role in this signalling cascade. However, to date there has been no research on mechanical load induced FAK phosphorylation in human cardiac fibroblasts.

It was alluded to earlier that formation of the focal adhesion involves many proteins, including FAK, which act as a bridge between integrins and signalling proteins. These include paxillin, $p130^{Cas}$, talin and tensin. In this section two of the main focal adhesion proteins are discussed, paxillin and $p130^{Cas}$.

1.8.4 Paxillin

Paxillin was initially purified from smooth muscle tissue. It is most abundant in muscle tissue, the highest being found in smooth muscle, but is present in lower amounts in skeletal and cardiac muscle. It is a multi-domain adaptor protein capable of interacting with several structural and signalling proteins including vinculin, FAK (Schaller & Parsons, 1995), Src and Crk. It contains binding sites for many structural and regulatory molecules (Turner & Miller, 1994). Some of these binding sites are available for interaction with their ligand only when phosphorylated. Paxillin can also directly bind to the β1 integrin subunit.

Structure

Paxillin is a 68kDa protein containing two distinct structural domains. The first comprises the amino-terminal 325 amino acids and contains binding sites for vinculin, FAK and the FAK related kinase, PYK2. Each of these binding sites contain a novel 10 amino acid repeating sequence, called the paxillin LD repeats (leucine-aspartate pairing occurs at the start of each sequence) (Figure 1.5). These sequences interact with common paxillin

binding subdomains (PBS) within vinculin and FAK (Tachibana *et al*, 1995). The amino terminal of paxillin also contains proline-rich domains that interact with the SH3 domains of Src and Crk family members (Weng, 1993). The carboxy terminus is composed of double zinc finger motifs, called LIM domains. The third LIM domain is essential for targeting the protein to focal adhesions (Brown *et al*, 1996).

Paxillin phosphorylation

Paxillin phosphorylation is prominent during rat embryo fibroblast adhesion to ECM components and is observed to occur to the same level of phosphorylation as FAK (Burridge *et al,* 1992), as well as following a similar time course of phosphorylation to that of FAK. Indeed, there have been many reports suggesting a direct association of FAK with paxillin (Tachibana *et al*, 1995, Turner *et al*, 1993, Hildebrand *et al*, 1995, Bellis *et al*, 1995, Slack, 1998). On the tyrosine residues present on FAK there is a primary site of paxillin phosphorylation. *In vitro* (Bellis *et al,* 1995) and *in vivo,* FAK is able to phosphorylate paxillin on tyrosine residues (Turner *et al*, 1993). Paxillin can also bind directly to the β1 subunit of integrins (Tanaka *et al*, 1996). Using peptides that mimick the intracellular tails of α5β1 to assay *in vitro* association with cytoskeletal proteins, it was observed that paxillin directly interacted with the intracellular region of the β1 subunit. This suggests that paxillin may function as a key intermediary in integrin-mediated signal transduction, along with FAK.

Paxillin itself recruits molecules to the plasma membrane for efficient processing of integrin- and growth factor-mediated signals derived from the extracellular environment, including

tensin and vinculin, both of which are cytosketetal proteins. Thus paxillin is important in both signal transduction from outside to inside the cell and also in cytoskeletal anchorage.

Paxillin phosphorylation by nonintegrin receptors

As with FAK, paxillin phosphorylation occurs by other receptors. Stimulation of vascular smooth muscle cells with angiotensin II and thrombin leads to paxillin phosphorylation and cytoskeletal reorganisation (Turner, 1994). These changes may regulate pathways leading to smooth muscle proliferation and migration associated with hypertension and atherosclerosis *in vivo*. Recently, insulin-like growth factor I has been shown to stimulate tyrosine phosphorylation of paxillin in Swiss 3T3 fibroblasts. VEGF, the neuropeptides bombesin and vasopressin and endothelin rapidly stimulate tyrosine phosphorylation of paxillin in both Swiss 3T3 cells and HUVECs (Zachary *et al*, 1993, Abedi & Zachary, 1997).

Evidence for paxillin phosphorylation by mechanical load

Paxillin phosphorylation, as with FAK is increased in response to mechanical load. This has been observed in airway smooth cells whereby the cells were grown on collagen type I and subjected to 30 min cyclic deformation and compared with identical cells not subjected to strain. Strain caused a rapid increase in tyrosine phosphorylation of paxillin, which remained elevated for 24 hrs (Smith *et al*, 1998). A similar result was obtained from cyclical mechanical loading of both aortic endothelial cells and human umbilical vein endothelial cells (Yano *et al*, 1996, Yano *et al*, 1997).

1.8.5 p130^{Cas}

p130^{Cas} (crk associated substrate) is a recently discovered protein that is thought to be associated with the focal adhesion complex, although its role in the focal adhesion is still not yet fully understood. p130^{Cas} was originally identified as a major tyrosine-phosphorylated protein in cells transformed by v-Crk. It was initially found to be highly tyrosine phosphorylated in Rous sarcoma virus (RSV)-transformed cells. Analysis of its functional domains has led to the hypothesis that it may act as an adaptor molecule in the focal adhesion complex.

Structure

p130^{Cas} contains an SH3 domain followed by a substrate domain, a proline-rich motif and several tyrosine residues near the C terminus (Sakai *et al*, 1994) (Figure 1.5). The SH3 domain is known to bind to FAK and FRNK (Lui *et al*, 1996). The substrate domain is rich in consensus SH2 binding sites, it has 15 potentially phosphorylated tyrosine residues, binds to v-Crk (Nakamoto *et al*, 1996) and several other proteins including Src, tensin, Abl, Grb2, PI 3-kinase and Nck. The proline rich sequence near the C terminus and Y762 provide the binding sites for the SH3 and SH2 domains of Src kinase, respectively. This data suggests that P130^{Cas} acts as an adaptor molecule, which can assemble and transmit cellular signals via interaction through the SH2 and SH3 domains of a wide variety of signalling molecules. The aforementioned molecules have been implicated in a variety of signalling pathways and their binding to p130^{Cas} could mediate the activation of these signalling pathways upon integrin dependent adhesion.

Role in the focal adhesion complex

Mutational analysis has shown that $p130^{Cas}$ is localised to the focal adhesions in 3T3 fibroblasts (Nakamoto *et al*, 1997). Its localisation to the focal adhesions occurs in part by the ability of its SH3 domain to bind to FAK, its substrate domain to bind with Abl and the Src binding domain to bind to c-Src and other Src family kinases including Fyn. Once at the focal adhesion, tyrosine phosphorylation of $p130^{Cas}$ occurs, either by FAK and/or other tyrosine kinases.

The interaction of $p130^{Cas}$ with FAK is likely to be functionally important in integrin-mediated signal transduction. *In vitro*, the SH3 domain of $p130^{Cas}$ forms a stable complex with FAK, whereas the other domains of $p130^{Cas}$ fail to associate with FAK (Harte *et al*, 1996, Polte & Hanks, 1997). More specifically it binds to the proline rich sequence P^{712} PKPSR, a typical type II SH3 consensus binding site present in the C terminus of FAK. FAK is likely to mediate $p130^{Cas}$ phosphorylation because it becomes phosphorylated upon adhesion with similar kinetics to that of $p130^{Cas}$. Tyrosine phosphorylation of $p130^{Cas}$ during cell adhesion to fibronectin indicates that the association of $p130^{Cas}$ and FAK may be functionally significant.

Further evidence for integrin-mediated $p130^{Cas}$ phosphorylation comes from adhesion studies of 3T3 fibroblasts to ECM substrates. Fibronectin, but not polylysine, and adhesion to immobilised anti-integrin antibodies results in elevated phosphotyrosine of $p130^{Cas}$ (Nijima *et al*, 1995). This tyrosine phosphorylation also coincides with tyrosine phosphorylation of FAK and requires organisation of the actin cytoskeleton (Vuori & Ruoslahti, 1995). This finding is consistent with the tyrosine phosphorylation being

mediated by integrins and suggests $p130^{Cas}$ plays a role in signalling pathways mediated by cell adhesion, and may amplify and propagate integrin-mediated signals by interacting with SH2-containing molecules, such as Grb2. Astier *et al* (1997) showed that the related adhesion focal tyrosine kinase RAFTK, which has a structure similar to FAK, is a target for β1-integrin mediated tyrosine phosphorylation in human B cells and can interact constitutively with $p130^{Cas}$. This is further supported by the observation that cell adhesion to two different anti-integrin antibodies also resulted in elevated tyrosine phosphorylation of $p130^{Cas}$. However, although FAK phosphorylation coincides with $p130^{Cas}$ phosphorylation, it remains to be seen whether tyrosine phosphorylation of $p130^{Cas}$ requires integrin-mediated FAK activation, or whether it is a result of separate, integrin-activated but FAK independent kinase pathway. $p130^{Cas}$ is however, regarded as one of the focal adhesion proteins which binds directly to FAK (Turner & Miller, 1994, Schaller & Parsons, 1995, Polte & Hanks, 1995) upon integrin:ECM interactions. Phosphorylation of $p130^{Cas}$ requires the presence of intact cytoskeleton (Nakamura *et al*, 1998), since cytochalasin D inhibits adhesion-induced phosphorylation.

<u>$p130^{Cas}$ phosphorylation by non-integrin receptors</u>

As with FAK, $p130^{Cas}$ can be phosphorylated by growth factors including bombesin, LPA, phorbol esters and PDGF (Casamassima & Rozengurt, 1997). Human growth factor (hGH) stimulates the tyrosine phosphorylation of $p130^{Cas}$ and CrkII, their association, and the association of multiple other tyrosine phosphorylated proteins to the focal adhesion complex (Zhu *et al*, 1998). $p130^{Cas}$ complexes with c-Crk in insulin-like growth factor I (IGF-I) stimulated quiescent Swiss 3T3 cells (Casamassima & Rozengurt, 1998) along with

FAK and paxillin. This complex is thought to play a novel role in IGF-I signal transduction.

To date, however, there is no evidence of $p130^{Cas}$ phosphorylation in response to mechanical load, neither in human cardiac fibroblasts or other cell types.

1.9 SIGNALLING COMPONENTS ACTIVATED BY FAK

The role of FAK in the activation of signalling pathways extends to mediating the recruitment of other signalling molecules to the focal adhesion complex. FAK is involved in communication between integrins and the nucleus by activating the mitogen-activated (MAP) kinase pathway (Schlaepfer *et al*, 1998). This occurs via recruitment of Src, which acts through the Grb2/Sos/Ras pathway to initiate the Raf/MEK/MAPK signalling pathway. Via its binding to PI 3-kinase, FAK has also been associated with the phospholipid signalling pathway (Figure 1.6).

MAP Kinase Pathway

The MAP kinase family is involved in signal transduction from a large number of growth and differentiation factors. Recent data has hinted at a connection between integrins, FAK and MAP kinase. MAP kinase is a serine/threonine protein kinase whose activity is rapidly stimulated by a number of external stimuli, including mechanical load (Yazaki *et al*, 1993), through mechanisms mediated by tyrosine-kinase receptors, non-receptor tyrosine kinases, and G protein-coupled receptors. MAP kinase is activated by phosphorylation on its threonine and tyrosine residues, a process which is carried out by a dual specificity protein

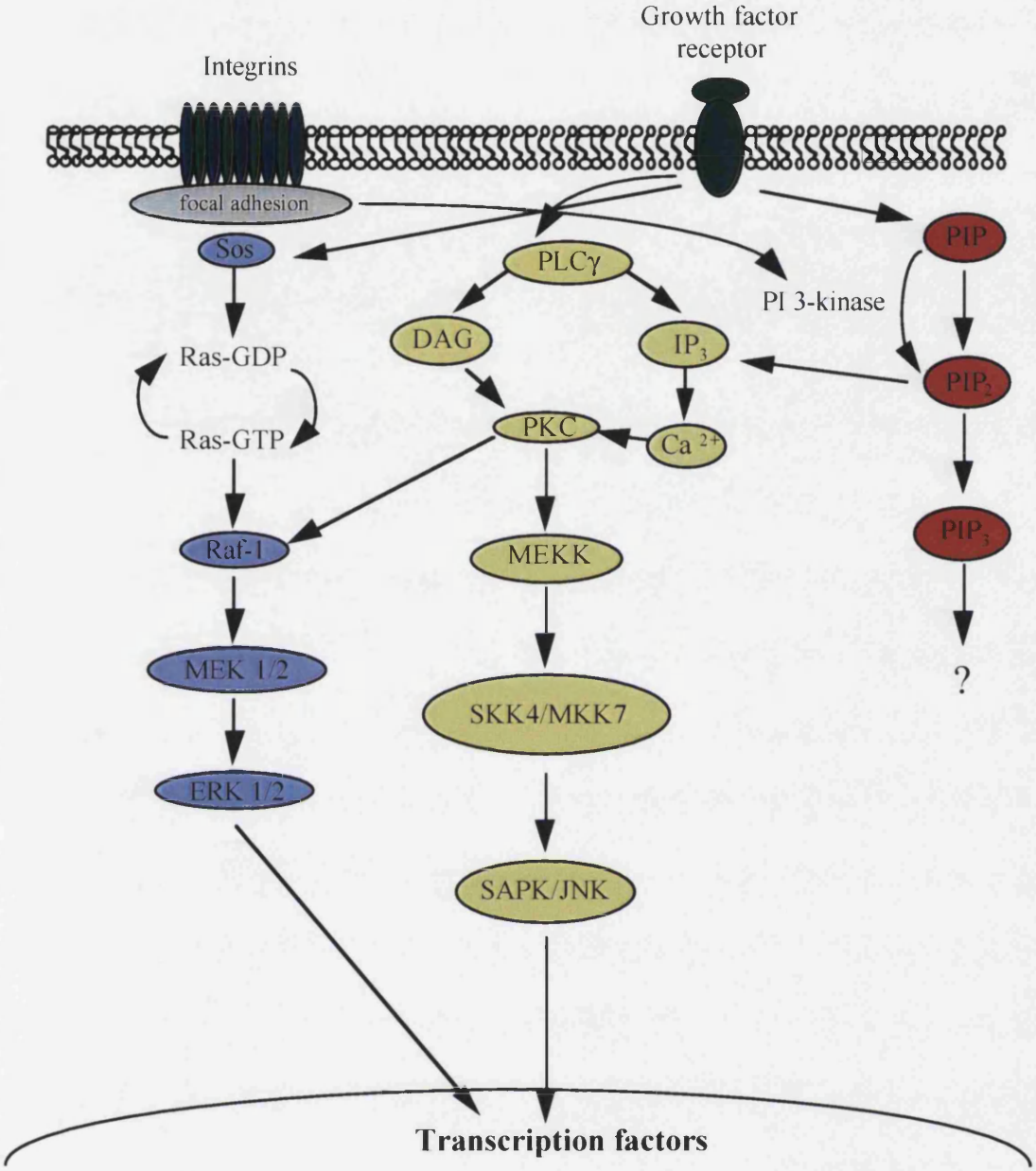


Figure 1.6 Signalling pathways activated downstream of the focal adhesion complex. Diagram also shows the pathways known to be activated by growth factor receptors.

kinase MAP kinase kinase. MAP kinases have been shown to phosphorylate and thereby activate many well studied regulatory proteins located in diverse cellular compartments, including nuclear transcriptional factors (Zhao *et al*, 1999). A function of the MAP kinases may therefore be to provide a link between transmembrane signalling and the nucleus.

Cell attachment to the ECM or growth factor receptor engagement leads to activation of the MAP kinase pathway. Adhesion of REF52 cells and 3T3 cells to either fibronectin or laminin or substrata coated with a synthetic peptide containing the RGD sequence results in the activation of MAP kinases (Chen *et al*, 1994). This activation appears to be dependent on integrin engagement rather than simply cell attachment as cells adherent to poly-D-lysine did not activate MAP kinase. Treatment of the cells with cytochalasin D blocked MAP kinase activation suggesting an important role for the cytoskeleton (Chen *et al*, 1994). A similar study using NIH-3T3 cells demonstrated that adhesion to fibronectin stimulated the activation of MAP kinases, and this was blocked by treatment with cytochalasin D (Zhu & Assoian, 1995). These data do not suggest how integrin activation leads to MAP kinase activation. Schlaepfer (1994) was however, able to co-immunoprecipitate FAK with the adaptor protein Grb2, which is thought to mediate signal transduction from membrane receptors to the Ras/MAP kinase pathway.

Phosphorylated FAK can activate MAP kinase in integrin-mediated cell adhesion (Schlaepfer *et al*, 1994). Src binding to FAK at position Y397 results in FAK phosphorylation (Figure 1.5). Phosphorylation by Src at other tyrosine residues, including Y925 which is in the consensus motif for Grb2 SH2 binding (Schlaepfer *et al*, 1994, Schlaepfer & Hunter, 1996) also leads to activation of the MAP kinase pathway. Grb2 is

an adaptor molecule that connects activated receptor tyrosine kinases to Ras/MAP kinase signalling pathways by binding to Sos, a guanine nucleotide exchange factor for Ras (Buday & Downward, 1993, Chardin *et al*, 1993, Egan *et al*, 1993). Cell adhesion to fibronectin leads to the activation of Ras (Clark & Hynes, 1996) and inhibition of Ras by a dominant-negative mutation blocks activation of MAP kinase stimulated by cell adhesion to fibronectin (Clark & Hynes, 1996). These data suggest that the association of FAK with Grb2/Sos in response to cell adhesion may lead to MAP kinase activation.

Alternatively there may be other mechanisms by which integrin signaling through FAK activates MAP kinase. This may include association of FAK with $p130^{Cas}$ and/or paxillin which leads to Ras activation by the adaptor molecule Crk. Crk has been shown to possess SH2 and SH3 domains which bind to paxillin *in vitro* (Birge *et al*, 1993). However, Chen *et al* (1996) reported that MAP kinase activation in cell adhesion was independent of Ras.

Mechanical stressing of cells activates MAP kinase in multiple cell types. In bovine aortic endothelial cells cyclic strain induces activation of ERK 1/2. Peak phosphorylation and activation of ERK 1/2 induced by 10% strain occurred at 10 min. A specific MEK inhibitor PD98059 (which acts as an upstream inhibitor of ERK 1/2 activation), inhibited their phosphorylation (Ikeda *et al*, 1999). ERK activation appeared to be independent of intracellular and extracellular calcium mobilisation but involved the activation of PKC and other tyrosine kinases. In a more recent study the same group determined the upstream members of the MAP kinase pathway activated in response to cyclical mechanical load. Cyclic strain induced a transient and rapid activation of p21ras by 1 min of strain. The PI 3-kinase inhibitors wortmannin and LY294002 inhibited strain-induced ERK 1/2 activation by

over 50% with LY294002 completely inhibiting ERK 1 activation. This suggests a possible involvement of p21ras and PI 3-kinase in the signal transduction leading to strain-induced ERK 1/2 activation. Mesangial cells cultured on type I collagen plates and exposed to a high-pressure cyclic strain for up to 120 min resulted in mesangial cell proliferation followed by activation of ERK 1/2 and p38 MAP kinases (Ingram *et al*, 1999), suggesting a role for these kinase cascades in glomerular capillary hypertension.

It has been demonstrated that acute hypertension and angioplasty rapidly induces MAP kinase activation in the arterial wall. Kinase activation is followed by an increase in c-*fos* and c-*jun* gene expression and enhanced transcription factor AP-1 DNA-binding activity. A similar MAP kinase activation can be mimicked in *in vitro* cultured smooth muscle cells stimulated by either shear stress or cyclic strain, suggesting direct effects of mechanical force on the MAP kinase signalling pathway (see Review Zou *et al*, 1998).

In response to shear stress bovine aortic endothelial cells, dual activation of ERK and JNK occurs (Li *et al*, 1997, Li *et al*, 1999). This study was able to identify a link between mechanical load, FAK and MAP kinase activation. Mutation of the phosphorylation site Tyr-397 (involved in assembly of c-src to FAK) on FAK attenuated the shear stress activation of both ERK and JNK. This suggests that FAK may be upstream of the activation of ERK, and because the mutated site is involved in the recruitment of other signalling molecules it also suggests c-src and perhaps other src family members may work in concert with FAK to regulate the downstream MAP kinases. Takahashi & Berk (1996, 1997) have also shown that shear stress activates c-src in endothelial cells. However, this study also showed that JNK can be activated by a FAK independent pathway since the

FAK mutation only partially attenuated shear stress JNK activation. Negative mutants of Ras, Rac and Cdc42 inhibits the shear stress activation of JNK. Thus, these may function together to regulate shear stress-induced JNK activation. *In vivo*, the activities of the MAP Kinases ERK 1/2, p38 and JNK are increased in myocardial ischaemia at the site of MI in Wistar rats (Shimizu *et al*, 1998). Increase in the activities of these MAP kinases was followed by enhancement of AP-1 and NF-kB DNA binding activity in areas of MI in rats. This in turn was followed by increases in increases in TGF-β, collagen I and III mRNA 1 week after MI. It is thought these signal transduction mechanisms may contribute to the myocardial ischaemia associated with MI by causing an increase expression of TGF-β mRNA and collagen types I and III in the area.

In summary, there is evidence to suggest that the MAP kinase pathway is activated by integrins, through FAK phosphorylation. Mechanical load has also been shown to activate MAP kinases resulting in collagen gene expression (Papakrivopoulou *et al*, 1999 submitted), thus MAP kinase may be a good candidate in the signaling pathway involved in mechanical load-induced stimulation of collagen synthesis.

Phospholipid Pathway

Signal transduction across cell membranes often involves the activation of components of the phospholipid pathway, including phospholipase C (PLC) and phosphatidyl inositol 3-kinase (PI 3-kinase). Activation of the phospholipid pathway often occurs in response to mitogenic factors and receptor binding.

Two signal transduction pathways utilising distinct phosphoinositide derivatives have been characterised. In one pathway, receptor ligand binding activates PLC, which in turn hydrolyses phosphatidlyinositol -4,5- bisphosphate (PIP_2) to generate the signalling molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG in turn stimulates membrane-bound phospholipid dependent and Ca^{2+}-dependent PKC, while IP_3 releases Ca^{2+} from endoplasmic reticulum stores (Putney, 1987, Ehrlich & Watras, 1988). In the other distinct pathway, phosphoinositides are phosphorylated at the D-3 position of the inositol ring by kinases to generate a family of lipid messengers, phosphatidyl inositol monophosphate (PIP), PIP_2 and phosphatidyl inositol trisphosphate (PIP_3). These are not substrates for PLC but are implicated in mitogenesis, intracellular trafficking and actin rearrangement. PIP_2 and PIP_3 are normally absent from resting cells but appear in seconds/minutes with stimulation of cells with growth factors or other cellular activators. In contrast, the concentration of PIP doesn't change substantially in response to stimulation therefore suggesting that PIP_2 and PIP_3 might function as intracellular messengers (Fry & Waterfield, 1993, Stephens *et al*, 1993).

PI 3-kinase is an enzyme required for the phosphorylation events that occur in the phospholipid signalling pathway and has also been implicated in downstream signalling events involving FAK (Chen & Guan, 1994, Rankin *et al*, 1996). PI 3-kinase forms a diverse family of lipid kinases. It consists of a p85 regulatory subunit which contains an N-terminal SH3 domain and two SH2 domains which are important in the binding of tyrosyl phosphopeptides, and a p110 catalytic subunit. The p85 subunit acts as an adaptor protein that allows the catalytic subunit to interact with growth factor receptors and tyrosine phosphorylated proteins. PI 3-kinase is found in cellular complexes with almost all ligand-

activated growth factor receptors and oncogene tyrosine kinases. It is important in mitogenesis, as cells transfected with mutant PDGF receptors that retain tyrosine kinase activity but that don't associate with or activate PI 3-kinase fail to show a mitogenic response to PDGF compared to wild type receptors (Coughlin *et al*, 1989).

FAK associates directly with PI 3-kinase by binding to the p85 subunit (Chen & Guan, 1994). PI 3-kinase activity associated with FAK occurs within the first 30 min of cell adhesion to fibronectin. FAK therefore may mediate signal transduction by integrins in a manner similar to that of receptor tyrosine kinases. Therefore integrin/ligand binding leads to tyrosine kinase activity of FAK, resulting in FAK autophosphorylation. This recruits PI 3-kinase and other signalling molecules to a multimolecular complex, possibly localised in focal contacts. Membrane translocation of PI 3-kinase and other signalling molecules including c-*src*, c-*yes* and Ras GTPase-activating protein have been observed to occur in an integrin dependent manner during platelet aggregation (Zhang *et al*, 1992). The redistribution of these signalling events could trigger downstream signalling events such as alteration of gene expression and reorganisation of cellular architecture. This stable association of FAK with PI3-kinase in NIH 3T3 mouse fibroblasts occurs through interaction of phosphotyrosine residues with SH2 domains (Chen & Guan, 1994). This association is dependent on cell adhesion and FAK autophosphorylation.

During cell adhesion, increased tyrosine phosphorylation of the p85 subunits of PI3-kinase and direct phosphorylation of p85 by FAK *in vitro* has been observed using co-immunoprecipitation studies. King *et al* (1997) demonstrated that engagement of fibronectin receptors in Cos 7 cells resulted in an accumulation of the PI 3-kinase products

PIP_2 and PIP_3. In addition PI 3-kinase was shown to be required for efficient adhesion and spreading on fibronectin as well as for activation of the MAP kinase ERK-2.

PI 3-kinase has been shown to be activated in response to different mechanical stimuli, including shear stress. An isoform of PI 3-kinase, PI 3-kinase γ shows an upregulation of activity in bovine aortic endothelial cells in response to shear stress. Addition of the PI 3-kinase inhibitor, wortmannin blocked the shear stress dependent activation of JNK (Go *et al*, 1998). This suggests a role for PI 3-kinase in mechanical load induced signalling.

Signalling pathways and cardiac hypertrophy

There is much evidence to suggest that the phospholipid signalling pathway is highly activated in the hypertrophied heart. The phospholipid pathway generates two second messengers, IP_3 and DAG, as described above. The accumulation of IP_3 and IP_4 after hormonal stimulation may play an important physiological role in the heart, but the accumulation of inositol phosphates may also be pathophysiological. Kawaguchi *et al* (1992, 1993) suggested that the phosphoinositol turnover pathways, which can be mediated by PIP_2 and DAG may play an important role in the development of hypertrophy in the hearts of rats with spontaneous hypertension.

Myocardial stretch can stimulate phosphatidylinositol turnover (von Harsdorf *et al*, 1989). The right atria of isolated rabbit hearts were labelled with ^{3}H-inositol and dilated by a volume of 75 μl for 1, 10 and 20 min. An increase in all three inositol phosphate fractions was observed after 1 min of dilatation. Larger increases were observed after 10 and 20

min. Smaller increases were also observed in other chambers of the heart after right atrial distension. It was initially thought that PI turnover in the heart was stimulated by norepinephrine and acetylcholine acting though α1-adrenoreceptors. However, addition of inhibitors did not influence PI turnover response to dilation indicating it is not mediated by these neurotransmitters. To date, no other signalling pathways have been implicated in cardiac hypertrophy.

In cultured ventricular myocytes, a variety of agonists are hypertrophic. These include agonists which couple to PLCβ (ET-1), α1-adrenergic agonists, constitutively active Gq or receptor tyrosine kinases. Mechanical stretch is hypertrophic and this may be mediated through the autocrine or other local release of AII from myocytes. Recent work on fibroblast has suggested that adhesion to ECM proteins may activate MAP Kinase through an integrin-linked pathway (Morino *et al*, 1995). This finding has implications for the hypertrophy induced by mechanical stretch. In summary, hypertrophy may be induced through Gq, then PLCβ which leads to the activation of PIP_2 and DAG and PKC. The integrin-linked pathway has not yet been studied in cardiac myocytes or fibroblasts.

The majority of research involved in activation of signalling pathways by mechanical load has been carried out in cardiomyocytes. Sadoshima *et al* (1996) have shown that signalling pathways activated in cardiomyocytes include PKC and MAP kinase. As discussed above, little is known of the pathways activated in cardiac fibroblasts.

AIMS OF THIS THESIS

Cardiac hypertrophy results from changes in the mechanical environment of the heart. An important biochemical event that is key in the development of cardiac hypertrophy is an increase in extracellular matrix deposition, mainly collagen. Although this process is well understood, the mechanisms by which cells in the heart detect changes in the mechanical environment resulting in increased collagen deposition are not well understood. In addition to this, the signalling pathways activated in response to mechanical load that lead to enhanced collagen gene expression have so far not been investigated. The aims of this thesis are therefore to understand the mechanisms of mechano-sensing by cardiac fibroblasts and the signalling processes involved in regulating collagen deposition induced by mechanical load.

The aims of this study were therefore:

- To investigate the influence of extracellular matrix composition in converting a mechanical signal into a stimulation of procollagen synthesis.
- To investigate the mechanism of mechano-signal transduction in human cardiac fibroblasts, in particular the role of integrins as mechano-sensors.
- To determine downstream signalling pathways activated by mechanical load and involved in mechanical load-induced procollagen synthesis.

The data presented here suggest that integrins are key in transducing the mechanical signal into a biochemical event. Proteins within the focal adhesion complex become phosphorylated, and in turn phosphorylate other signalling molecules thus activating signal

cascades that regulate procollagen synthesis.

2.1 MATERIALS

All chemicals were of reagent grade and purchased from BDH/Merck (Lutterworth, UK) unless otherwise stated. All solutions used for the preparation of buffers, solvents used for the preparation of HPLC buffers and solutions and HPLC columns and pre-columns were made up using ddH_2O MilliQ-grade. Sterile tissue culture plasticware was obtained from Falcon and Bibby-Sterilin, Marathon Laboratory Supplies (London, UK). Sterile cryotubes, 8-well chamber slides (Nunc), Dulbecco's Modification of Eagles Medium (DMEM), trypsin, antibiotics, L-glutamine and fetal calf serum (FCS) were all purchased from GIBCO-BRL (Paisley, Scotland, UK). The same batch of FCS was used for all the experiments described. BSA, holo-transferrin, DMSO, L-ascorbic acid, proline, 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBDCl), monoclonal mouse anti-myosin (smooth), anti-α smooth muscle actin (α-SMA) and anti-vimentin antibodies were all purchased from Sigma Chemical Company (Poole, Dorset, UK). Monoclonal mouse anti-desmin and rabbit anti-human von Willebrand Factor antibodies were purchased from Dako Ltd (High Wycombe, Bucks, UK). Type II collagenase was obtained from Lorne Laboratories (Twyford, Reading, Berkshire, UK). Carbon dioxide (CO_2) and helium gases were purchased from BOC (London, UK). Activated charcoal was purchased from Hopkins and Williams, (Essex, UK). Vacuum filtration unit and all filters were purchased from Millipore (Watford, Hertfordshire, UK). Titertek multiscanner (microtitre plate reader) was purchased from ICN Flow (High Wycombe, Bucks, UK). Sample concentrator (Savant Speedvac Plus SC1 10 AR) was obtained from Life Science International (Basingstoke, Hampshire, UK). All Western blotting apparatus was purchased from Novex Electrophoresis GmbH (Frankfurt, Germany).

CHAPTER 2: MATERIALS & METHODS

2.2 ISOLATION, CULTURE AND CHARACTERISATION OF HUMAN CARDIAC FIBROBLASTS

Transplanted human cardiac tissue was collected in cardioplegic solution (kindly donated by the Harefield Hospital, Uxbridge, Middlesex, UK) during heart transplant operations. Transplant tissue was obtained from recipient patients, with tissue samples from the left and right ventricle and left and right atrium. For each tissue sample obtained there was a brief medical history relating to the heart condition. Normal tissue samples were also obtained. Under sterile conditions, the tissue sample was minced finely and rinsed several times in media to remove traces of blood. The tissue was digested with type II collagenase (purified from *Clostridium histolyticum*) (3000 units/ml) in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and BSA (0.1 % w/v). The tissue minces were incubated in a humidified atmosphere of 10 % CO_2 in air at 37°C for 3 hrs 30 min in six-well plates. Digests were then transferred to a 50 ml centrifuge tube, spun at 220 *g* for 7 min and the supernatant discarded. The pellet was resuspended in wash solution of DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and BSA (0.1 % w/v). The cell suspension was then re-centrifuged, the supernatant discarded, cells re-suspended in 10 % FCS/DMEM containing penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (4 mM) and FCS (10 %). Cells were seeded into a 75cm^2 tissue culture flask and incubated in a humidified atmosphere of 10 % CO_2 in air at 37°C for 30 min. During this period, fibroblasts became attached to the culture flasks. The medium containing mostly non-adherent myocytes was discarded. The cells were maintained under standard tissue culture conditions, described below. Upon reaching visual confluence the cells were designated to be passage 1.

2.2.1 Routine cell culture

Cells were routinely grown in $75cm^2$ tissue culture flasks in DMEM/10 % FCS. The cultures were incubated in a humidified atmosphere of 10 % CO_2 in air at 37°C. Each cell line was tested every 3 months for mycoplasma contamination using a commercially available mycoplasma detection kit. Culture medium was changed every four days and cells were passaged 1:2 or 1:4 upon reaching visual confluence.

Cell passage was performed by removing the culture medium, washing once with 10 ml PBS, and adding 2 ml trypsin/EDTA (trypsin 0.05 % w/v, EDTA 0.02% w/v) to the cell layer. The cells were then incubated at 37°C until detachment from the tissue culture plasticware occurred. Detachment from the plasticware was observed under an inverted light microscope (Axioscop 20, Carl Zeiss, Germany). 8 ml DMEM/10 % FCS was then added to the flask to inactivate the trypsin. The cell suspension was transferred to a 50 ml sterile tube and centrifuged at 220 *g* for 5 min at 24° C. The supernatant was then discarded and the cell pellet agitated to form a suspension again. 10 ml 10% FCS/DMEM was added to the cell suspension and mixed thoroughly. 5 ml (1:2 passage) or 2.5 ml (1:4 passage) of this suspension was added to fresh culture flasks. The volume was made up to 12 ml by further addition of 10 % FCS/DMEM.

2.2.2 Storage of primary cell cultures

Freezing of cells

Primary cultures were frozen for storage and thawed as needed. All fibroblasts for storage were trypsinised by addition of 2 ml trypsin/EDTA to the cell layer in a humidified chamber

of 10 % CO_2 at 37°C until the fibroblasts had detached from the tissue culture flask. The trypsinisation reaction was quenched by addition of 8 ml 20 % FCS/DMEM to each flask. The contents from each flask was transferred to a sterile 50 ml centrifuge tube and centrifuged at 220 *g* for 5 min. The supernatant was discarded and the cell pellet gently agitated to produce a cell suspension. To each tube containing cell suspension an equal volume of DMEM/20 % FCS containing 1 % DMSO was added dropwise to the cells. A 1.5 ml aliquot of this mixture, representing one half of a confluent flask, was added to sterile cryotubes and frozen overnight at -70°C in a polystyrene container packed with paper towels to permit slow freezing. Tubes were then removed and stored in liquid nitrogen.

Thawing of cells

A single tube of cells was removed from the liquid nitrogen and thawed rapidly at 37°C in a water bath. The contents were transferred to a sterile 50 ml centrifuge tube. 8 ml DMEM/20 % FCS was added dropwise to the cell suspension with continual agitation. The cells were then spun down at 220 *g* for 5 min, the supernatant discarded, the cells re-suspended in 10 ml DMEM/10 % FCS and seeded into a 75cm^2 culture flask. Routine cell culture was then carried out as described above. Passage numbers were increased as appropriate to the thawed cells.

2.2.3 Immunofluorescent characterisation of primary fibroblast cultures

The fibroblastic nature of primary cells was determined by immunofluorescent staining of

cell layers with various antibodies to cytoskeletal and surface proteins. At confluence cells were trypsinised as described above and plated into 8-well chamber slides at a density of 10, 000 cells per well in 10 % FCS/DMEM and incubated until reaching 70-80 % confluency. Cultures were washed twice with PBS, fixed in cold (-20°C) methanol at -20°C for 30 min (cytoskeletal proteins) or paraformaldehyde (4 % w/v) (surface proteins) at room temperature for 3 min. Cells were then washed three times with PBS and stored at 4°C in PBS prior to staining. Non-specific binding was blocked with non-antigenic serum (which was prepared from the species in which the secondary antibody was made). The cells were then incubated with individual primary antibodies diluted in PBS/2 % BSA for 45 min at room temperature: anti-vimentin (1:40, clone V9), anti-αSMA (1:1000, clone 1A4), anti-desmin (1:100, clone DE-R-11). anti-myosin (1:1000, clone hSM-V), anti-von Willebrand Factor (1:10, 000) and cytokeratin (1:100, clone LP34). Cells were subsequently incubated with a secondary antibody conjugated to the fluorochrome FITC (against the animal to which the primary antibody was raised) for 60 min at room temperature. Cells were washed twice in PBS-T (Tween 20, 0.1% v / v) for 5 min and once in PBS for 5 min and mounted with Immun-Mount containing anti-fade. Cells were visualised under an inverted microscope (Axioscop, Germany). Positive control cells were also set up to confirm staining obtained with the human cardiac fibroblasts. These controls included: rat smooth muscle cells (A10s) (positive control for α smooth muscle actin) and epithelial cells (MDCKs) (positive control for cytokeratin).

2.3 MECHANICAL LOADING OF FIBROBLASTS

The loading device

Fibroblasts were mechanically loaded using the Flexercell FX3000™ cell straining device, a computer-controlled system that applies a defined mechanical load to adherent cell monolayers (Figure 2.1). The negative pressure applied beneath the culture plates, via a vacuum pump, is monitored by a pressure transducer and permits equivalent levels of pressure to be applied in each experiment. Eight 6-well plates fit into the manifold, allowing simultaneous assessment of replicate samples and the direct comparison of different conditions in a single experiment.

The Flexercell FX3000™ cell straining device works by applying a regulated strain to cultured cells by growing them on an elastic surface and deforming the surface with negative pressure. Deformation of the surface sets up a strain gradient across the membrane. Radial and circumferencial strain measurements indicate low strain in the well centre and maximum strain at the well periphery (Figure 2.2).

Plating of cells

For all loading experiments (unless otherwise stated) cells were grown to confluency in 75cm^2 tissue culture flasks, and incubated in a humidified atmosphere of CO_2 at 37°C with trypsin/EDTA until the cells detached. 8 ml of 10 % FCS/DMEM was added to the culture flask to stop the trypsinisation. The medium was transferred to a 50 ml tube and centrifuged at 220 *g* for 5 min.

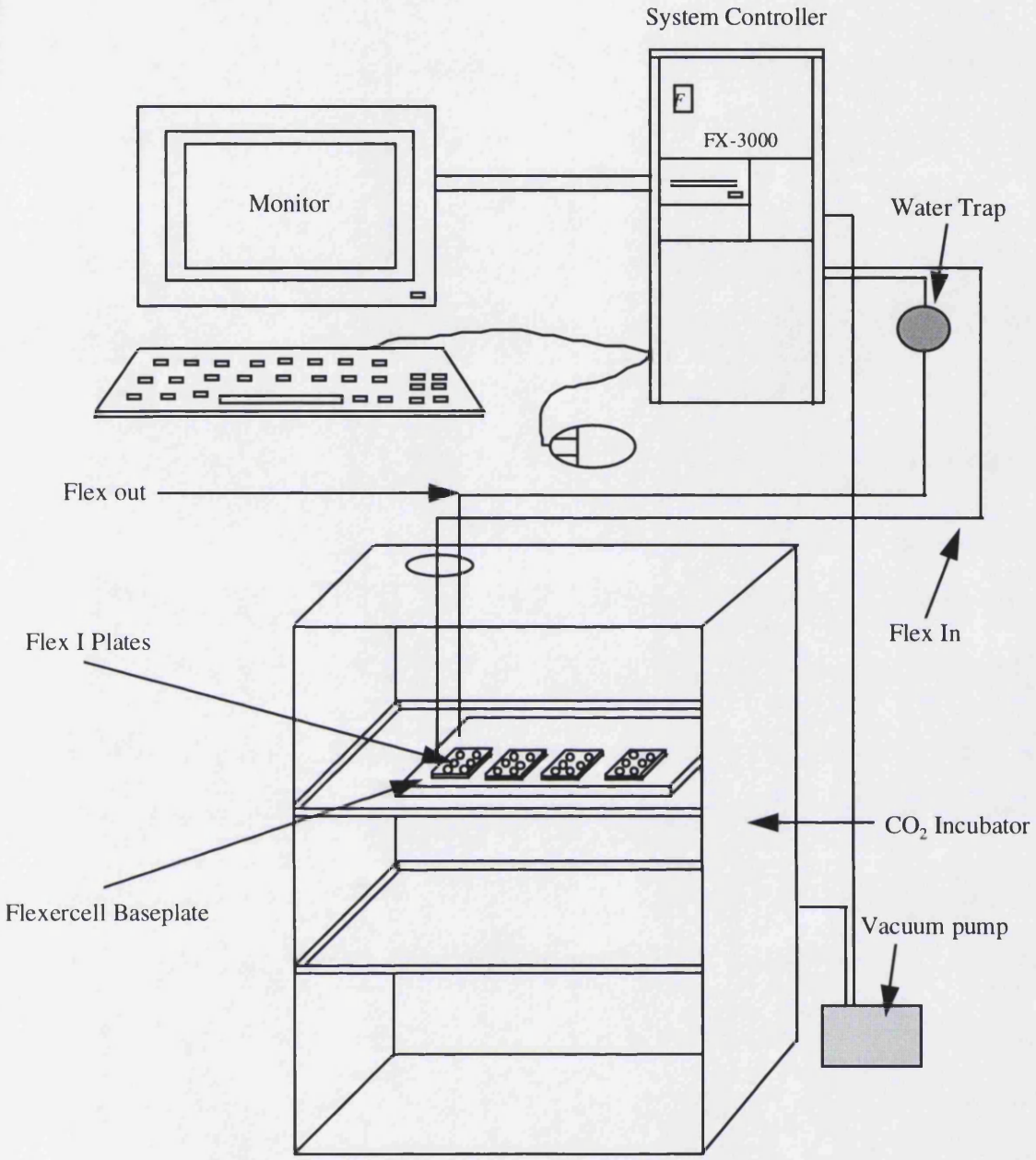


Figure 2.1. Schematic diagram showing the Flexercell Strain Unit (FX-3000) set-up.

The Strain Unit comprises a computer which controls the level of application of strain to the Flex I® plates, a baseplate which sits in the incubator and a vacuum pump.

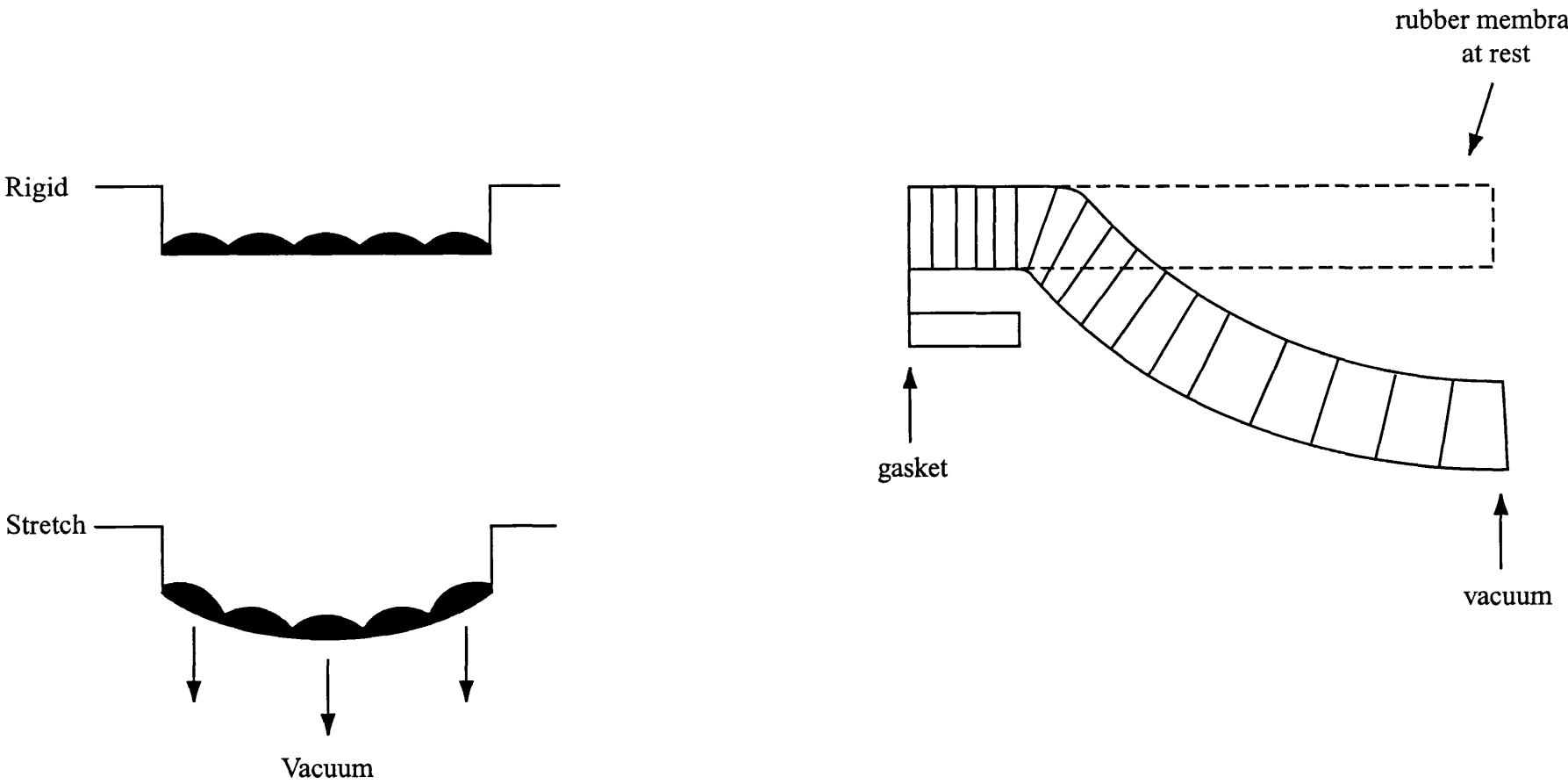


Figure 2.2. Schematic diagram showing the vacuum induced cycling of a Flex I® culture plate

The supernatant was discarded and the cell pellet resuspended in 10 ml 10 % FCS/DMEM. From this cell suspension, 10 µl was removed and the number of cells counted using a hemocytometer. From this, the volume of cell suspension needed for each experiment was calculated and the cell suspension was diluted to give a cell density of $5x10^4$ cells/ml.

For each experiment cells were seeded in 2 ml 10 % FCS/DMEM at a density of $1x10^5$ cells/well into collagen, elastin or fibronectin pre-coated silicone elastomer-bottomed 6-well culture plates. The plates were incubated in a humidified atmosphere of CO_2 at 37°C until reaching visual confluency. Cells were then quiesced for 24 hrs before the application of mechanical load.

2.3.1 Application of load

The cyclic mechanical load applied to each plate was a 20 % maximum radial strain at a frequency of 1.5 Hz. Cells were mechanically loaded using this regime in order to reproduce the levels of strain cells experience *in vivo* in the cardiovascular system (Winlove *et al*, 1995). Cyclical strain regimes have been used as they represent the effect of the beating heart. For static load experiments, cells were loaded into the manifold and stretched with a constant radial strain of 20 % introduced over a period of up to 1 hr. Static load was applied immediately to the plates. Physiologically static load may not exist within the cardiovascular system, however this type of load was used to determine if data obtained from measurements of phosphorylation levels in response to a cyclic mechanical load, were due to the nature of the load applied *in vitro*.

2.4 FIBROBLAST PROCOLLAGEN METABOLISM EXPERIMENTS

2.4.1 Principle of the assay

Procollagen metabolism by cultured fibroblasts was assessed by measuring hydroxyproline in protein (OHpro) in both the cell layer and medium using an HPLC method previously developed in this laboratory (Campa *et al*, 1990, McAnulty *et al*, 1991). The principle steps involved in measuring procollagen metabolism are given in detail below.

OHpro represents approximately 12 % of the primary sequence of procollagen (Laurent *et al*, 1981) and is essential for the formation of the collagen triple helix. This imino acid, however, is not present to a significant level in any other protein with the exception of elastin (2 % OHpro), C1q component of complement (4.3 % OHpro, Reid, 1979), acetylcholinesterase (5 % OHpro, Mays & Rosenberry, 1981), surfactant apolipoproteins A and D (Hawgood, 1989). These proteins have not been demonstrated to be produced in significant amounts by isolated fibroblasts in culture and therefore measuring OHpro content from the fibroblast cultures *in vitro* is an excellent determinant of procollagen synthesis.

2.4.2 Cell preparation for procollagen measurements

Cells were seeded onto collagen, elastin and fibronectin plates as previously stated. Plates were incubated until they became visually confluent (4-5 days). Medium was then replaced with 1 ml DMEM supplemented with 50 μg/ml ascorbic acid (ascorbate), 0.2 mM proline, BSA (1 mg/ml) and transferrin (1 μg/ml), penicillin and streptomycin (100 μg/ml) and glutamine (4 mM) (described collectively as preincubation medium). Absence of serum

leads to quiescence of fibroblasts, proline, ascorbate and glutamine provide suitable conditions for the synthesis of collagen. After 24 hrs the medium was replaced with identical medium or medium containing 10 % FCS. The plates were then loaded onto the Flexercell FX3000™ and stretched for 24 and 48 hrs, with the media being supplemented with ascorbic acid (10 µl of 500 µg/ml) after the first 24 hrs to the remaining plates to maintain the linear kinetics of procollagen synthesis. Ascorbic acid is essential for collagen synthesis. Ascorbate is a cofactor for the enzymatic activity of prolyl hydroxylase which hydroxylates prolyl residues in procollagen. Ascorbate in low concentrations is essential for production of collagen, since a minimum of 35% of the prolyl residues in collagen need to be hydroxylated for the collagen molecule to maintain its triple-helical formation (Steinmann *et al*, 1981) at physiologic temperatures. Incubation of cells in the absence of ascorbate leads to underhydroxylation of procollagen. Proline is required in the culture medium for the hydroxylation of proline residues to form hydroxyproline. Transferrin acts as a cofactor and is required by the enzymes prolyl 4-hydroxylase and lysyl hydroxylase for the hydroxylation of hydroxyproline and hydroxylysine. Glutamine is an essential amino acid required for the normal growth of cells.

Identical plates were set up as unloaded controls. After 24 and 48 hrs of mechanical load, the plates were frozen at -20°C for 24 hrs, then thawed and the cell layer from each well scraped into the medium. The medium and cell layer from individual wells was transferred into separate 7 ml Sterilin tubes. Each well was further washed with 1 ml PBS to remove remaining protein and added to the corresponding tube. All proteins were precipitated by addition of absolute ethanol to a final concentration of 67 % to each tube and left at 4°C

overnight. Proteins were separated from free amino acids and small peptides (<3 amino acids) by filtration through a 0.45 μm pore filter (Millipore, Watford, UK) using a vacuum unit. The protein on the filter was washed with 2 ml 67% ethanol. The filter was hydrolysed in 6 M HCl at 110°C for 16 hrs. Hydrolysates were mixed with a small spatula-load of charcoal (to decolourise the solution), and filtered through a 0.66 μM pore filter. 100 μl of each hydrolysate was aliquoted into a 1.5 ml eppendorf tube and evaporated to dryness under vacuum on a sample concentrator prior to analysis by reverse phase high pressure liquid chromatography (HPLC).

OHpro was isolated and measured by reverse phase HPLC of 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) derivitized hydrolysates (Campa *et al*, 1992). Each dried sample was re-dissolved in 100 μl HPLC grade water, buffered with 100 μl 0.4 M potassium tetraborate (pH 9.5) and reacted with 100 μl NBD-Cl in methanol, incubated in the dark at 37°C for 20 min. 50 μl of 1.5 M HCl was added to stop the reaction and 150 μl 167 mM sodium acetate (FSA, Loughborough, UK) in acetonitrile (26 % v/v) was then added to the samples to make them compatible with the initial running buffer. Samples were filtered (Millipore, type GV, pore size 0.22 μm) and a 100 μl aliquot loaded onto the HPLC column and eluted with an acetonitrile gradient described below.

Instrumentation and chromatographic conditions

Derivitised samples were separated on a Beckman System Gold HPLC (Beckman, High Wycombe, UK) with a reverse phase cartridge column (LiChroCART LiChrospher 250 x 4 mm, 5 μm particle size, 100RP-18) protected by a directly coupled pre-column

(LiChrosorb, 4 x 4 mm, 5 μm particle size, 100RP-18). The columns were continuously maintained at 40°C in a heated column oven. At the beginning of each day, buffers were degassed with helium and the HPLC equilibrated in running buffer A (Table 2.1) for 40 min. The first two samples run each day were OHpro standard solutions (equivalent to 50 pmol in the peak) used in the quantitation of the samples.

NBDCl derivatives in samples and standards were eluted with an acetonitrile gradient, increasing the concentration of buffer B (Table 2.1) over time. The chromatographic conditions employed are summarised. Post-column detection was achieved by monitoring absorbance at 495 nm using a flow-through variable wavelength monitor and the signal was processed using an on-line chromatography computing integrator (Beckman Systems Gold Integrator) for quantitative analysis. A typical HPLC chromatogram obtained is shown (Figure 2.3). OHpro elutes from the column at ~6 min after injection. The remaining amino acid derivatives in the sample were eluted as the proportion of the organic buffer (acetonitrile) was increased. The total running and column regeneration time was 25 min.

Calculation for measuring procollagen synthesis

The OHpro content in each sample was determined by comparing the peak area of the sample obtained on a chromatogram to those generated from standard solutions, derivitized and separated under identical conditions. All values obtained for EtOH-insoluble fractions were corrected for the amount of OHpro in the protein-bound OHpro measured in the cell layer and the culture medium at the onset of the incubation period by subtracting the EtOH-insoluble fraction of the t0 sample, with the t0 sample representing the level of procollagen synthesised before the application of mechanical load.

BUFFER A	BUFFER B
160 ml acetonitrile	750 ml acetonitrile
1840 ml ddH_2O	250 ml ddH_2O
13. 13.6g Na acetate (3-hydrate) pH 6.7 with orthophosphoric acid	

TIME (min)	BUFFER A	BUFFER B
0.00	100%	0%
5.00	95%	5%
12.50	20%	80%
24.75	100%	0%
25.00	100%	00%

Table 2.1. The top table shows the composition of the HPLC buffers A and B. The bottom table shows changes in the acetonitrile gradient required to elute the samples.

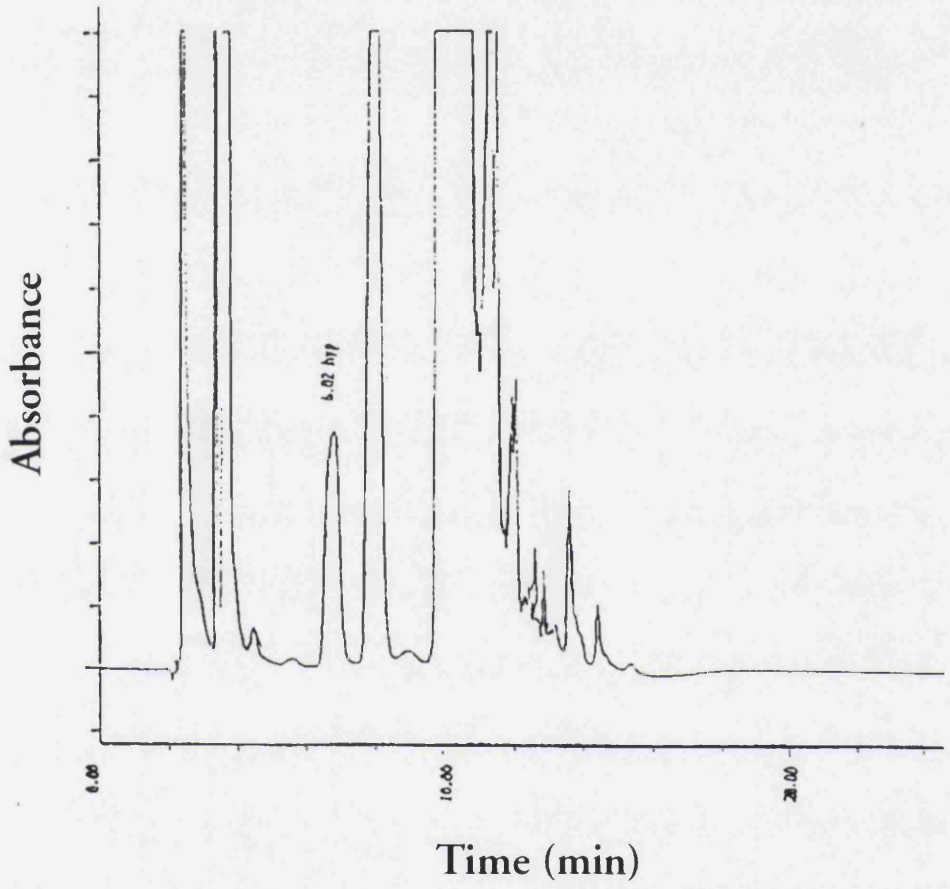


Figure 2.3. Typical chromatogram obtained in the separation and measurement of OHpro.

Calculation for measuring procollagen synthesis

$$\frac{\text{total µl acid}}{\text{volume aliquot dried}} \times \frac{\text{500 µl reaction mix}}{\text{100 µl loaded on column}} \times \text{HPLC number} \times \frac{1}{1000} = \text{nmols OHpro}$$

to correct for cell number:

$$\frac{\text{nmols OHpro}}{\text{cell number}} \times 10^4$$

These values represent procollagen synthesised over the 24 or 48 hr incubation period. Parallel identical cultures were included to obtain cell counts in order to calculate procollagen synthesis per cell.

Setting up cultures for obtaining cell counts

For all collagen synthesis experiments parallel identical plates were set up to obtain cell counts, including a t0 plate representing cell number before the application of mechanical load. The medium from each well was discarded and the wells washed twice with PBS. 300 µl trypsin/EDTA was added to each well and the plates were incubated in a humidified chamber at 37°C until detachment of the cells from the silicone-elastomer membrane. The reaction was stopped by addition of an equal volume of 10 % FCS/DMEM to each well. The cell suspension was passed through a pipette several times to ensure a single suspension of cells. Cell suspension from each well was transferred to separate tissue culture 13 ml Sterilin tubes. From this, 10 µl of cell suspension was removed and the cell number was

measured using a hemocytometer (BDH, UK). Thus, for each plate six cell counts were obtained. The average number of cells per plate was calculated by taking the mean of the cell counts from the six wells, multiplying by the dilution factor (x 0.6). Data is expressed as procollagen synthesis with units of nmols OHpro/10^5 cells.

Effect of soluble fibronectin on procollagen metabolism

To answer the question of the importance of the cell:matrix interaction in mechanical load induced procollagen synthesis, an attempt was made to block the fibronectin integrin:matrix interactions. This was carried out by the addition of soluble fibronectin to the culture media. This method has previously been used by Wilson *et al* (1995) to determine the involvement of fibronectin integrins in VSM cell mitogenic response to mechanical load. Cells were plated as described above on collagen, elastin or fibronectin coated silicone-elastomer Flex I® plates. 2 hrs before the application of mechanical load individual wells were incubated with soluble fibronectin (25 μg/ml). Plates were mechanically loaded for 48 hrs in incubation medium, and frozen prior to preparation for procollagen synthesis measurements. Identical plates were set up as rigid controls and for cell counts of all conditions.

Effect of integrin blocking antibodies on procollagen metabolism

To determine the effect of blocking integrin antibodies on procollagen synthesis in response to mechanical load, cells were seeded onto Flex I® plates in 2 ml 10 % FCS/DMEM per well at a density of 1 x 10^5 cells/well. Cells were incubated in a humidified atmosphere of 10 % CO_2 in air at 37°C until reaching visual confluence. The media was removed and

replaced with incubation medium. Plates were incubated for a further 24 hrs. This medium was then replaced with identical medium or medium containing 10% FCS. One hour prior to the application of mechanical load individual wells were incubated with the integrin blocking antibodies anti-$\alpha 5\beta 1$, (1:100 - 1:1000), anti- $\alpha v\beta 3$ (1:500) and anti- $\alpha v\beta 1$ (1:500). The plates were then loaded for 24 or 48 hrs. After the application of mechanical load, plates were frozen prior to cell preparation for procollagen synthesis measurements. Procollagen was measured as described above. Identical plates were set up for unloaded controls and for cell counts of all conditions.

Effect of PI 3-kinase inhibitors on procollagen metabolism

Cells were seeded onto fibronectin coated Flex I® plates as described in "Mechanical Loading of Cells." One hour prior to the application of mechanical load two phosphatidylinositol-3 kinase (PI 3-kinase) inhibitors, wortmannin (used at two doses of 100 nM or 1 μM) and LY 294002 (10 μM) or vehicle (DMSO) were added to individual wells. The higher dose of wortmannin used in these experiments has, in the literature, been shown to selectively inhibit PI 4-kinase and not PI 3-kinase (Meyers & Cantley, 1997, Balla *et al*, 1997). Plates were mechanically loaded for 24 hrs in serum free medium, frozen and prepared for procollagen measurements. Identical plates were set up for unloaded controls and for cell counts of all conditions. Due to the unstable nature of wortmannin in culture, the compound was added every half an hour.

Effect of tyrphostin 25 on procollagen metabolism

Tyrphostin 25 is a tyrosine kinase inhibitor that prevents the phosphorylation of signalling

molecules on tyrosine residues. This renders the molecule inactive, preventing it from phosphorylating other downstream signalling molecules. Tyrphostin 25 in a non-specific tyrosine kinase inhibitor, but has been used to inhibit FAK phosphorylation (Yano *et al*, 1996). Cells were seeded onto fibronectin coated silicone elastomer Flex I® plates in 10 % FCS/DMEM. Upon reaching visual confluence cells were quiesced in incubation medium for 24 hrs. The medium was replaced with identical medium and individual wells were supplemented with different concentrations of the tyrosine kinase inhibitor tyrphostin 25 (5 μM-20 μM) or vehicle (DMSO). All plates were incubated in a humidified atmosphere of 10 % CO_2 at 37°C for 4 hrs prior to the application of mechanical load. After the 4 hr preincubation period, plates were loaded for 24 hrs, and frozen prior to the preparation for procollagen synthesis measurements. Identical plates were set up for unloaded controls and for cell counts of all conditions.

Effect of cytochalasin D on procollagen metabolism

Cells were seeded onto fibronectin coated silicone elastomer Flex I® plates in 10 % FCS/DMEM. Upon reaching visual confluence cells were quiesced in incubation medium for 24 hrs. The medium was replaced with identical medium and individual wells were supplemented with cytochalasin D (0.1 μM - 4 μM) which disrupts the actin cytoskeleton, or vehicle (DMSO). The plates were then incubated in a humidified atmosphere of 10 % CO_2 at 37°C for 2 hrs prior to the application of load. Plates were mechanically loaded for 24 hrs, and frozen prior to the preparation for procollagen synthesis measurements. Identical plates were set up for unloaded controls and for cell counts of all conditions.

2.4.3 Assessment of cytochalasin D and tyrphostin 25 on cell viability

Due to the high toxicity of cytochalasin D, a cell viability assay - the lactate dehydrogenase assay - was carried out to determine the effect of this compound on cell viability. This assay was also carried out on tyrphostin 25 treated cells.

Principle behind the assay

The CytoTox 96® Cytotoxicity Assay (Promega, Southampton, UK) is a colourimetric assay that quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability, thus release of LDH is an accurate test for cytotoxicity. Released LDH in culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt (INT) into a red formazan product, with the LDH acting as the catalyst for the reaction.

Cell plating and mechanical loading

Cells were plated as described in section 2.3 - "Plating of cells". Upon reaching visual confluency the medium was removed and replaced with pre-incubation medium for 24 hrs. This was removed and replaced with identical medium +/- cytochalasin D (4 μM) or +/- DMSO (vehicle) and either mechanically loaded for up to 24 hrs or left as rigid controls. To determine the cytotoxic effect of tyrphostin 25 on fibroblasts, plates were set up as for collagen metabolism experiments, the media replaced with pre-incubation medium for 24

hrs. This was removed and replaced with identical medium +/- tyrphostin 25 (20 μM) or +/- DMSO and incubated for 2 hrs prior to the application of mechanical load. Control plates were also set up according to the assay instructions. These included: a control to adjust for the presence of phenol red in the tissue culture medium, and a control to adjust for the presence of any endogenous LDH activity arising from animal serum supplements to the tissue culture medium.

Preparation of samples for the assay

After the application of load the medium was aspirated from each well of one plate, pooled and stored at 4°C. The cell monolayer was washed once with ice-cold PBS and aspirated. 1 ml of lysis buffer (0.05% Triton X-100 in PBS) was added to each well and the cell layer was scraped into the lysis buffer. The lysates from each well of one plate were collected and pooled and stored at 4°C prior to carrying out the assay.

Assay procedure

50 μl of medium or cell lysate from each plate was added to one well of a 96-well plate. 50 μl of reconstituted substrate was added to each well. The plate was covered with foil and incubated for 30 min at room temperature. 50 μl of stop solution (1M acetic acid) was then added to each well and the absorbance measured at 492nm with a plate reader (ICN Flow, High Wycombe, Bucks, UK). Values obtained were subtracted from the control values thus adjusting for the presence of phenol red in the culture medium. Values were expressed as a percentage of LDH release.

2.5 FIBROBLAST REPLICATION EXPERIMENTS

Principle of the assay

Fibroblast replication in response to the presence or absence of serum, mechanical load and adhesion to the extracellular matrix proteins was assessed by measuring incorporation of radiolabelled thymidine into cell nuclei. The assay works by incorporating the radiolabelled thymidine in DNA newly synthesised by the cells. The assay is very sensitive, and because radioactivity is added only 4 hrs prior to the end of the experiment, it only measures newly synthesised DNA.

Cell plating and incubation period

Human cardiac fibroblasts were grown to confluency in 75cm^2 tissue culture flasks, trypsinised and reseeded onto collagen, elastin, and fibronectin coated Flex I® plates at a density of 1x10^5 cells in 2 ml 10 % FCS/DMEM for 24 hrs. The plates were incubated in a humidified atmosphere of 10 % CO_2 in air at 37°C. After 24 hrs, the medium was removed and replaced with 1 ml serum free DMEM containing 50 μg/ml ascorbic acid, 0.2 mM proline, BSA (1 mg/ml) and transferrin (1 μg/ml) to quiesce the cells. After 3 days the medium was removed and replaced with identical medium +/- 10 % FCS, +/- human plasma fibronectin (25 μg/ml). The cells were mechanically loaded or left unloaded as rigid controls for 24-96 hrs.

Radiolabelling cells and precipitation of proteins

^{3}H-methylthymidine was added to each well to a final concentration of 1 μCi/ml 4 hrs prior to ending the experiment. At the end of the experiment the medium was removed from the

plates and each well washed extensively with ice cold PBS. 1 ml of 10 % trichloroacetic acid (TCA) was added to each well, into which the cell layer was scraped. BSA (100 µl, 10 mg/ml) was also added to each well to act as a carrier of the precipitated protein. The TCA from each well was removed and transferred to 1.5 ml eppendorfs. The eppendorfs were centrifuged at 13 000 *g* for 2 min and the supernatant aspirated. The pellet was re-dissolved in 0.5 ml 0.5 M NaOH and a 250 µl aliquot was added to 3 ml scintillation fluid in scintillation vials and counted for 10 min using a beta-counter (Minaxi β 4000 series, United Technologies, Downers Grove, IL, USA). Incorporation of ^{3}H-methylthymidine was expressed as disintegrations per minute (dpm)/well.

2.6 ANALYSIS OF PROTEIN PHOSPHORYLATION IN RESPONSE TO MECHANICAL LOAD

Principle behind the technique

Western blotting allows the separation of proteins of different molecular weights by gel electrophoresis. Electrophoretically separated components are transferred from a gel to a solid support and probed with antibodies that react with specific antigenic epitopes displayed by the target protein attached to the solid support. Western blotting is extremely useful for the identification and quantitation of specific proteins in complex mixtures of proteins.

Cell plating and mechanical loading

Confluent cultures of human cardiac fibroblasts were detached from the tissue culture flask by addition of 2 ml trypsin/EDTA to the cell monolayer. Detached cells were resuspended

in 8 ml 10 % FCS/DMEM and centrifuged at 220 *g* for 5 min. The supernatant was discarded and the cell pellet resuspended in 10 ml 10 % FCS/DMEM. The cells were then seeded on to fibronectin Flex I® plates at a density of $1x10^5$ cells in 2 ml 10 % FCS/DMEM per well. Plates were incubated in a humidified atmosphere of 10 % CO_2 in air at 37°C until reaching visual confluence. Cells were then quiesced by removal of the medium and addition of 1 ml incubation medium. After 24 hrs, cells were treated with DMEM containing 0 % FCS.

For experiments incorporating the kinase or cytochalasin D inhibitors, individual plates were incubated for either 2 hrs or 4 hrs prior to the application of mechanical load with different inhibitors: wortmannin (1 μM or 100 nM) and LY294002 (10 μM) (both PI 3-kinase inhibitors), tyrphostin 25 (tyrosine kinase inhibitor, 5 μM, 10 μM or 20 μM) and cytochalasin D (0.1 μM - 4 μM). The plates were loaded in the manifold and mechanically loaded for up to 4 hrs. For each experiment an identical plate was set up and left as the rigid control, allowing the comparison of loaded versus unloaded.

For integrin blocking experiments, cells were plated as previously described on fibronectin coated Flex I® plates. 2 hrs prior to the application of mechanical load individual plates were incubated with the α5β1 blocking antibody (10^{-10} M) at 37°C in a humidified atmosphere. The plates were mechanically loaded for up to 4 hrs. An identical plate was set up and left as the rigid control, allowing the comparison of loaded versus unloaded.

Preparation and electrophoresis of cell samples

After the application of mechanical load cells were lysed under either native or denaturing conditions depending on the primary antibody used.

Cell lysis under denaturing conditions

After the application of mechanical load, plates were immediately transferred onto ice. Medium from each well was discarded and the cell layer washed extensively with ice cold PBS. This was discarded and the cells were lysed by addition of 150 µl of boiling lysis buffer (10 mM Tris (pH 7.4), 1 % SDS and 1 mM DDT) to each well into which the cell layer was scraped. Lysates from each well of one plate were pooled (i.e 6 wells) and transferred to a 1.5 ml eppendorf tube and boiled for 5 mins. Lysates were clarified by sonication followed by centrifugation at 15 000 *g* at 4°C for 5 mins. The supernatants were transferred to fresh eppendorfs for protein measurement followed by immunoprecipitation.

Cell lysis under native conditions

After the application of mechanical load, plates were immediately transferred onto ice. The medium was discarded and the wells washed twice with 1 ml ice-cold PBS. This was discarded and the cells lysed with 150 µl/well of ice-cold radioimmunoprecipitation buffer (RIPA buffer) (50 mM Tris-HCl (pH 7.4) (buffering agent which prevents protein denaturation), 150 mM NaCl (prevents non-specific protein aggregation), 1 % NP-40 (non-ionic detergent to extract proteins), 0.25 % sodium deoxycholate (ionic detergent to extract proteins), 1 mM EDTA (calcium chelator). Phosphatase inhibitors were freshly added to the lysis buffer before cells were lysed (1 mM sodium orthovanadate, 1 mM sodium

fluoride). A Complete™, Mini protease inhibitor cocktail tablet (Boehringer Mannheim, UK) was also added immediately to the buffer prior to use (1 tablet/10 ml RIPA buffer: to give final concentrations of protease inhibitors, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin). The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to the RIPA buffer immediately before use (final concentration of 1mM) due to its unstable nature in aqueous solutions. In such solutions it has a half life of approximately 30 min and was added thereafter to the RIPA buffer as required. The adherent cells were scraped into the lysis buffer using a plastic cell scraper and the cell suspension from each well of one plate was pooled (i.e. 6 wells) and transferred to a 1.5 ml eppendorf. The eppendorfs were gently agitated on an orbital shaker for 15 min at 4° to lyse cells. The lysates were then centrifuged for 15 min at 14 000 *g* at 4°C. The supernatants were immediately transferred to fresh eppendorfs for protein measurement followed by immunoprecipitation. Samples were stored at -70°C prior to protein measurements.

2.6.1 Protein measurement

The protein content of each of the extracted samples was measured prior to immunoprecipitation and loading on to SDS-polyacrylamide gels. A range of standard dilutions (2000 μg/ml-50 μg/ml) of bovine serum albumin (BSA) was prepared using the BCA Protein Assay Kit (Pierce, Rockford, USA), diluted in the same diluent as the sample (i.e. lysis buffer). This kit was used to measure protein concentrations in the samples.

Principle behind the assay

The principle behind the Pierce protein assay is based on the reaction that occurs between copper ions (Cu^{2+}) in the presence of protein, whereby the copper ions becomes oxidised (Cu^{1+}), and in turn a blue/purple colour is formed. Bicinchoninic acid (BCA) is a selective detection reagent for Cu^{1+}. The presence of protein in combination with Cu^{2+} (in an alkaline medium) leads to the formation of Cu^{1+}. The purple product, formed by the interaction of two molecules of BCA with one Cu^{1+} ion exhibits a strong absorbance at 562mn. This allows the spectrophotometric quantitation of protein in aqueous solutions.

Reaction that occurs between the BCA reagents and samples

$$\text{Protein (peptide bonds)} + Cu^{2+} \rightarrow \text{tetradentate-}Cu^{1+}\text{ complex}$$

$$Cu^{1+} + 2\text{ bicinchoninic acid (BCA)} \rightarrow \text{BCA-}Cu^{1+}\text{ complex (purple coloured)}$$

A mixture of the solutions BCA Reagent A (containing Na_2CO_3, $Na_4(CO_3)_2$, bicinchoninic acid and sodium tartrate in 0.2N NaOH) and BCA Reagent B (4 % cupric sulphate) was prepared (1:50 respectively). 25 µl of each standard or sample was pipetted into separate wells of a 96-microwell plate (GIBCO-BRL, Paisley, UK). 200 µl of solution A/B was added to each well and the plate was agitated on a plate shaker (Luckham R100, Luckham Ltd., Sussex, UK) for 30 seconds to combine all the solutions. The plate was covered and incubated at 37°C for 30 min. After incubation the plate was cooled to room temperature and the absorbance at 580 nm of all standards and samples was measured on a plate reader (ICN Flow, High Wycombe, Bucks, UK). A standard curve was plotted for the standards

and the concentration of the protein in each sample was determined by the standard curve. Once the protein concentrations had been determined, 500 µg of protein from each sample was aliquoted into an eppendorf and made up to 300 µl with lysis buffer. Lysates were stored at -20°C prior to immunoprecipitation.

Immunoprecipitation of protein

Immunoprecipitations were routinely performed by initially preclearing lysates with rabbit IgG and 10 µl protein G-plus agarose beads (Autogen Bioclear, Wiltshire, UK). Lysates were centrifuged at 7 000 *g* for 5 min at 4°C. The supernatants were transferred to fresh eppendorfs and incubated, rotating, with 3 µg/ml antibody for 2 hrs at 4°C. Antibodies used were raised against focal adhesion proteins: anti-focal adhesion kinase monoclonal antibody (FAK), anti-p130^{Cas} monoclonal antibody and anti-paxillin monoclonal antibody (all from Affiniti Research Products Ltd, Exeter, UK), anti-FAK polyclonal antibody, recognising the carboxyl terminal and anti-phosphotyrosine antibody PY20 or PY99 (both from Autogen Bioclear, Wiltshire, UK). Immunocomplexes were collected by incubating lysates with protein G Plus-agarose beads for 1 hr-overnight at 4°C.

After incubation with the agarose beads the eppendorfs were centrifuged for 5 min at 15 000 *g*. Immunoprecipitates were then washed three times with lysis buffer by centrifugation for 5 min at 15 000 *g* and the supernatant discarded to leave the agarose:Ab conjugated pellet. Proteins bound to the agarose beads were extracted with 2 x SDS-PAGE sample buffer (200 mM Tris (pH 6.8), 6 % SDS, 2 mM EDTA, 10 % w/v glycerol, 4 % β-mercaptoethanol, 0.01 % bromophenol blue) and boiled for 5 min at 90°C.

Immunoprecipitates were further analysed by SDS polyacrylamide gel electrophoresis followed by Western blotting.

SDS-polyacrylamide gel electrophoresis

The Novex gel apparatus, XCell II™ Mini-Cell (Novex Electrophoresis GmbH, Frankfurt, Germany) was used for all polyacrylamide gel electrophoresis. For most proteins Mw ranging from 100 to 200 kDa (e.g. FAK and $p130^{Cas}$) 7.5 % acrylamide separating gels were overlaid with a 4 % acrylamide stacking gel. For proteins with a Mw 40-100kDa (e.g. paxillin) 10 % acrylamide separating gels were used. The separating gel (50 % Acrylamide/Bis, 1 M Tris-HCl (pH 8.8), 10 % SDS, 50 % sucrose, 75 μM ammonium persulphate, TEMED and water) was routinely poured first into the gel cassette and overlaid with t-amyl alcohol (Sigma, Poole, UK) and allowed to polymerize for at least 1 hr. The t-amyl alcohol was removed and the gel surface washed extensively with ddH_20. The stacking gel (50 % Acrylamide/Bis, 0.375 M Tris-HCl (pH 6.8), 10 % SDS, TEMED, ammonium persulphate and water) was poured on top of the separating gel and the combs were positioned at the top of the stacking gel immediately after pouring. The stacking gel was allowed to polymerise for 1 hr. The gel apparatus was assembled and running buffer (24 mM Tris, glycine, 10 % SDS, ddH_20) was poured into the gel tank. Samples to be electrophoresed were boiled for 5 min prior to loading onto the gel. The gels were routinely run at 125 v -130 v for 2 hrs. For each gel, 3 μl of a molecular weight marker was also loaded ("See Blue" marker, Novex Electrophoresis GmbH, Frankfurt, Germany).

Western blotting

Once the samples had reached 1 cm from the bottom of the gel, the gel was removed from the gel cassette and the separated proteins were transferred from the gel to Hybond™ ECL™ nitrocellulose membrane (Amersham Life Science, Bucks, UK) using the Novex XCell II™ Mini-Cell. The cassette was assembled with the gel sponged between 3MM paper (Whatman, UK) and the nitrocellulose membrane. The nitrocellulose membranes and the 3MM paper were pre-wetted in transfer buffer (12 mM Tris, 96 mM glycine, ddH_20, pH 8.3) for 10 s. The membrane was laid on top of the gel on the side nearest the positive electrode. Any air bubbles formed between the gel and the membrane during assembly of the cassette were rolled out (Figure 2.4). The proteins were transferred at 25 v for 2 hrs in transfer buffer. After transfer, the cassette was dismantled and the membrane was stained with Ponceau Red (Sigma Chemicals, Poole, UK) to ensure complete and even transfer of all proteins. The membrane was rinsed with ddH_2O to remove excess Ponceau Red dye. The membranes were blocked in 5 % w/v non-fat milk protein (Marvel) in TBS (pH 7.6) containing 1.0 % w/v Tween 20 (Sigma Chemicals, Poole, UK) (TBS-T) or for phosphotyrosine antibodies, 1 % w/v BSA with 1 % milk w/v in TBS-T for 1 hr at room temperature or overnight at 4°C. After incubation, the blocking solution was discarded and the membranes were rinsed twice in TBS-T then incubated with protein-specific antibodies (anti-phosphotyrosine antibody, PY20 or PY99, Autogen Bioclear, Wiltshire, UK) or focal adhesion protein antibodies in 5 % w/v Marvel dissolved in TBS-T or 1 % w/v BSA / 1 % w/v Marvel for 1 hr at room temperature or overnight at 4°C. This was discarded and the membranes were washed 3 x 5 min in TBS-T. The membranes were then incubated with horseradish peroxidase (HRP) conjugated anti-mouse IgG (Sigma Chemicals, Poole, UK) at

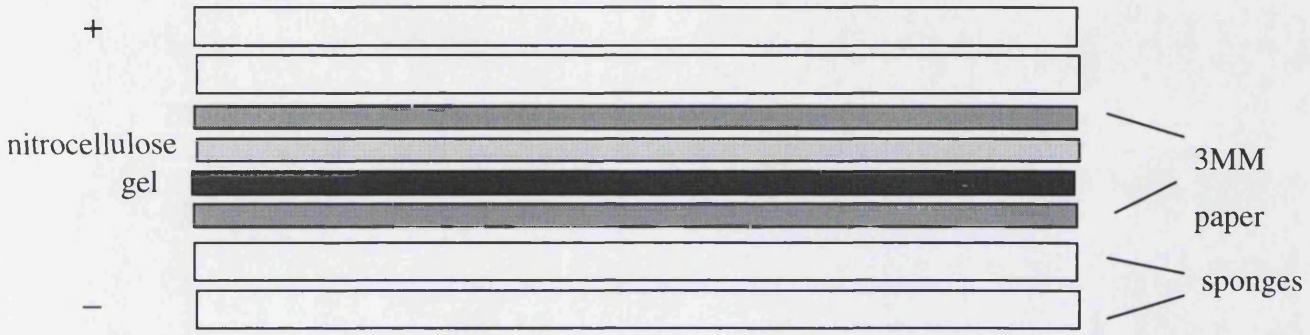


Figure 2.4. Assembly of gel cassette for transfer of proteins from the gel to nitrocellulose membrane.

a 1:5000 dilution in 5 % w/v Marvel dissolved in TBS-T for 1 hr at room temperature. Finally the membranes were washed in the same way as described above. Immunoreactive bands were visualised by incubating the membrane in ECL (Amersham Life Science, Bucks, UK) reagent for 1 min and then exposing the membrane to autoradiography film (Hyperfilm™, ECL™-high performance chemiluminescence film, Amersham Life Science, Bucks, UK) for 30 s and 1 min.

Films were immediately developed using a Velopex MD2000 developer (Medivance Ltd., UK). ECL western blotting is a light emitting non-radioactive method for detection of immobilised specific antigens conjugated with HRP-labelled antibodies.

Principles of ECL detection

Chemiluminescence involves the excitation of chemicals effected by a chemical reaction. HRP (to which the secondary antibody is conjugated) oxidises luminol (present in the ECL reagents) in the presence of chemical enhancers e.g. phenol. Immediately following oxidation, luminol is in an excited state. Luminol decays to ground state via a light emitting pathway. The presence of phenol on the reaction has the effect of increasing the light output approximately 1000 fold and extending the time of light emission. The light produced by luminol returning to ground state peaks after 5 - 20 min and decays slowly thereafter with a half life of 60 min. The maximum light emission is at a wavelength of 428 nm which can be detected by a short exposure to blue-light sensitive autoradiography film.

2.7 IMMUNOFLUORESCENT STAINING OF FOCAL ADHESION PROTEINS

Cell plating and mechanical loading

Human cardiac fibroblasts were grown to confluency in tissue culture flasks in the presence of 10 % FCS/DMEM. Cells were trypsinised, pelleted and resuspended in 10 % FCS/DMEM. The cells were plated on to fibronectin coated silicone-elastomer Flex I® plates at a density of $1x10^4$ cells/well in 10 % FCS/DMEM and allowed to reach 70-80 % confluence. The media from each well was removed and replaced with preincubation medium for 24 hrs. Cells were then either stretched or left as rigid controls, in serum free medium. After the application of load, media was removed from the wells and each well was washed twice with PBS. After the final PBS wash, cells were fixed in 4 % paraformaldehyde for 10 min at room temperature, washed 3 times with PBS and stored at 4°C in PBS containing 0.05 % sodium azide prior to staining.

Cell permeabilisation and immunofluorescent staining

The cells were permeabilised on ice with permeabilisation buffer (20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5 % Triton X-100, 0.05 % sodium azide, PBS) for 5 min. After permeabilisation, wells were washed three times with PBS then non-specific background was blocked by addition of 5 % FCS/PBS to each well for 30 min. This was aspirated and each well was then washed three times with PBS. The wells were incubated with individual primary antibodies diluted in 5 % FCS/PBS for 1 hr at room temperature: monoclonal anti-α-actinin (1:200, clone BM-75.2), monoclonal anti-human vinculin (1:200, clone hVIN-1) (Sigma Immunochemicals, Poole, UK), monoclonal anti-FAK (1:75, clone 77), monoclonal anti-$p130^{Cas}$ (1:75, clone 21), monoclonal anti-paxillin (1:100, clone 349)

(all from Transduction Labs, Affiniti Research Products Ltd, Exeter, UK) or the phosphotyrosine antibody PY99 (1:500) (Autogen Bioclear, Wiltshire, UK).

Primary antibody was removed and the wells were washed with PBS and subsequently incubated with secondary antibody: anti-mouse conjugated to FITC (1:200 dilution) (Sigma Immunochemicals, Poole, UK) and phalloidin conjugated to rhodamine (1:200) (Cambridge Bioscience, Cambridge, UK) to detect actin stress fibres. These were added either separately or together to each well and incubated for 1 hr at room temperature in a dark humidified chamber. Secondary antibody was removed and each well rinsed three times with PBS. Each well was removed from the plate and mounted with a coverslip using Immun-Mount (containing anti-fade) (Shandon, UK). Wells were stored at 4°C in the dark prior to analysis using the Leica confocal microscope. Images were obtained using the Leica TCS NT software (Leica, Germany).

Confocal Microscopy

Scanning laser confocal microscopy (Leica Lasertechnik GmbH, Heidelberg, Germany) was carried out to assess the localisation of focal adhesions in human cardiac fibroblasts in response to mechanical load and in rigid control cells. Fluorescent images were sequentially collected in 1 μm steps through cardiac fibroblasts for FITC and tetramethylrhodamine isothiocyanate (TRITC) fluorochromes at 488- and 568-nm emission wavelengths, respectively. These images were accumulated together to produce an image made up of a composition of the cell taken at different sections through the cell. The images were analysed with image packages loaded onto the Leica NTS.

Effect of cytochalasin D on the actin cytoskeleton and paxillin

To determine the importance of the actin cytoskeleton on mechanical load induced formation of focal adhesions, cytochalasin D was added to disrupt the actin cytoskeleton. Cells were seeded onto fibronectin coated Flex I® plates in 2 ml 10 % FCS/DMEM at a density of $1x10^4$ cells /well and incubated in a humidified atmosphere at 37°C until 70-80 % confluent. Cells were quiesced for 24 hrs with incubation medium. Individual wells were incubated with 1 μM cytochalasin D 2 hrs prior to the application of mechanical load. Plates were mechanically loaded for 1 hr or left as rigid controls. Wells were prepared for immunofluorescence as described above and stored at 4°C in the dark prior to analysis.

2.8 INTEGRIN CELL SURFACE BIOTINYLATION AND IMMUNOPRECIPITATION

Cell plating and mechanical loading

Cells were grown to visual confluence in 75 cm^2 tissue culture flasks, detached from the flasks by addition of trypsin/EDTA and seeded on fibronectin or collagen coated Flex I® plates at a density of $1x10^5$ cells in 10 % FCS/DMEM. Upon reaching confluency, the medium was replaced with serum free medium for 24 hrs. After 24 hrs this was replaced with either identical medium or medium containing 10 % FCS. Plates were mechanically loaded for either 24 or 48 hrs. At the end of the strain regimen, cells were washed with PBS and detached from the well membranes with 300 μl trypsin/EDTA per well in a humidified atmosphere of 10 % CO_2 at 37°C for 5 min. The cells were pelleted at 1 000 *g* for 5 min and washed with PBS. Sulfo-NHS-Biotin (Pierce & Wariner, UK) was added to

a final concentration of 100 μg/ml to the cells and the cell suspension was incubated for 1hr, rotating. The cells were subsequently washed with PBS and lysed with lysis buffer (RIPA) for 15 min at 4°C. Lysates were then centrifuged at 15 000 *g* for 10 min. Lysates were stored at -20°C prior to measuring protein levels using the BCA Protein Assay Kit (see Protein Measurement section for description of assay).

Immunoprecipitation of integrin subunits

For immunoprecipitation of the integrins, aliquots of supernatants containing equal amount of protein were incubated with anti-α5 integrin, anti-α2 integrin or anti-β1 integrin (all from GIBCO-BRL, Paisley, UK) for 2 hrs at 4°C, rotating. 20 μl of Protein G-plus agarose beads was added to each aliquot to bind the antibody, and aliquots were incubated overnight at 4°C. The agarose beads conjugated to antibody was collected from each sample by centrifugation at 12 000 *g* for 5 min. The supernatant from each sample was discarded and the beads washed with lysis buffer and centrifuged at 12 000 *g* for a further 5 min. This was repeated again twice. After the final spin the supernatant was removed and 40 μl of electrophoresis buffer was added to each sample. The samples were then boiled for 5 min at 90°C, and the supernatant from each sample was analysed by gel electrophoresis and Western blotting.

SDS-polyacrylamide gel electrophoresis

The Novex gel apparatus was used for all polyacrylamide gel electrophoresis. After boiling for 5 min, the supernatant from each sample was loaded and run on SDS 7.5 % polyacrylamide gels (see SDS polyacrylamide gel electrophoresis section for recipe)

overlaid with a 4 % acrylamide stacking gel (see SDS polyacrylamide gel electrophoresis section for recipe). Gels were run for 2 hrs at 125 v. The separated proteins were transferred to nitrocellulose membranes for 2 hrs at 30v.

Western blotting

Once the samples had run, the gel was removed from the cassette and the separated proteins were transferred from the gel as described above. After staining with Ponceau Red (Sigma, Poole, UK) the membranes were incubated with 5 % non fat milk protein (Marvel) in TBS (pH 7.6) containing 1.0 % w/v Tween 20 (TBS-T) for 1 hr at room temperature or overnight at 4°C to block non-specific binding. Membranes were washed 3 x 5 min in TBS-T, then incubated with horseradish peroxidase conjugated streptavidine (DAKO, Bucks, UK) at a 1:5000 dilution in 5 % milk/TBS-T for 1 hr at room temperature. The membranes were then washed 3 times in TBS-T. Immunoreactive bands were visualised by incubating the membrane with ECL (enhanced chemiluminescence) reagent for 1 min and then exposing the membrane to autoradiography film for 30 s and 1 min. Autoradiography film was immediately developed using the Velopex MD2000 developer (Medivance Ltd., UK).

2.9 MEASUREMENT OF INOSITOL LIPIDS IN RESPONSE TO MECHANICAL LOAD

Cell plating and mechanical loading

Human cardiac fibroblasts were seeded onto fibronectin Flex I plates in 10 % FCS/DMEM and allowed to reach confluency. 48 hrs before the application of load cells were incubated

in DMEM containing 10 μCi/ml *myo*-[2-^{3}H] inositol (Amersham, Bucks, UK). 10 min before the application of load, 10 mM LiCl was added to each well to quench the reaction. The fibroblasts were loaded for 1 or 5 min. Unloaded control plates were treated in an identical fashion.

Separation of lipid layer

After the application of mechanical load the medium was removed and 1.25 ml 100 % methanol was added to each well, into which the cell layer was scraped. This was transferred to separate glass tubes and chloroform was added to each tube. The tubes were vortexed and chloroform and ddH_20 were added to each tube. The tubes were vortexed again and the samples spun for 5 min at 5 000 *g*, 4°C. Three layers formed, of which the top layer was pipetted off and loaded onto a Dowex 1-X8 anion exchange resin column (formate form).

Separation of inositol lipids

Total inositol phosphate was separated from free inositol and glycerophosphoinositol (GPI) by passage through the Dowex column. Free ^{3}H-inositol was eluted by addition of 6 ml ddH_20 onto the column and discarded. The column was then washed with 6 ml 5 mM sodium tetraborate/5 mM sodium formate to elute GPI which was discarded. To elute total inositol, 3 ml 1 M ammonium formate/0.1 M formic acid was added to the column. The elution was collected directly into scintillation vials. 5 ml of scintillant was added to each vial and the radioactivity was measured using a beta counter.

To separate further the total inositol into inositol monophosphate (IP), inositol bisphosphate (IP_2), and inositol trisphosphate/tetraphosphate ($IP_{3/4}$), elutions were carried out as described above using a Dowex column with varying salt concentrations.

STATISTICAL ANALYSIS

All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired students t-test for single group comparisons. Differences were considered statistically significant at $P<0.05$.

RESULTS

CHAPTER 3

HUMAN CARDIAC FIBROBLAST PROCOLLAGEN SYNTHESIS IN RESPONSE TO MECHANICAL LOAD AND EXTRACELLULAR MATRIX COMPOSITION

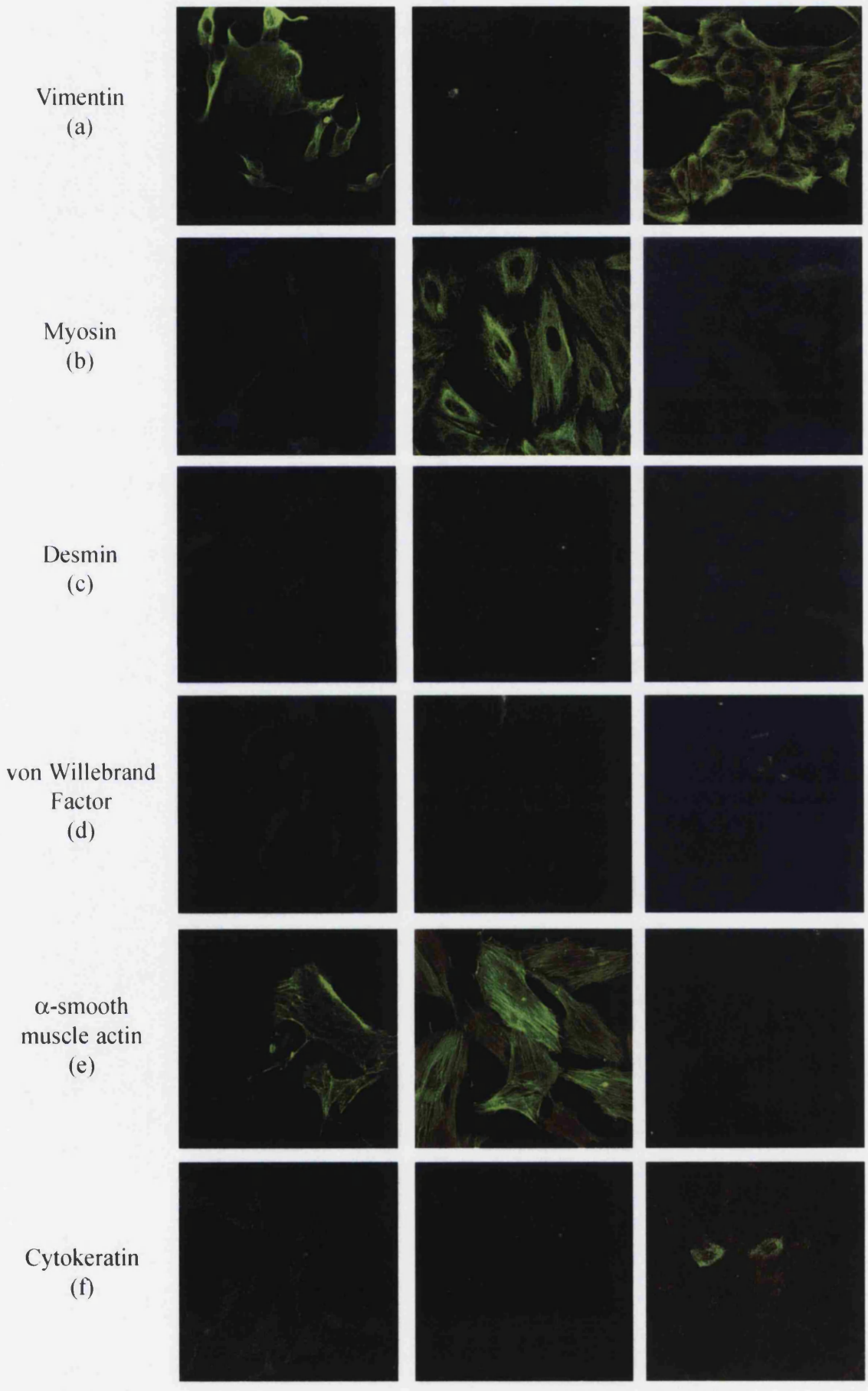
3.1 IMMUNOFLUORESCENT CHARACTERISATION OF FIBROBLASTS

For each different fibroblast isolation used, the fibroblastic nature of the primary cell isolation was determined by FITC conjugated immunostaining of cell layers with various antibodies to cytoskeletal and surface proteins. The staining pattern exhibited by the primary cells was compared with that obtained in various cell lines employed as controls.

A representative example of the staining pattern exhibited by the primary cell isolations is shown in Figure 3. Primary cells in culture stained positive for vimentin (Figure 3.1a.) and negative for myosin, desmin and von Willebrand Factor (Figure 3.1b, c., d.). In addition some cells in all the cultures stained positive for α-SMA (Figure 3.1e.). Fibroblasts also stained negative for cytokeratin (Figure 3.1f).

For comparison and confirmation of antibody specificity, a human smooth muscle cell line (A10), and a mesenchymal cell line (MDCK) were stained alongside the primary cells. A10s stained negatively for vimentin (Figure 3.1a.), and positively for myosin (Figure 3.1b). A10s stained negatively for desmin (Figure 3.1c.) and von Willebrand Factor (Figure 3.1d.) and positive for α-SMA (Figure 3.1e.). They also stained negative for cytokeratin (Figure 3.1f). MDCKs stained positively only for vimentin and cytokeratin (Figure 3.1a, f.) and negative for all other antibodies tested (Figure 3.1b, c., d,e.). On the basis of these results it was concluded pure cultures of cardiac fibroblasts had been obtained, with some of the fibroblasts transformed to the myofibroblast phenotype.

Figure 3.1 Immunofluorescent characterisation of human cardiac fibroblasts. Human cardiac fibroblasts were stained for various cytoskeletal and surface proteins to determine their fibroblastic phenotype. Two other cell lines, A10 and MDCK were used as positive controls. Images shown are representative of 3 individual experiments.


Human cardiac fibroblasts
A10
MDCK
Vimentin (a)
Myosin (b)
Desmin (c)
von Willebrand Factor (d)
α-smooth muscle actin (e)
Cytokeratin (f)

3.2 HUMAN CARDIAC FIBROBLAST PROCOLLAGEN SYNTHESIS IN RESPONSE TO MECHANICAL LOAD AND EXTRACELLULAR MATRIX COMPOSITION

3.2.1 Effect of load and ECM composition on procollagen synthesis in the presence and absence of serum

It has been previously demonstrated in our laboratory that rat cardiac fibroblasts grown on elastin coated Flex I® plates and subjected to a cyclical mechanical load for 48 hrs in the presence of 10% serum increase their levels of collagen synthesis (Butt *et al*, 1997).

To investigate the effect of mechanical load on human cardiac fibroblasts procollagen synthesis and to address the hypothesis that ECM composition may affect the fibroblasts' response to mechanical load, fibroblasts were grown on collagen, elastin or fibronectin Flex I® plates. Human cardiac fibroblasts were subjected to a cyclical biaxial load for 24 or 48 hrs. Further, to determine whether serum is required in these cells under these conditions, fibroblasts were loaded in the presence or absence of 10% FCS. To determine whether separate fibroblast isolations responded differently to mechanical load, different isolations were tested for their response to mechanical load on all the matrices.

After 48hrs, procollagen synthesis levels were increased above rigid control on all matrices in the presence of serum (collagen, 73 ± 2 %, elastin, 45 ± 12 %, fibronectin 97 ± 0.2 % above rigid controls (Figure 3.2)). After 24hrs, only those fibroblasts

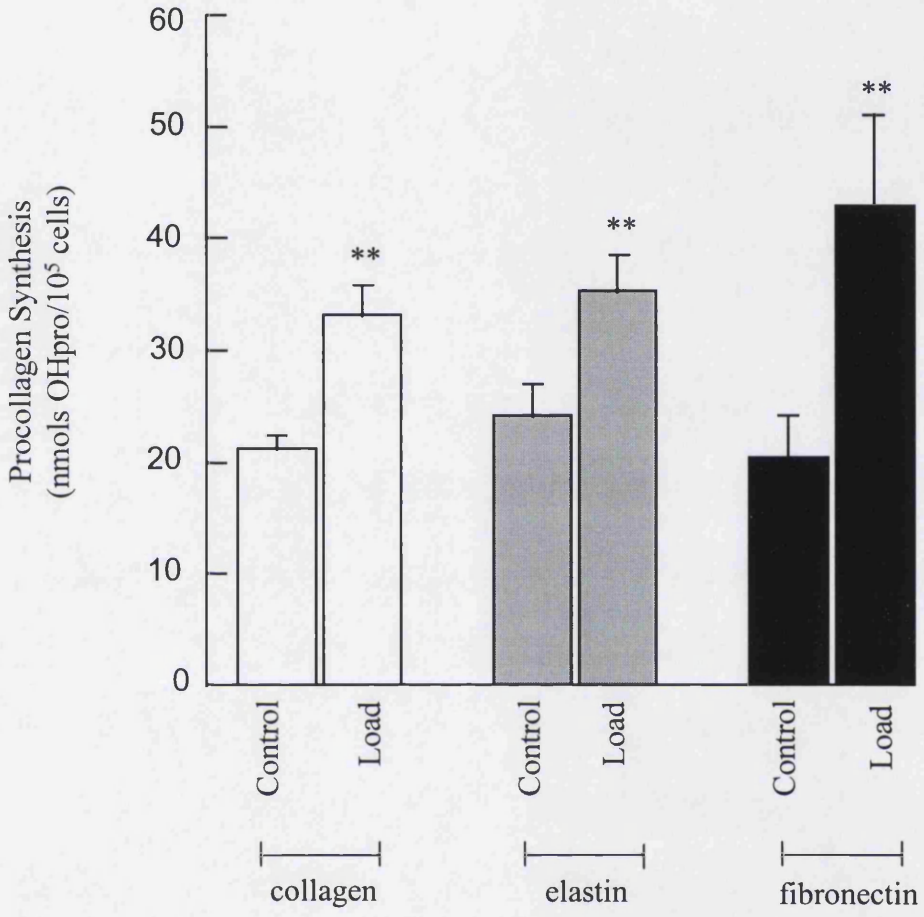


Figure 3.2. The effect of 48 hrs mechanical load on procollagen synthesis. Procollagen synthesis levels were measured in fibroblast cultures grown on collagen, elastin, or fibronectin after 48 hrs of mechanical load in the presence of 10 % serum. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

loaded on fibronectin Flex I® plates showed an increase in procollagen synthesis (81.0 ± 0.1 % vs. rigid control, Figure 3.3). There was no significant increase in procollagen synthesis by those fibroblasts grown on collagen or elastin Flex I® plates. Table 3.1 shows the reproducibility of the experiment

In the absence of serum, at both 24 (Figure 3.4) and 48 hrs (Figure 3.5) only those fibroblasts loaded on fibronectin resulted in increased procollagen synthesis levels above rigid controls in response to load, (24hrs, 50 ± 9 %, 48hrs, 157 ± 0.4 % vs. rigid control). No significant increase in procollagen synthesis was obtained with fibroblasts grown on collagen and elastin. Table 3.2 shows the reproducibility of the experiment.

3.2.2 Effect of mechanical load and matrix composition on fibroblast cell number at the end of the procollagen synthesis assay

In order to ensure that the effects of mechanical load and matrix composition on procollagen synthesis were not being influenced by changes in cell number, cell counts were performed in parallel cultures from 3 separate experiments for confluent cultures exposed to 24 and 48hrs of mechanical load and different matrices. Results are shown in Table 3.3 and indicate that mechanical load did not cause a significant increase in cell number at either time point on any matrix.

3.2.3 Effect of mechanical load and ECM composition on human cardiac fibroblast replication

Cell replication was measured by radiolabelled thymidine incorporation over a period

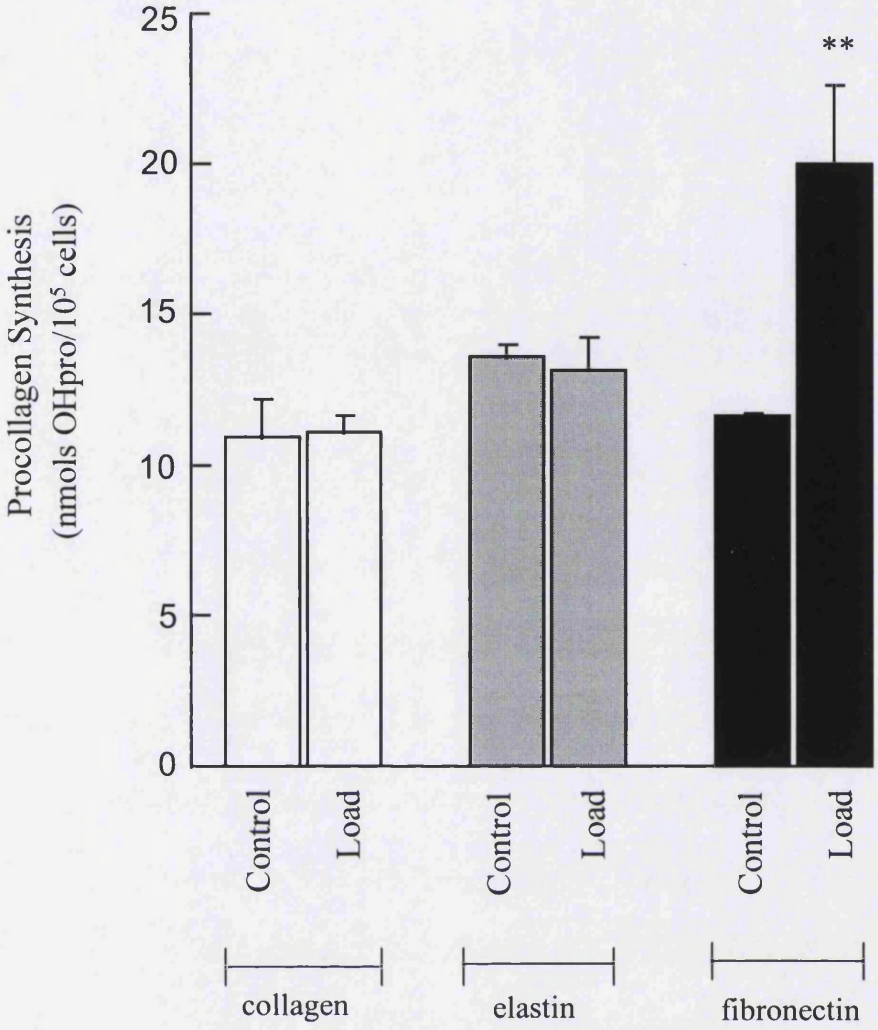


Figure 3.3. The effect of 24 hrs of mechanical load on procollagen synthesis in the presence of 10 % serum. Procollagen synthesis was measured in fibroblast cultures grown on fibronectin, collagen or elastin. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

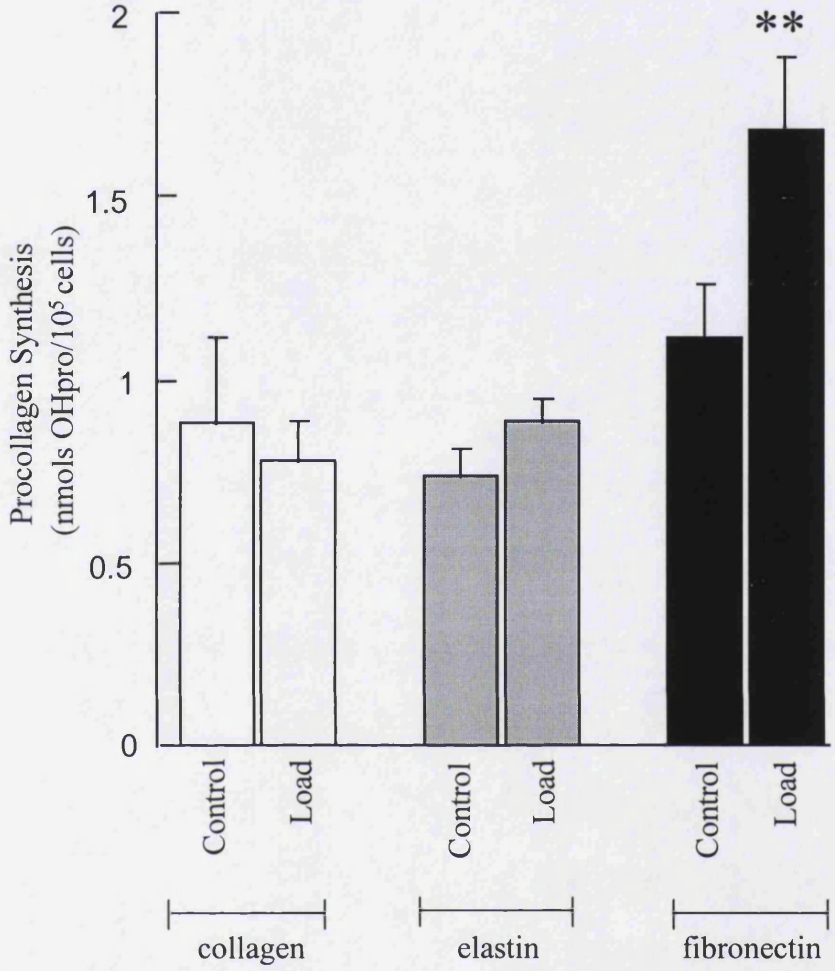


Figure 3.4. The effect of 24 hrs mechanical load on procollagen synthesis in the absence of serum. Procollagen was measured in cultures of fibroblasts grown on collagen, elastin or fibronectin after 24 hrs mechanical load. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

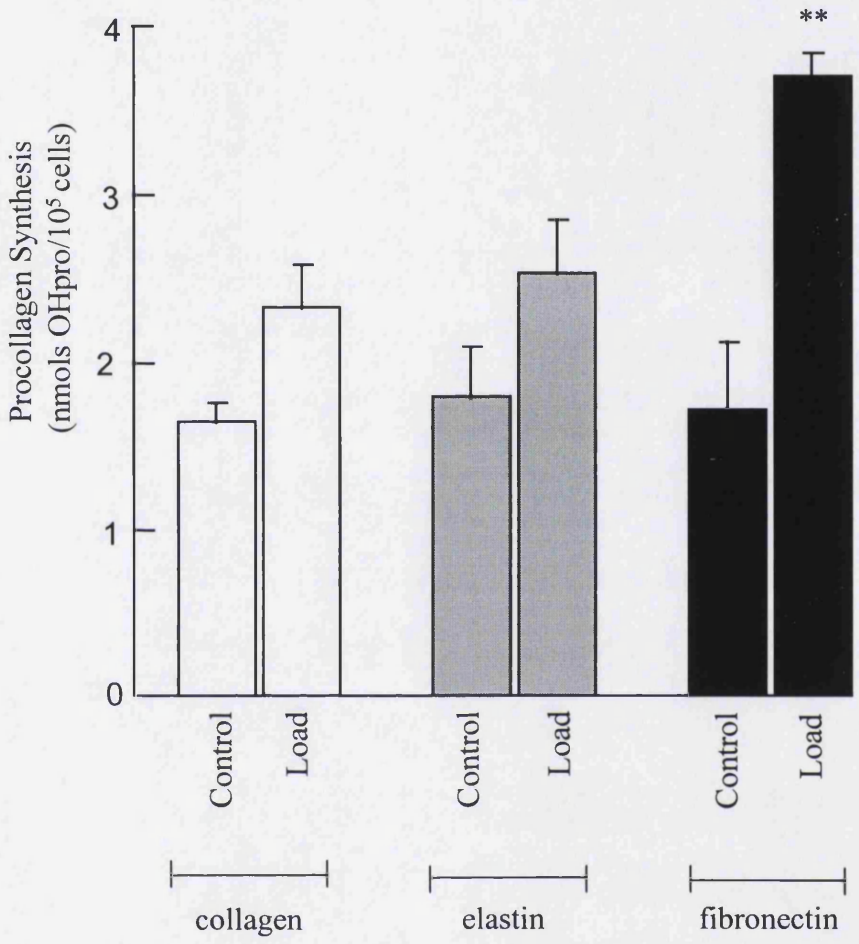


Figure 3.5. The effect of 48 hrs mechanical load on procollagen synthesis in the absence of serum. Procollagen synthesis levels were measured in fibroblast cultures on collagen, elastin or fibronectin after 48 hrs mechanical load. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

Effect of 10 % FCS and 24 hrs mechanical load on procollagen synthesis

10 % FCS / 24hrs	#1	#2	#3
Collagen rigid	10.95 ± 1.20	0.78 ± 0.24	9.79 ± 0.26
Collagen load	11.10 ± 0.53	0.98 ± 0.11	11.81 ± 1.35
Elastin rigid	13.60 ± 0.42	2.87 ± 0.76	12.68 ± 1.63
Elastin load	13.15 ± 1.14	2.21 ± 0.60	15.54 ± 0.55
Fibronectin rigid	11.46 ± 0.03	1.66 ± 0.03	17.50 ± 4.57
Fibronectin load	19.35 ± 2.63**	9.97 ± 2.61**	31.70 ± 0.37**

Effect of 10 % FCS and 48 hrs mechanical load on procollagen synthesis

10 % FCS/ 48 hrs	#1	#2	#3
Collagen rigid	21.43 ± 1.10	11.23 ± 1.10	15.43 ± 2.31
Collagen load	33.16 ± 2.38**	23.29 ± 2.60**	32.17 ± 2.84**
Elastin rigid	24.18 ± 2.59	14.28 ± 2.91	20.45 ± 3.22
Elastin load	35.21 ± 3.65**	27.38 ± 3.19**	38.49 ± 1.78**
Fibronectin rigid	20.04 ± 3.88	12.40 ± 3.82	28.01 ± 3.57
Fibronectin load	42.96 ± 8.00**	36.95 ± 6.50**	50.58 ± 4.16**

Table 3.1. The effect of 10 % FCS and mechanical load on procollagen synthesis on different matrices from 3 individual experiments. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM. **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

Effect of 0 % FCS and 24 hrs mechanical load on procollagen synthesis

0 % FCS / 24 hrs	#1	#2	#3
Collagen rigid	0.88 ± 0.24	1.31 ± 0.11	1.08 ± 0.03
Collagen load	0.78 ± 0.11	1.68 ± 0.05	1.24 ± 0.17
Elastin rigid	0.74 ± 0.07	1.02 ± 0.37	1.11 ± 0.14
Elastin load	0.89 ± 0.06	1.11 ± 0.09	1.40 ± 0.09
Fibronectin rigid	1.12 ± 0.14	1.42 ± 0.14	1.09 ± 0.12
Fibronectin load	1.68 ± 0.26**	2.15 ± 0.21**	1.92 ± 0.20**

Effect of 0 % FCS and 48 hrs mechanical load on procollagen synthesis

0 % FCS / 48 hrs	#1	#2	#3
Collagen rigid	1.65 ± 0.11	2.43 ± 0.05	2.00 ± 0.15
Collagen load	2.33 ± 0.26	2.78 ± 0.22	2.14 ± 0.14
Elastin rigid	1.80 ± 0.29	2.11 ± 0.17	1.80 ± 0.07
Elastin load	2.54 ± 0.31	2.89 ± 0.24	2.09 ± 0.06
Fibronectin rigid	1.72 ± 0.40	2.00 ± 0.13	2.30 ± 0.11
Fibronectin load	3.71 ± 0.14**	3.98 ± 0.17**	4.05 ± 0.27**

Table 3.2. The effect of 0 % FCS and mechanical load on procollagen synthesis on different matrices from 3 individual experiments. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM. **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

Effect of mechanical load and 10 % serum on cell number

10% FCS	T0	24 hr rigid	24 hr load	48 hr rigid	48 hr load
collagen	2.64±0.28	2.84±0.24	2.88±0.13	3.0±0.06	2.89±0.21
elastin	2.49±0.16	2.66±0.36	2.45±0.07	2.80±0.11	2.74±0.05
fibronectin	2.90±0.44	2.87±0.32	2.99±0.26	2.84±0.08	2.92±0.13

Effect of mechanical load and 0 % serum on cell number

0 % FCS	T0	24 hr rigid	24 hr load	48 hr rigid	48 hr load
collagen	3.21±0.53	2.73±0.17	2.37±0.09	2.73±0.25	2.48±0.38
elastin	3.05±0.48	2.38±0.15	2.00±0.28	2.35±0.17	2.32±0.17
fibronectin	2.50±0.45	2.10±0.04	2.10±0.18	2.50±0.04	2.20±0.09

Table 3.3 The effect of cell number on collagen, elastin or fibronectin in the presence (top table) or absence (bottom table) of serum after 24 and 48 hrs of mechanical load. Identical plates were set up as for procollagen synthesis experiments and cell number counted on confluent cultures. Values are expressed as cell number (x 10^5). Values represent mean ± SEM (n=6).

of 96 hrs in subconfluent cultures. Fibroblast replication in response to mechanical load under serum free conditions was not significantly increased on any matrix at both 24 and 48 hrs (Figures 3.6 and 3.7). In fact, there appeared to be a slight decrease, although not statistically significant, in fibroblast replication on mechanically loaded collagen plates.

In the presence of serum, an increase in thymidine incorporation was observed after 24 hr mechanical load on all matrices (collagen, 2365 ± 100 vs. 1247 ± 95; elastin, 2410 ± 110 vs. 1240 ± 153; fibronectin, 2857 ± 101 vs. 1777 ± 239) (Figure 3.8)

After 48 hrs mechanical load, fibroblasts plated on elastin and fibronectin showed a greater increase in thymidine incorporation compared to fibroblasts grown on collagen (collagen 1400 ± 93 vs. 795 ± 100, elastin, 2273 ± 200 vs. 592 ± 24; fibronectin, 2665 ± 362 vs. 1294 ± 234) (Figure 3.9). At 72 and 96 hrs very little increase in replication was observed in response to mechanical load on all the matrices suggesting the response was complete by this time (data not shown).

3.2.4 Effect of soluble fibronectin on procollagen synthesis in response to mechanical load

Soluble fibronectin (25 μg/ml) blocked mechanical load induced procollagen synthesis on all matrices (collagen, 11.5 ± 3.2 with sFn, 22.9 ± 2.4 without sFn; elastin, 3.3 ± .4 with sFn, 24.1 ± 4.1 without sFn;

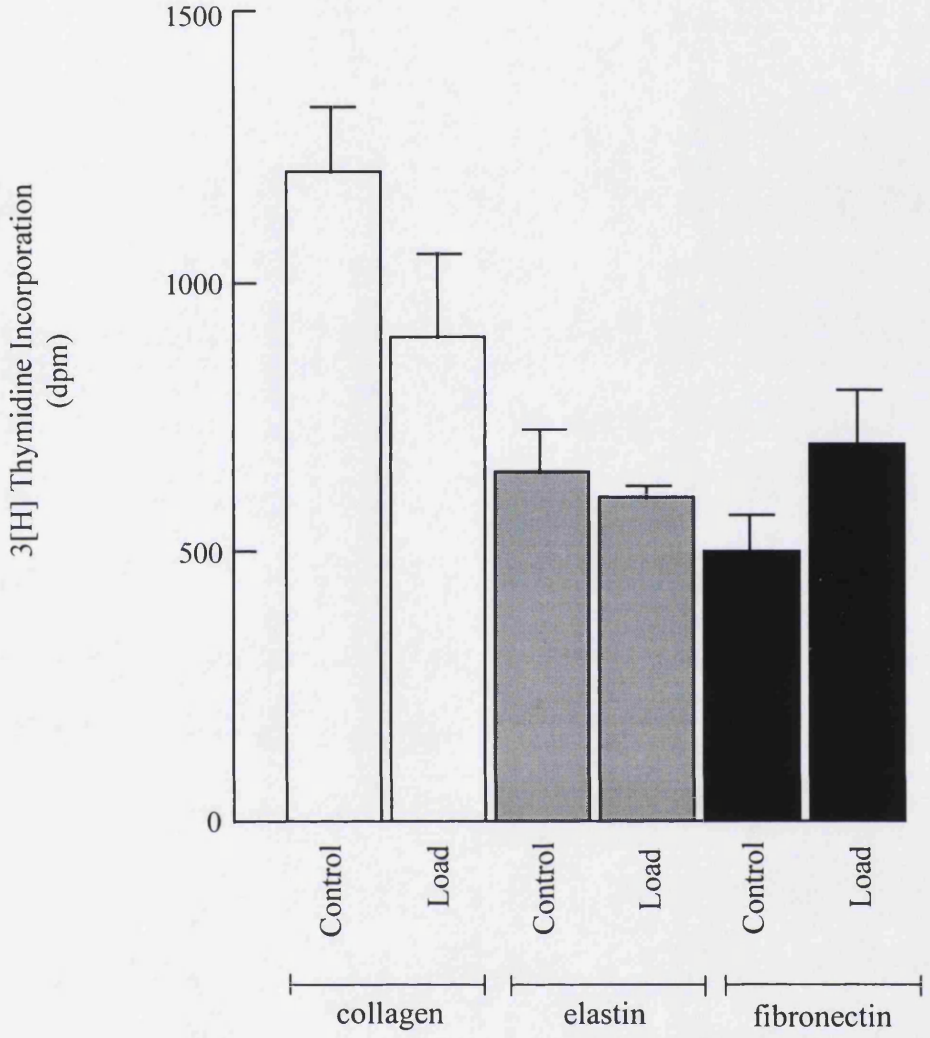


Figure 3.6. The effect of 24 hrs mechanical load and 0% serum on fibroblast replication. Thymidine incorporation was measured in fibroblast cultures grown on collagen, elastin or fibronectin. Values are expressed as disintegrations per minute (dpm). Values represent mean ± SEM (n=6). Graph shown is representative of 3 individual experiments.

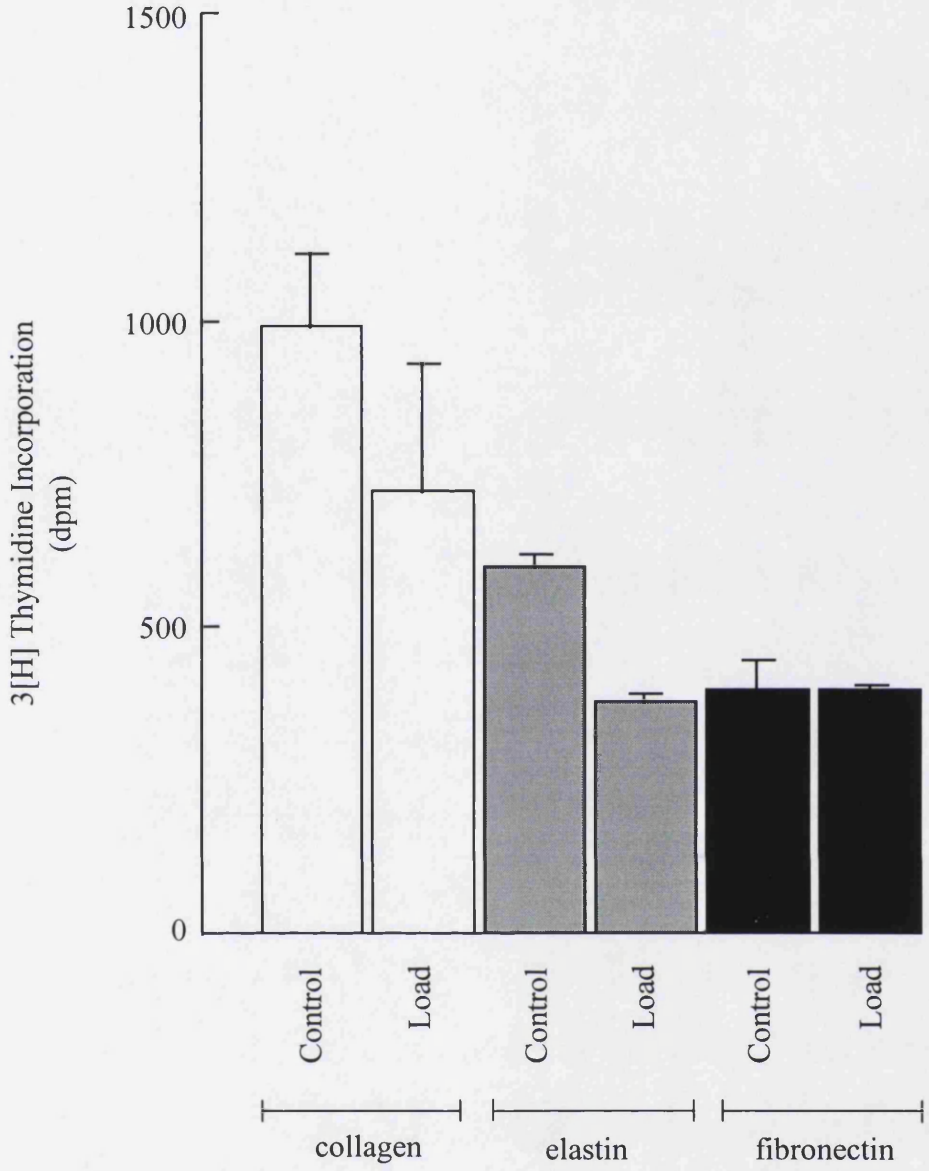


Figure 3.7. The effect of 48 hrs mechanical load and 0% serum on fibroblast replication. Thymidine incorporation was measured in fibroblast cultures grown on collagen, elastin or fibronectin. Values are expressed as dpm. Values represent mean ± SEM (n=6). Graph shown is representative of 3 individual experiments.

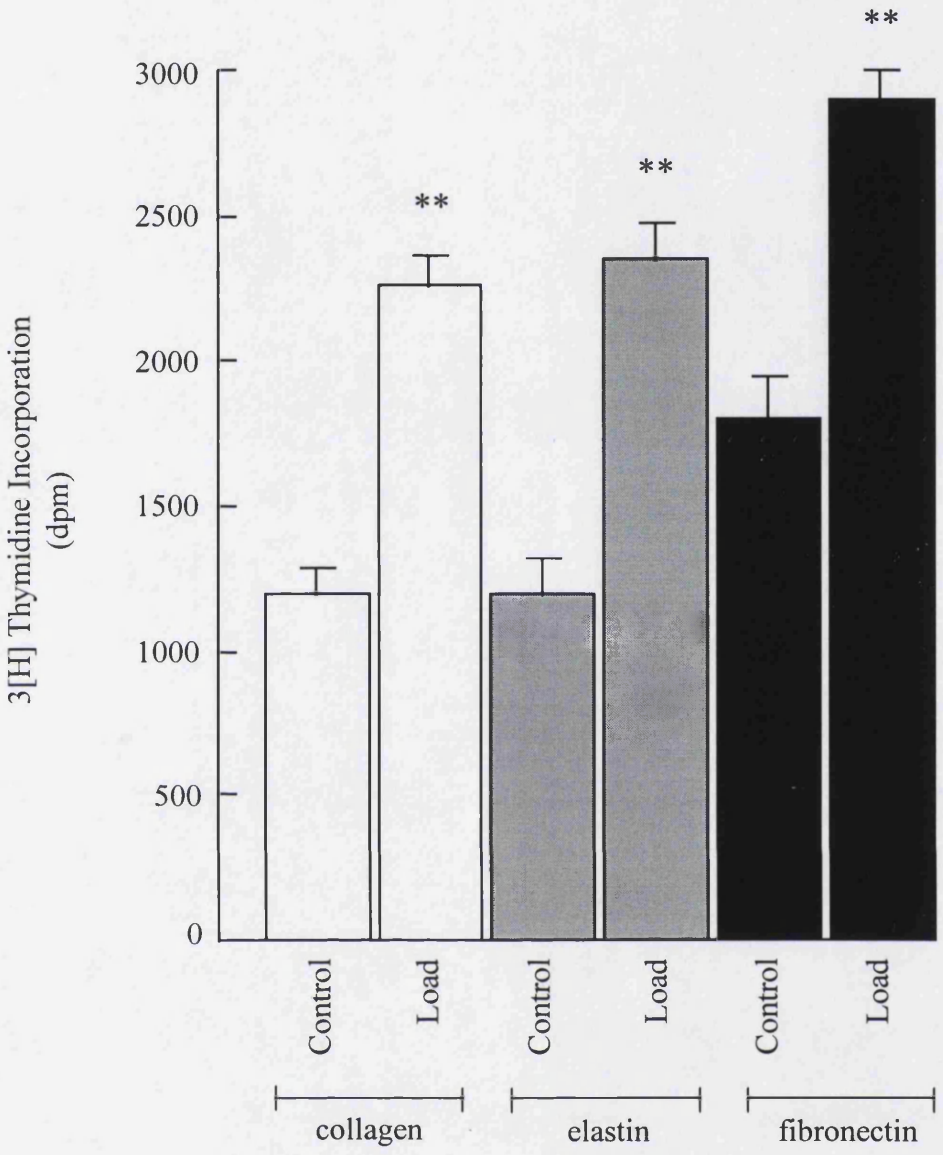


Figure 3.8. The effect of 24 hrs mechanical load and 10 % serum on fibroblast replication. Thymidine incorporation was measured in fibroblast cultures grown on collagen, elastin or fibronectin. Values are expressed as dpm. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples. Graph shown is representative of 3 individual experiments.

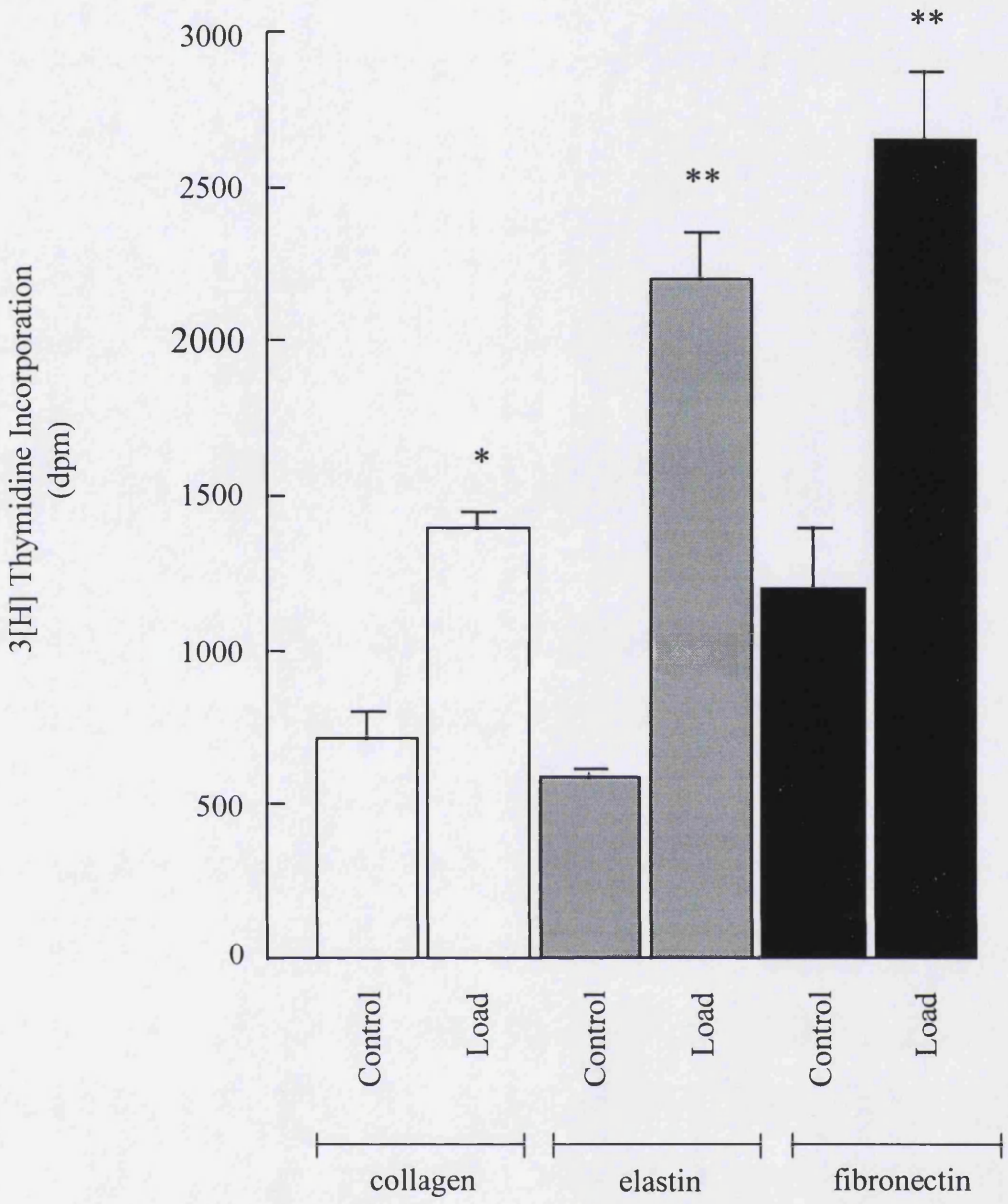


Figure 3.9. The effect of 48 hrs mechanical load and 10 % serum on fibroblast replication. Thymidine incorporation was measured in fibroblast cultures grown on collagen, elastin or fibronectin. Values are expressed as dpm. Values represent mean ± SEM (n=6). *P<0.05, **P<0.01 is the level of significance of the difference between loaded and rigid control samples. Graph shown is representative of 3 individual experiments.

fibronectin, 11.3 ± 4.1 with sFn, 28.4 ± 1.9 without sFn, all $p<0.01$) (Figure 3.10).

A parallel experiment was set up to determine the effect of soluble fibronectin on cell number in response to mechanical load. Table 3.4 shows that cell number did not significantly change by addition of soluble fibronectin.

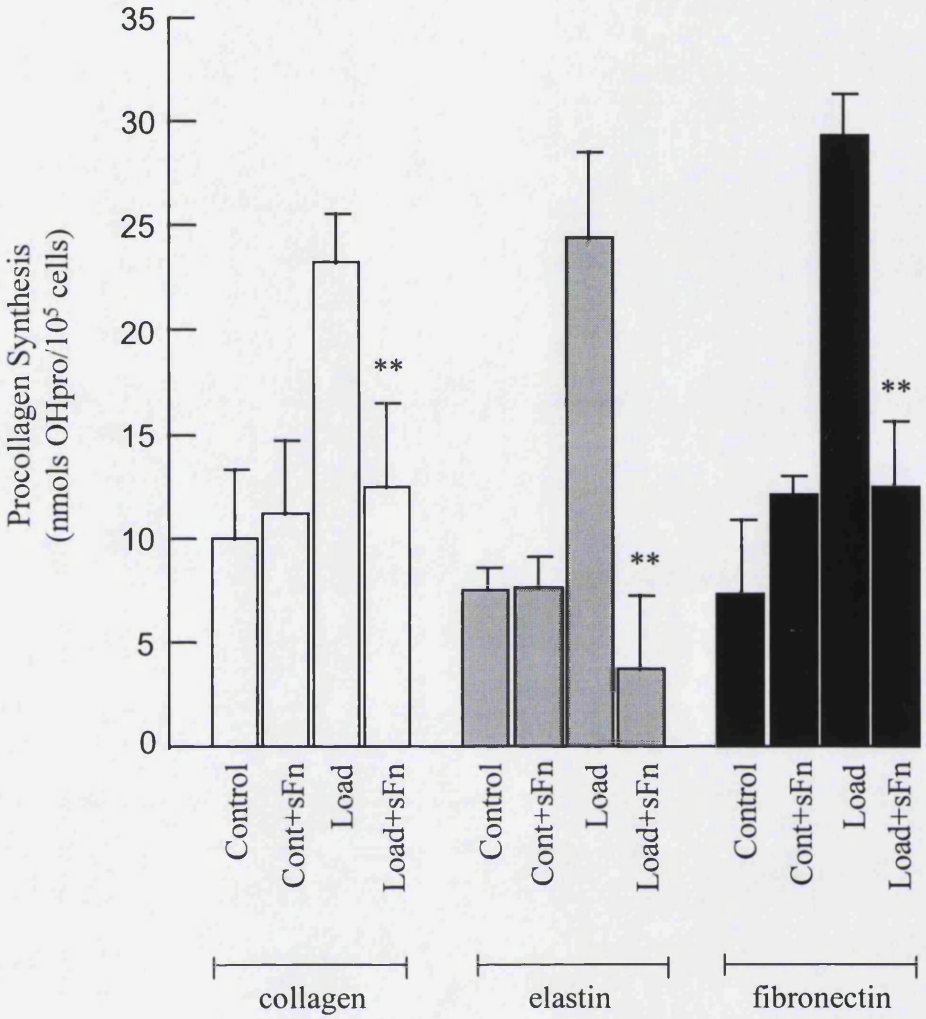


Figure 3.10. The effect of soluble fibronectin on procollagen synthesis. Procollagen synthesis levels were measured in cultures of fibroblasts grown on all three different matrices. Fibroblasts were mechanically loaded for 48 hrs in 10 % serum, in the presence or absence of soluble fibronectin. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01is the level of significance of the difference between loaded and loaded + sFn samples. (sFn=soluble fibronectin). Graph shown is representative of 3 individual experiments.

	T0	48 hr rigid	+ sFn	48 hr load	+ sFn
collagen	2.33±0.27	2.49±0.21	2.52±0.08	3.19±0.06	2.93±0.05
elastin	2.58±0.67	2.52±0.08	2.48±0.19	2.30±1.15	2.30±0.04
fibronectin	2.51±0.34	2.60±0.31	2.30±0.04	3.75±0.26*	3.36±0.32

Table 3.4. The effect of soluble fibronectin (sFn) and mechanical load on cell number in cultures of fibroblasts grown on collagen, elastin or fibronectin. Values are expressed as cell number (x 10^5). Values represent mean ± SEM (n=6). *P<0.05 is the level of significance of the difference between loaded and unloaded samples.

3.2.5 Summary

The results described in this section, examining the effect of extracellular matrix composition on human cardiac fibroblast procollagen synthesis and replication in response to mechanical load, showed that:

a) Mechanical load stimulates procollagen synthesis in human cardiac fibroblasts.

b) In the presence of serum, procollagen synthesis is enhanced after 48 hrs of mechanical load by fibroblasts grown on all matrices.

c) In the presence of serum, after 24 hrs mechanical load, only those fibroblasts grown on the fibronectin matrix showed an increase in procollagen synthesis.

d) In the absence of serum, procollagen synthesis is only increased by load in cells grown on the fibronectin matrix. This occurs at both 24 and 48 hrs.

e) Fibroblast replication in response to mechanical load is not affected by matrix composition, and is increased on all matrices at 24 and 48 hrs. Serum is required, on all matrices, for this response.

f) Soluble fibronectin blocks mechanical load induced procollagen synthesis on all matrices.

CHAPTER 4

THE ROLE OF INTEGRINS IN MECHANO-SIGNAL TRANSDUCTION IN HUMAN CARDIAC FIBROBLASTS

4.1 Recent evidence in the literature has suggested that integrins may function as mechano-signal transducers in certain cell types. They may act to transduce signals from the extracellular matrix to the interior of the cell. This in turn acts to trigger specific signalling pathways. Integrins have been implicated as mechanotransducers in HUVECs (Ishida *et al*, 1996), osteoblasts/osteoclasts (Pavalko *et al*, 1998) and cardiac myocytes (Sadoshima *et al*, 1992). At the time of writing, there is no evidence in the literature implicating integrins as mechanotransducers in human cardiac fibroblasts.

Results from the previous chapter suggested that fibronectin enhances the effect of mechanical load on procollagen synthesis at both 24 and 48 hrs over other matrix components tested. This lead to the hypothesis that fibronectin integrins act as mechanotransducers in these cells. In order to examine the role of two fibronectin integrins, $\alpha5\beta1$ and $\alpha v\beta3$, and one collagen integrin, $\alpha2\beta1$, as mechanotransducers, these "receptors" were blocked by addition of anti-integrin blocking antibodies.

4.1.1 Effect of anti-$\alpha5\beta1$ and anti-$\alpha v\beta3$ antibody on fibroblast procollagen synthesis on fibronectin Flex I® plates

A dose response for the anti-$\alpha5\beta1$ blocking antibody showed that maximal blocking occurred with the highest dose of the blocking antibody (0.1 μg/ml) (Figure 4.1 and 4.2). Mechanical load induced procollagen synthesis by fibroblasts cultured on fibronectin was blocked by addition of the anti-$\alpha5\beta1$ blocking antibody (Figure 4.3.a.). Cell number was not affected by addition of the blocking antibody (Table 4.1).

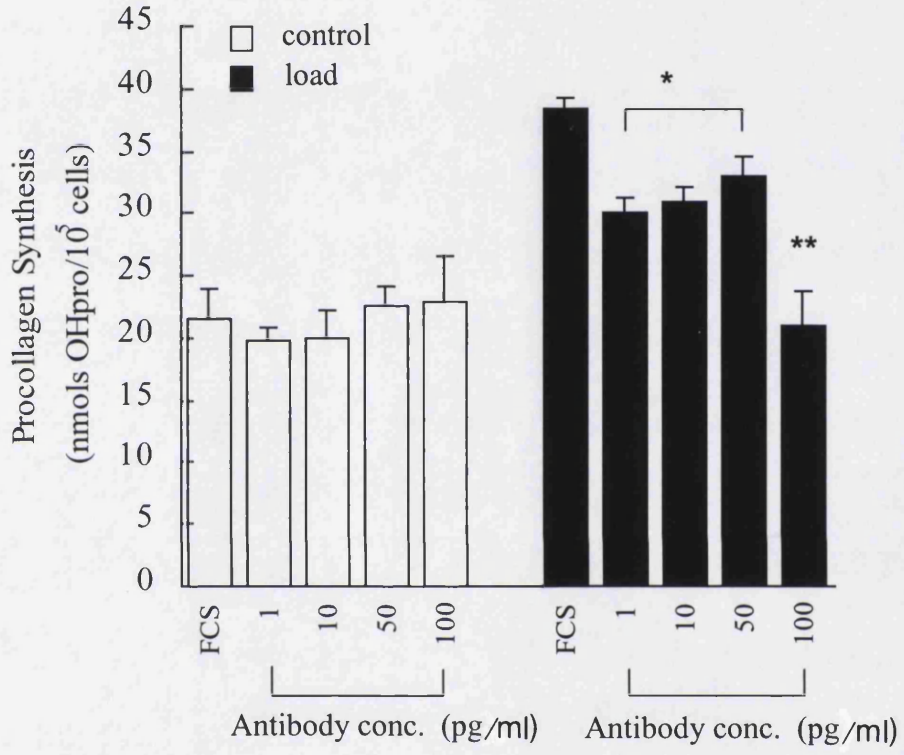


Figure 4.1. Dose response for anti-α5β1 after 24 hrs mechanical load. Anti-α5β1 blocked procollagen synthesis in a dose dependent manner in the presence of serum on fibronectin Flex I® plates. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and loaded + antibody samples.

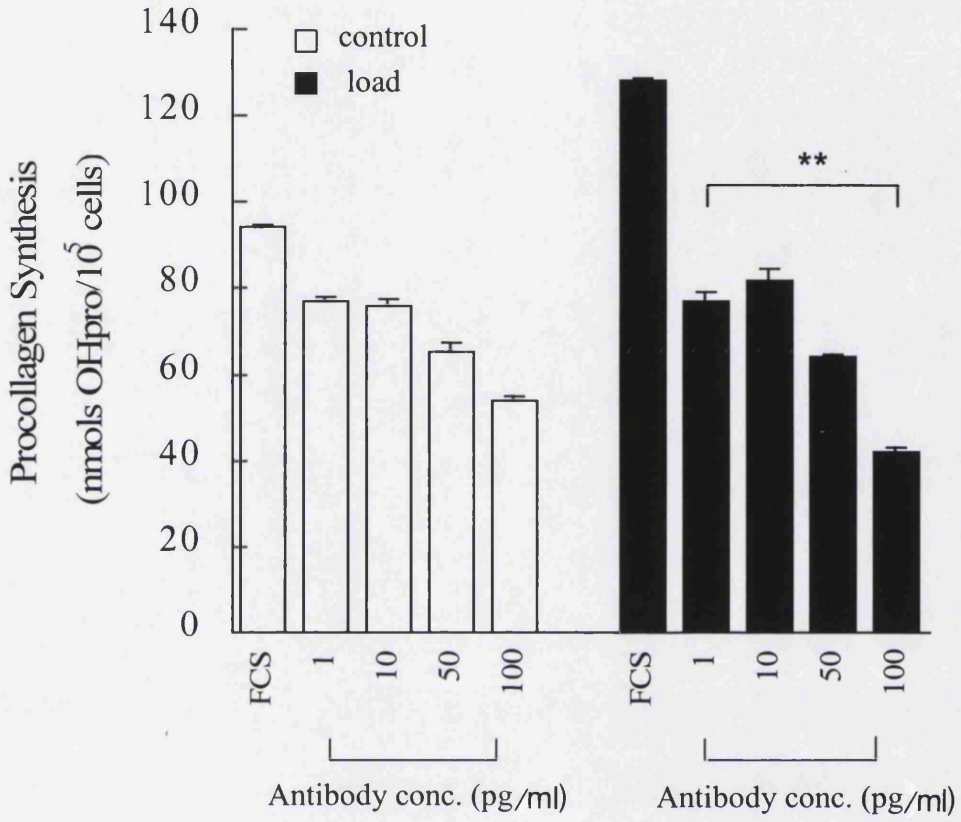


Figure 4.2. Dose response of anti-α5β1 after 48 hrs mechanical load. Anti-α5β1 inhibited mechanical load induced procollagen synthesis in a dose dependent manner on fibronectin coated Flex I® plates. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and loaded + antibody samples.

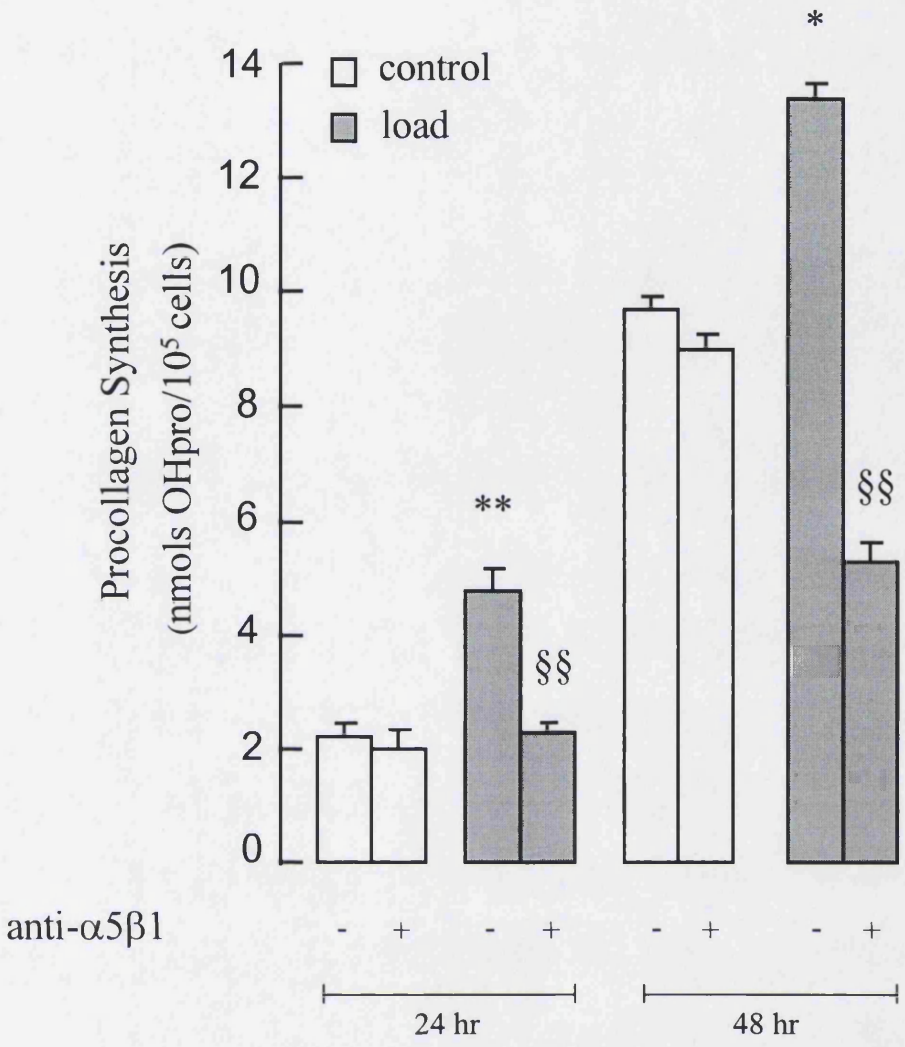


Figure 4.3a. The effect of anti-α5β1 (0.1 μg/ml) antibody on mechanical load induced procollagen synthesis on fibronectin plates. Fibroblasts were mechanically loaded for 24 and 48 hrs in the absence of serum. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples. §§P<0.01 is the level of significance of the difference between loaded and loaded + antibody samples. Graph shown is representative of 3 individual experiments.

	T0	24 hr rigid	24 hr load	48 hr rigid	48hr load
- antibody	3.53 ± 0.26	3.20 ± 0.13	3.91 ± 0.17	3.75 ±0.21	3.50 ± 0.32
anti- α5β1	NA	3.48 ± 0.16	3.50 ± 0.07	3.65 ± 0.07	3.50 ± 0.04
- antibody	2.94 ± 0.06	2.61 ± 0.18	2.85 ± 0.43	2.48 ± 0.21	2.29 ± 0.05
anti- αvβ3	NA	2.45 ± 0.13	2.49 ± 0.23	2.60 ± 0.17	2.68 ± 0.13
anti- α2β1	NA	2.60 ± 0.17	2.80 ± 0.12	2.47 ± 0.22	2.10 ± 0.13

Table 4.1. The effect of the anti-α5β1, αvβ3 and α2β1 and mechanical load on cell number on fibronectin plates. Fibroblasts were mechanically loaded in the absence of serum. Values are expressed as cell number (x 10^5). Values represent mean ± SEM (n=6). NA= not applicable.

Anti-αvβ3 antibody had a similar effect on mechanical load induced procollagen synthesis to anti-α5β1 (Figure 4.3.b.). Cell number was not significantly affected by addition of the antibody to the cell cultures (Table 4.1).

4.1.2 Effect of anti-α2β1 blocking antibody on procollagen synthesis on fibronectin Flex I® plates

Addition of anti-α2β1 to fibroblasts did not significantly affect mechanical load induced procollagen synthesis (Figure 4.4). Cell number was not affected by addition of the blocking antibody (Table 4.1).

4.1.3 Effect of anti-α5β1 blocking antibody on procollagen synthesis on collagen Flex I® plates

Addition of anti-α5β1 to fibroblasts inhibited mechanical load induced procollagen synthesis after 48 hrs (Figure 4.5) (in the presence of serum). After 24hrs there was no increase in procollagen synthesis when comparing rigid versus loaded samples (Figure 4.5). Cell number was not significantly affected by the addition of the antibodies to the culture medium (Table 4.2).

4.1.4 Effect of anti-α2β1 blocking antibody on procollagen synthesis on collagen Flex I® plates

Addition of anti-α2β1 did not inhibit mechanical load induced procollagen synthesis at 48 hr (Figure 4.6). As indicated above there was no effect of mechanical load on procollagen synthesis at 24 hrs (Figure 4.6). Cell number was not significantly affected by the addition of the antibodies to the culture medium (Table 4.2).

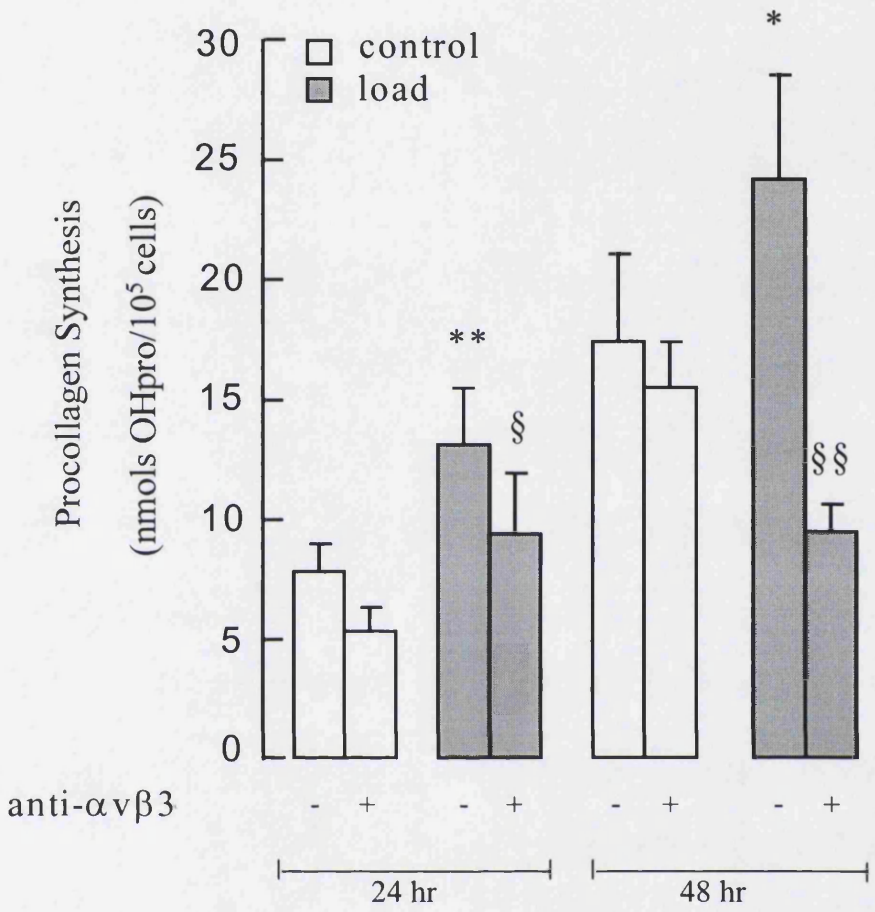


Figure 4.3b. The effect of anti-αvβ3 antibody on mechanical load induced procollagen synthesis on fibronectin plates. . Fibroblasts were mechanically loaded for 24 and 48 hrs in the absence of serum. Values represent mean ± SEM (n=6). **P<0.01 and *P<0.05 is the level of significance of the difference between loaded and rigid control samples. §§P<0.01 and §P<0.05 is the level of significance of the difference between loaded and loaded + antibody samples. Graph shown is representative of 3 individual experiments.

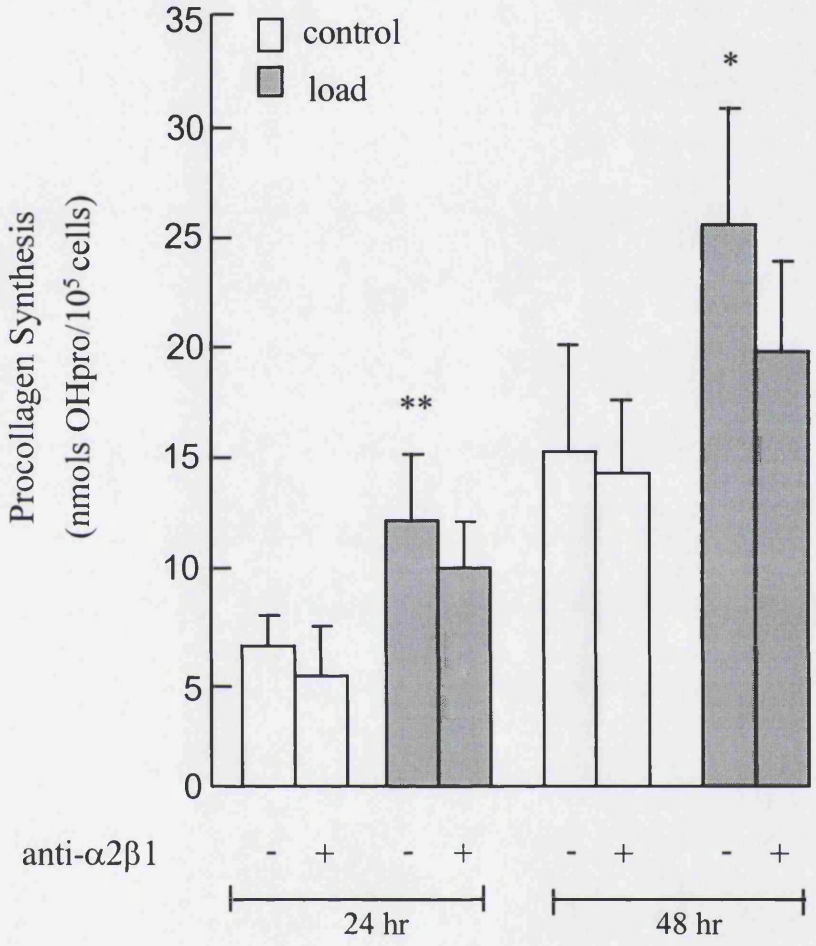


Figure 4.4. The effect of the collagen integrin blocking antibody for α2β1 on mechanical load induced procollagen synthesis. Fibroblasts were loaded on fibronectin plates in the absence of serum for 24 or 48 hrs . Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 and *P<0.05 is the level of significance of the difference between loaded and rigid control samples.

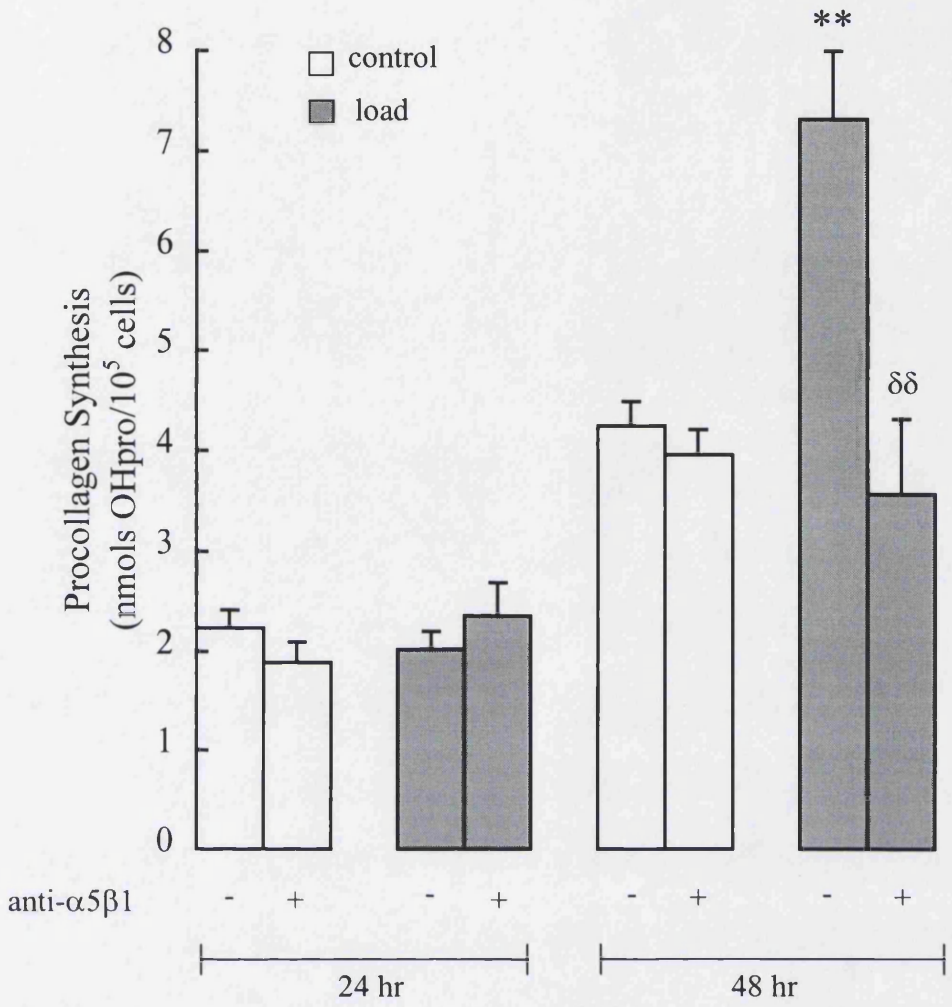


Figure 4.5. The effect of anti-α5β1 on mechanical load induced procollagen synthesis on collagen plates in the presence of 10 % FCS. Fibroblasts were mechanically loaded for 24 and 48 hrs and procollagen synthesis was measured. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between rigid and loaded samples. $^{\delta\delta}$P<0.01 is the level of significance of the difference between loaded and loaded + antibody samples.

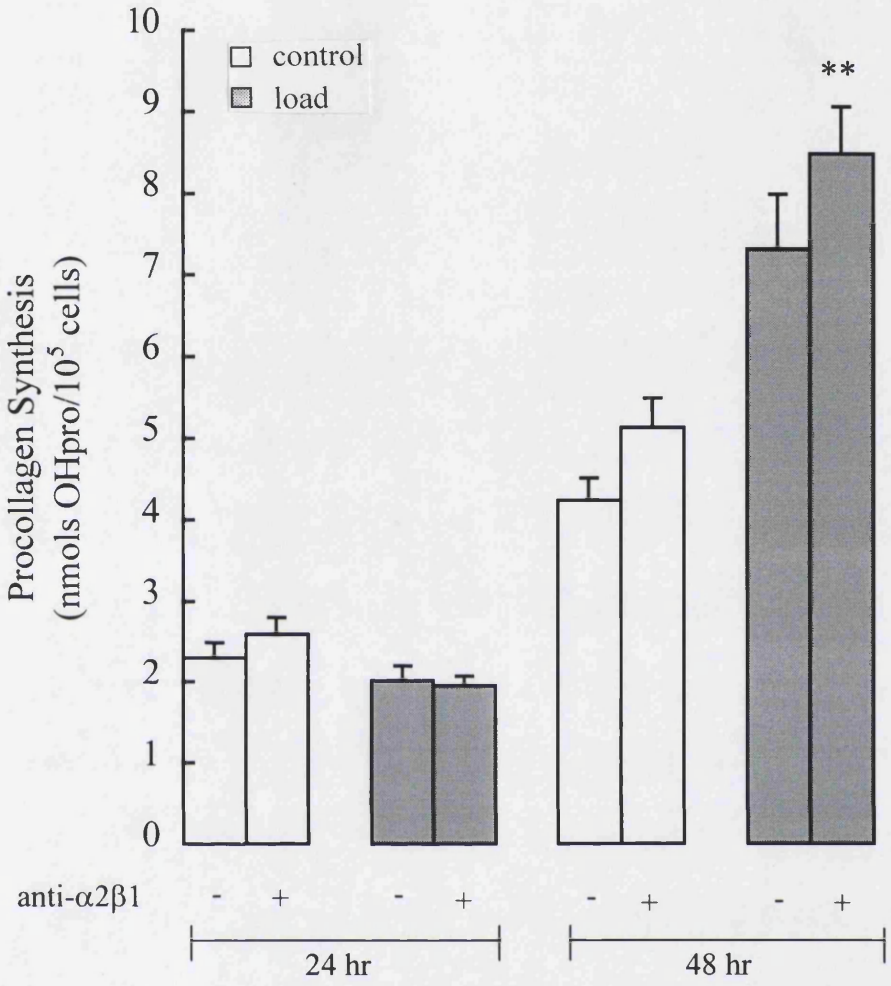


Figure 4.6. The effect of anti-α2β1 on mechanical load induced procollagen synthesis in the presence of 10 % FCS. Fibroblasts were mechanically loaded for 24 and 48 hrs on collagen Flex I®plates. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between rigid and loaded samples.

	T0	24 hr rigid	24 hr load	48 hr rigid	48 hr load
- antibody	2.44 ± 0.08	2.58 ± 0.14	2.42 ± 0.16	2.50 ± 0.11	2.53 ± 0.14
+ anti-α5β1	NA	2.49 ± 0.06	2.60 ± 0.11	2.86 ± 0.11	2.86 ± 0.12
+ anti-α2β1	NA	2.54 ± 0.18	2.63 ± 024	2.55 ± 0.07	2.45 ± 0.17

Table 4.2. The effect of the anti-α5β1 and α2β1 and mechanical load on cell number. Fibroblasts were mechanically loaded on collagen Flex I® plates and mechanically loaded in the presence of serum. Values are expressed as cell number ($x10^5$). Values represent mean ± SEM (n=6).

4.1.5 Integrin expression in response to mechanical load

Fibroblasts were mechanically loaded on fibronectin Flex I® plates in the absence of serum for 24 or 48 hrs and on collagen Flex I® plates in the presence of serum for 24 or 48 hrs. Cells were biotinylated and the integrin subunits α5, β1 and α2 immunoprecipitated, followed by Western blot analysis. α5 integrin subunit expression did not change in response to mechanical load at either time point examined, whereas α2 integrin subunit expression increased in response to load on the fibronectin plates (Figure 4.7). Integrin expression on collagen Flex I® plates followed a similar pattern to those fibroblasts grown on fibronectin. An increase in α2 integrin expression was only observed in response to mechanical load on the collagen plates.

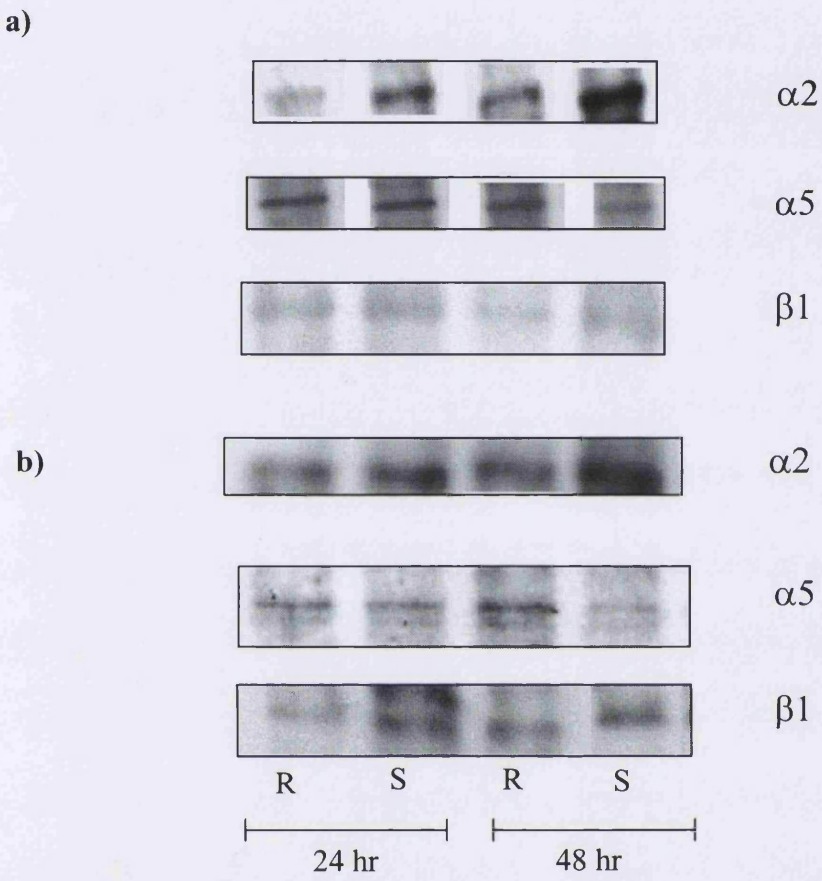


Figure 4.7. Western blots showing the effect of mechanical load on integrin expression. Fibroblasts were mechanically loaded on fibronectin (a) or collagen (b) Flex I® plates. Integrin subunits α5, α2 and β1 were immunoprecipitated and the samples analysed by Western blotting. Blots shown are representative of 2 individual experiments. R=rigid, S=stretch.

4.1.6 Summary

The results from this chapter suggest that in human cardiac fibroblasts, integrins can act as mechanotransducers converting the mechanical signal into a stimulation of procollagen synthesis. Observations from the experiments in this chapter show that:

a) Blocking fibronectin integrins $\alpha5\beta1$ or $\alpha v\beta3$ inhibits mechanical load induced procollagen synthesis on fibronectin plates. Blocking the collagen integrin $\alpha2\beta1$ has no effect on mechanical load induced procollagen synthesis.

b) Blocking the fibronectin integrin $\alpha5\beta1$ inhibits mechanical load induced procollagen synthesis on collagen plates. Blocking the collagen integrin $\alpha2\beta1$ does not inhibit mechanical load induced procollagen synthesis on collagen plates.

c) Integrin expression of $\alpha5$ does not change in response to mechanical load on either fibronectin or collagen plates.

d) Integrin expression of $\alpha2$ is affected by mechanical load, with an increase after 24 hrs on fibronectin plates and an increase after 48 hrs on collagen plates.

CHAPTER 5

ACTIVATION OF THE FOCAL ADHESION COMPLEX BY MECHANICAL LOAD-ROLE IN MECHANO-SIGNAL TRANSDUCTION

5.1 Upon integrin activation, clustering occurs leading to the formation of focal adhesion complexes (Astier *et al*, 1997, Kornberg *et al*, 1992, Kornberg *et al*, 1991). These may contain a combination of tyrosine kinases and actin binding proteins that are essential for the conversion of extracellular signals into intracellular biochemical signals. This chapter focuses on the potential signalling pathways activated in response to mechanical load immediately after integrin/ligand binding and activation, i.e. the role of some of the proteins that constitute the focal adhesion complex.

For all the experiments described in this and following chapters, human cardiac fibroblasts were cultured on a fibronectin matrix in the absence of serum, and mechanically loaded for either 24 hrs (for procollagen synthesis experiments) or up to 1 hr (for tyrosine phosphorylation experiments) unless otherwise stated.

5.1.1 Focal Adhesion Kinase (FAK) phosphorylation in response to mechanical load

Tyrosine phosphorylation levels of the major focal adhesion complex protein, FAK, were determined in response to a cyclic mechanical load for various times (1-120 mins) (Figure 5.1). FAK was immunoprecipitated and the samples run on an SDS PAGE gel. This was then blotted using a phosphotyrosine antibody (PY99). Mechanical load caused an increase in FAK phosphorylation after 1 min (80 % increase above rigid control). After 5 min of mechanical load FAK phosphorylation levels were 400 % greater than rigid control levels. This decreased after 10 min of mechanical load. At 20 min, phosphorylation levels increased (80 % increase above rigid control). At 30, 60 and 120 min, levels remain at basal FAK levels.

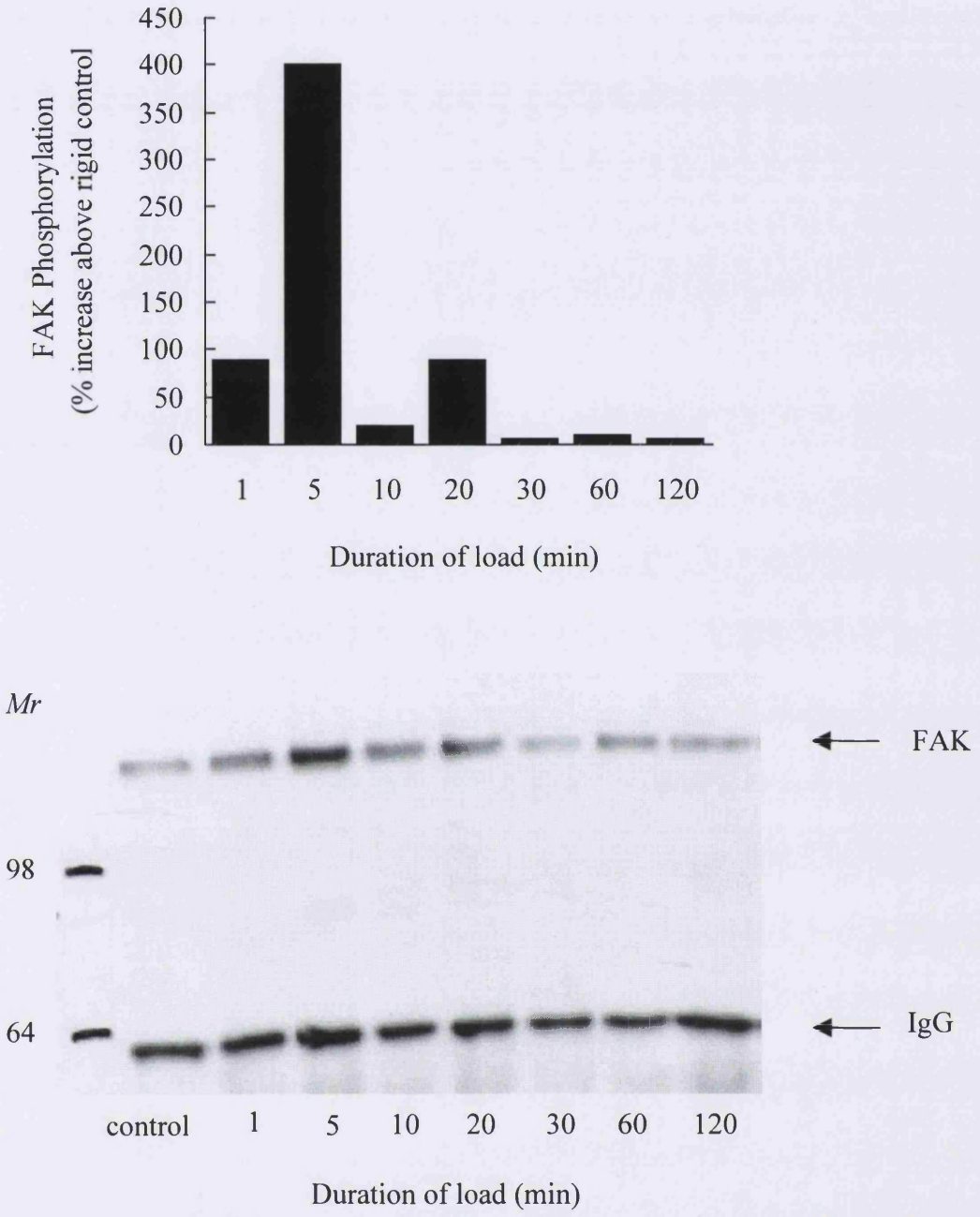


Figure 5.1. The effect of mechanical load on FAK phosphorylation. Graph represents blot shown above. Blot shown is representative of 4 individual experiments. Values are expressed as FAK phosphorylation (% increase above rigid control). Molecular weights (*Mr*) are indicated down the left-hand side of the gel. The lower band represents the heavy chain IgG band.

Table 5.1 shows the reproducibility of the experiments. To determine whether total protein levels of FAK change in response to mechanical load, fibroblasts were mechanically loaded for the same times used above. Samples were subjected to SDS PAGE and the gel was blotted using a FAK antibody. Total FAK levels were not significantly affected by mechanical load compared to the rigid control (Figure 5.2).

5.1.2 Paxillin phosphorylation in response to mechanical load

Paxillin phosphorylation in response to mechanical load responds in a different pattern to that of FAK (Figure 5.3). Mechanical load induced a 42 % increase in paxillin phosphorylation levels above the rigid control after 1 min. However after 5 min, paxillin phosphorylation levels fell (5% increase above rigid control) then increased again after 10 min (41 % increase) and 20 min (37 % increase) of mechanical load. Phosphorylation levels then remain constant to 120 min mechanical load. Table 5.2 shows the reproducibility of the experiments.

Total paxillin levels were not affected by mechanical load (Figure 5.4). There is no significant change in the paxillin protein levels in response to mechanical load.

5.1.3 $p130^{Cas}$ phosphorylation levels in response to mechanical load

Mechanical loading lead to an increase in $p130^{Cas}$ tyrosine phosphorylation levels after 1 min of mechanical load (Figure 5.5) (238 % increase above rigid control). This was decreased after 5 min (100 % increase above rigid control) then decreased dramatically at 10 min to below rigid control levels.

Time of load	# 1	# 2	# 3	# 4
control	0.97	0.96	1.11	0.70
1 min	1.53	1.80	_____	5.93
5 min	0.98	3.86	3.28	3.65
10 min	1.47	1.12	1.18	4.55
20 min	0.88	1.81	1.91	1.03
30 min	1.36	0.54	2.13	2.50
60 min	2.71	0.89	1.85	2.26
120 min	0	0.62	1.18	3.52

Table 5.1. Data from 4 individual experiments examining the effect of mechanical load on FAK phosphorylation. Values are expressed as densitometric units (arbitrary).

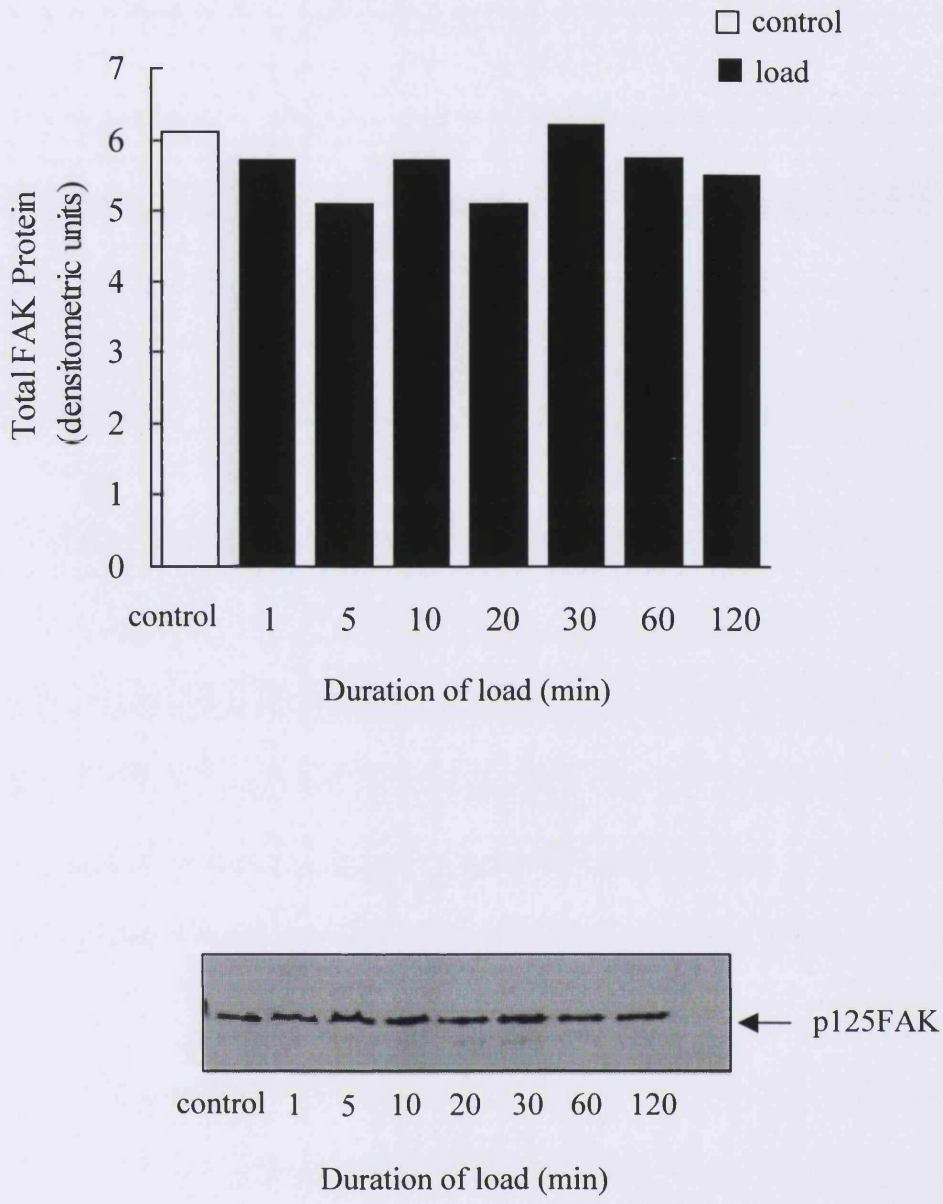


Figure 5.2. The effect of mechanical load on total FAK protein levels. Graph represents data from blot shown above. Values are expressed as total FAK protein (densitometric units). Blot shown is representative of 5 individual experiments.

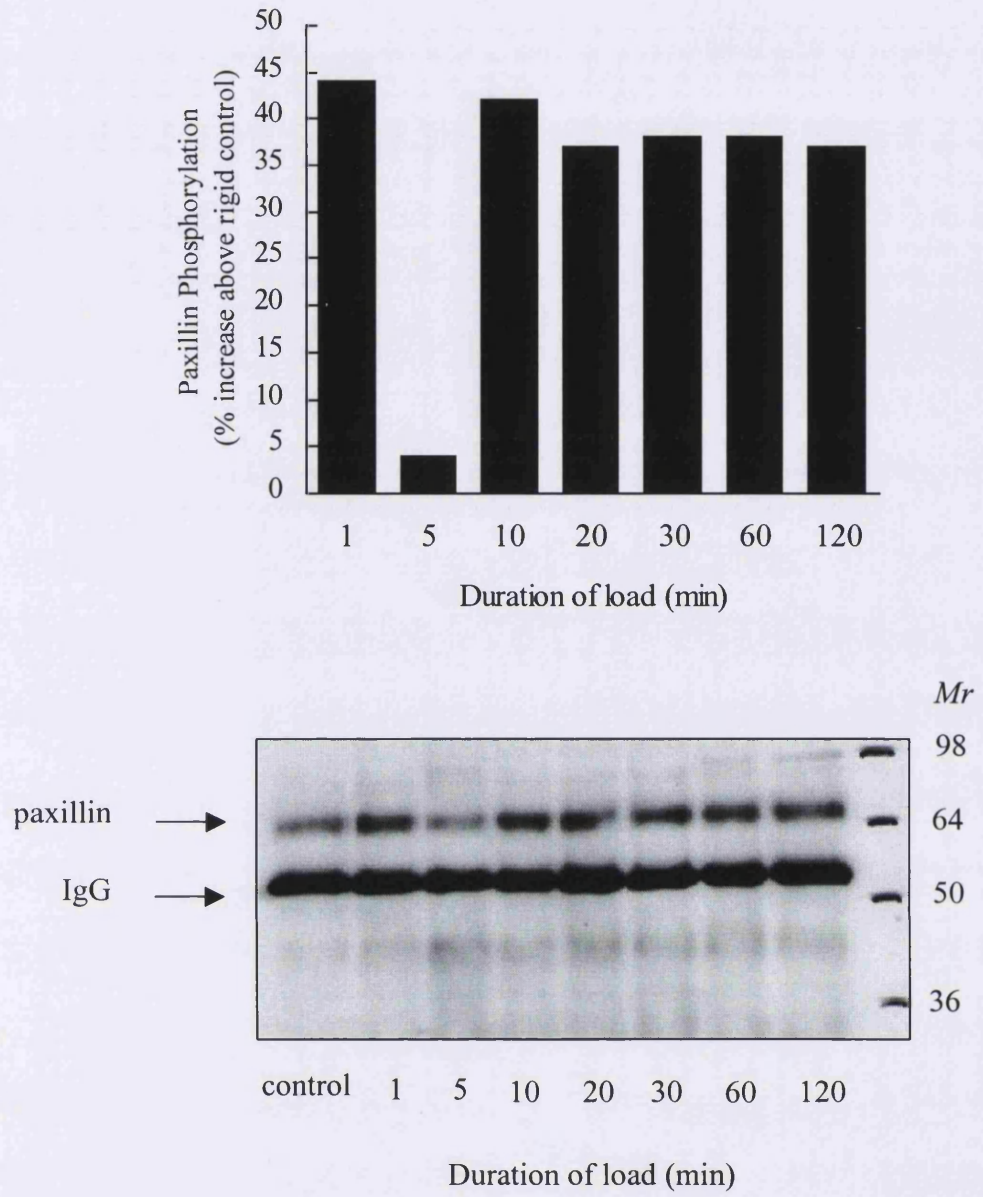


Figure 5.3. The effect of mechanical load on paxillin phosphorylation. Graph represents data from blot shown above. Values are expressed as paxillin phosphorylation (% increase above rigid control). Blot shown is representative of 3 individual experiments. Molecular weights (*Mr*) are indicated down the right-hand side of the gel.

Time of load (min)	#1	#2	#3
control	5.88	7.39	3.79
1	6.88	9.03	5.64
5	5.14	7.52	3.88
10	7.30	8.63	2.10
20	3.68	8.97	7.17
30	3.90	5.97	6.36
60	3.80	5.66	6.66
120	6.26	6.99	8.22

Table 5.2. The effect of mechanical load on paxillin phosphorylation from 3 individual experiments. Values are expressed as densitometric units (arbitrary).

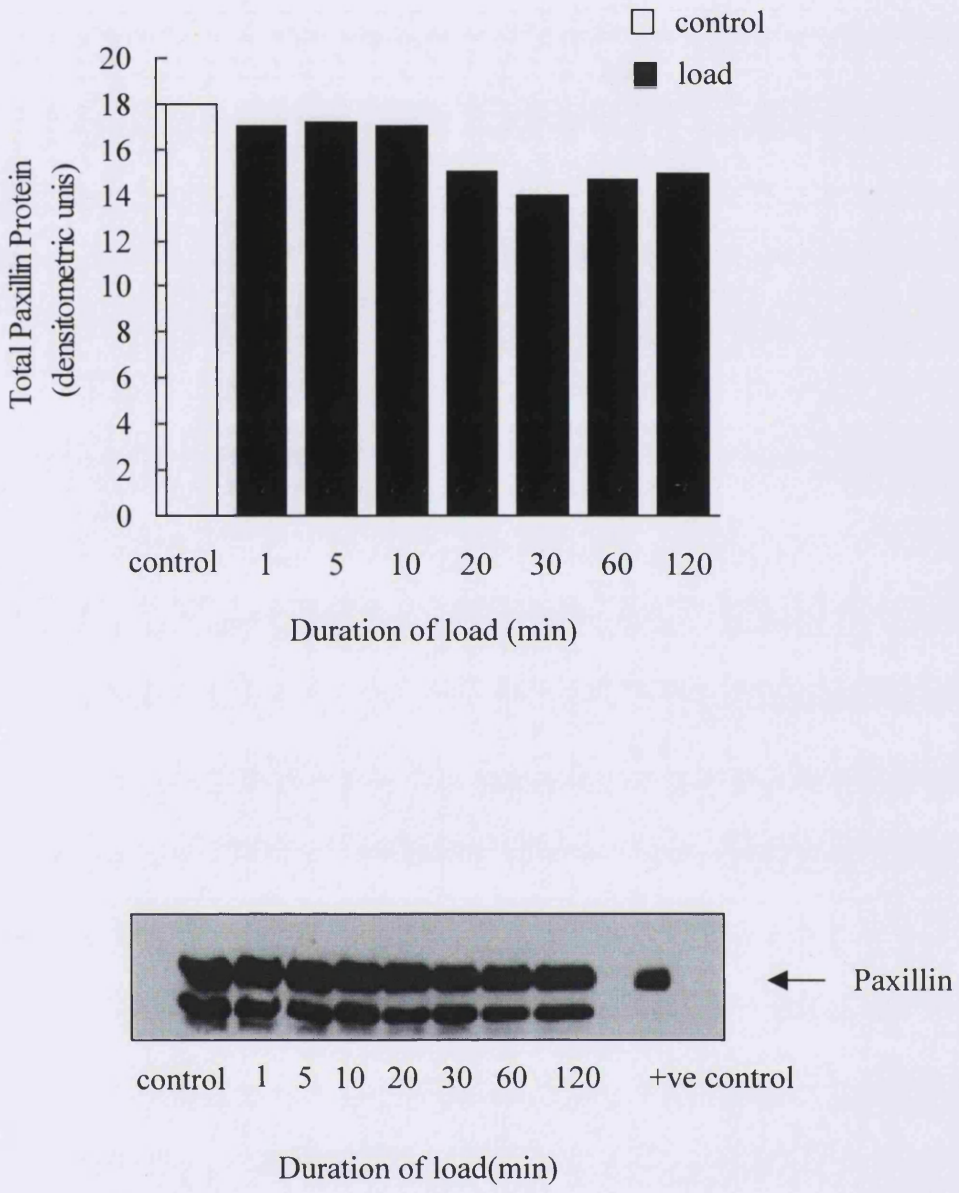


Figure 5.4. The effect of mechanical load on total levels of paxillin protein. Graph represents data from blot shown. Values are expressed as total paxillin protein (densitometric units). Blot shown is representative of 4 individual experiments. The lower band at ~55 kDa represents the heavy chain of IgG.

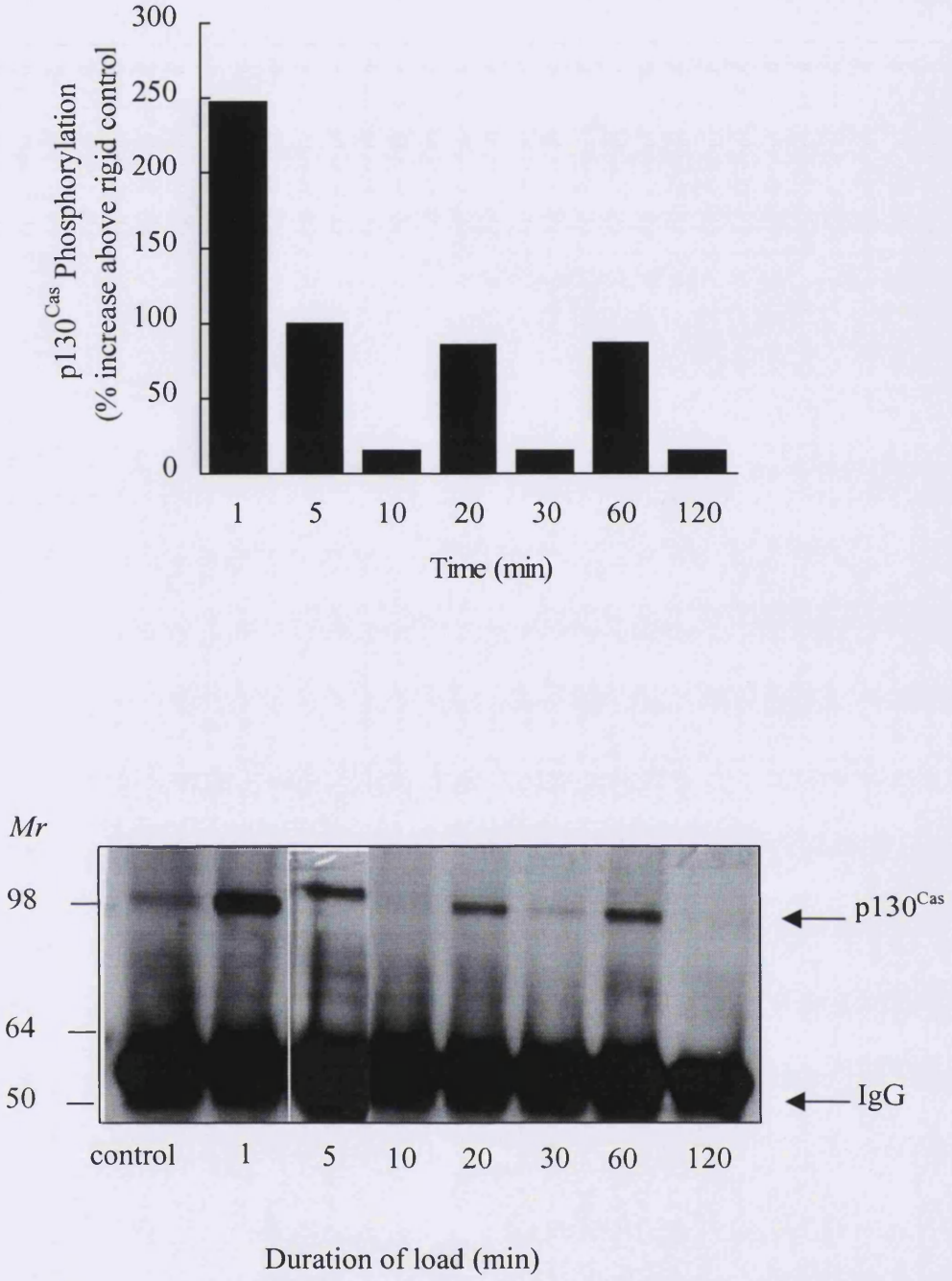


Figure 5.5. The effect of mechanical load on $p130^{Cas}$ phosphorylation. Graph represents data from blot shown above. Values are expressed as $p130^{Cas}$ phosphorylation (% increase above rigid control). Blot shown is representative of 3 individual experiments. Molecular weights (*Mr*) are indicated down the left-hand side of the gel.

A cyclical pattern of phosphorylation is then observed with increases at 20 min (75 % increase above rigid control) and 60 min (76 % increase above rigid control). At both 30 and 120 min $p130^{Cas}$ phosphorylation levels were not significantly different from the rigid control. Table 5.3 shows the reproducibility of the experiments.

Total levels of $p130^{Cas}$ appear not to be affected by mechanical load (Figure 5.6), with no significant changes in total protein levels compared to rigid control.

5.1.4 Effect of anti-α5β1 antibody on mechanical load induced FAK phosphorylation

The anti-α5β1 integrin antibody was added to fibroblast cultures to determine whether FAK phosphorylation by mechanical load requires integrin activation. Addition of the antibody prevented the mechanical load induced FAK phosphorylation (Figure 5.7) after 5 mins mechanical load.

5.1.5 Confocal microscopy of focal adhesion proteins: determining the effect of mechanical load on focal adhesion formation

To examine the effect of mechanical load on focal adhesion formation in fibroblasts, cells were stained with antibodies to various focal adhesion proteins: vinculin, paxillin, α-actinin, FAK, $p130^{Cas}$ as well as the actin cytoskeleton. In response to mechanical load, an increase in paxillin (Figure 5.8) and vinculin (Figure 5.9) staining was observed. The number of focal adhesions number increased dramatically compared to the rigid controls. The focal adhesions appeared to cover the whole cell

Time of load (min)	#1	#2	#3
control	1.95	2.06	3.10
1	8.07	8.78	12.25
5	0.57	7.33	6.40
10	3.77	3.10	5.24
20	0.60	5.10	6.66
30	1.68	2.80	3.92
60	2.48	5.06	5.66
120	2.50	1.69	4.34

Table 5.3. The effect of mechanical load on p130^{Cas} phosphorylation of 3 individual experiments. Values are expressed as densitometric units (arbitrary).

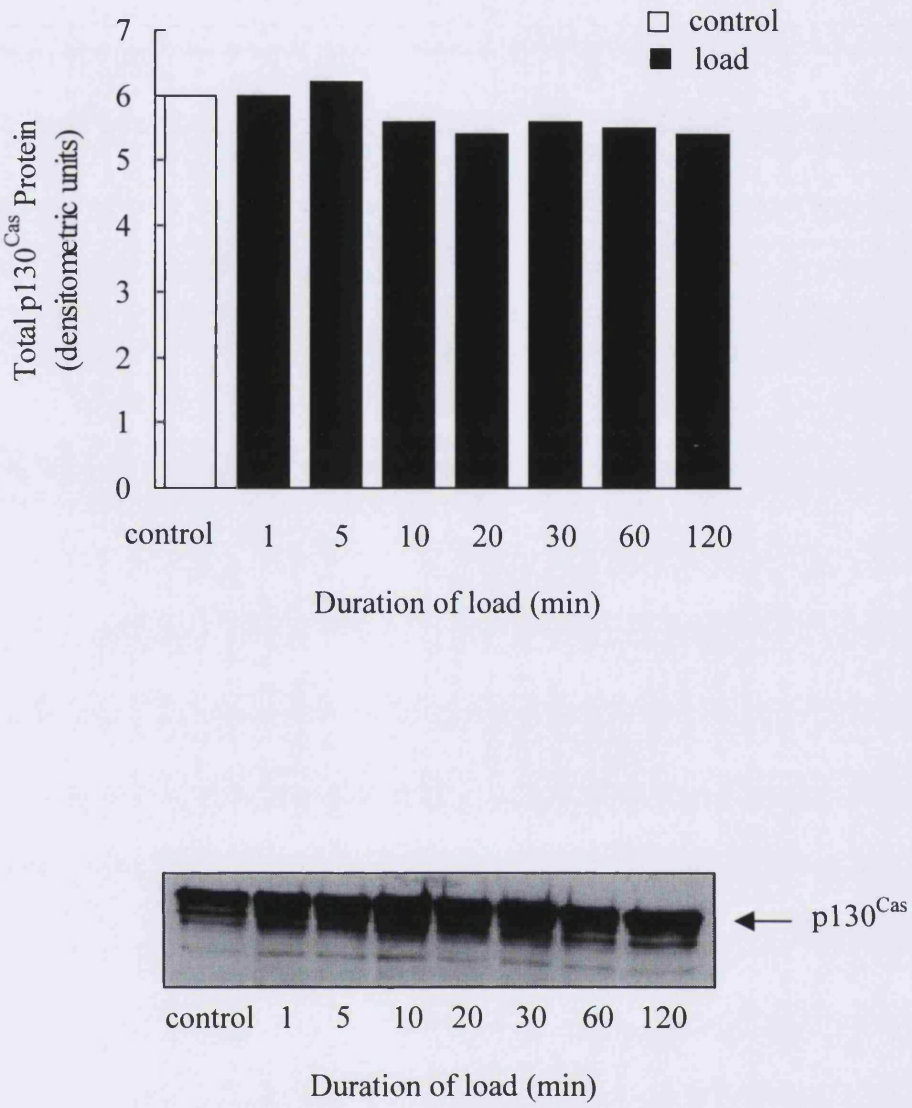


Figure 5.6. The effect of mechanical load on total levels of p130^{Cas} protein. Graph represents data from blot shown above. Values are expressed as total p130^{Cas} protein (densitometric units). Blot shown is representative of 4 individual experiments.

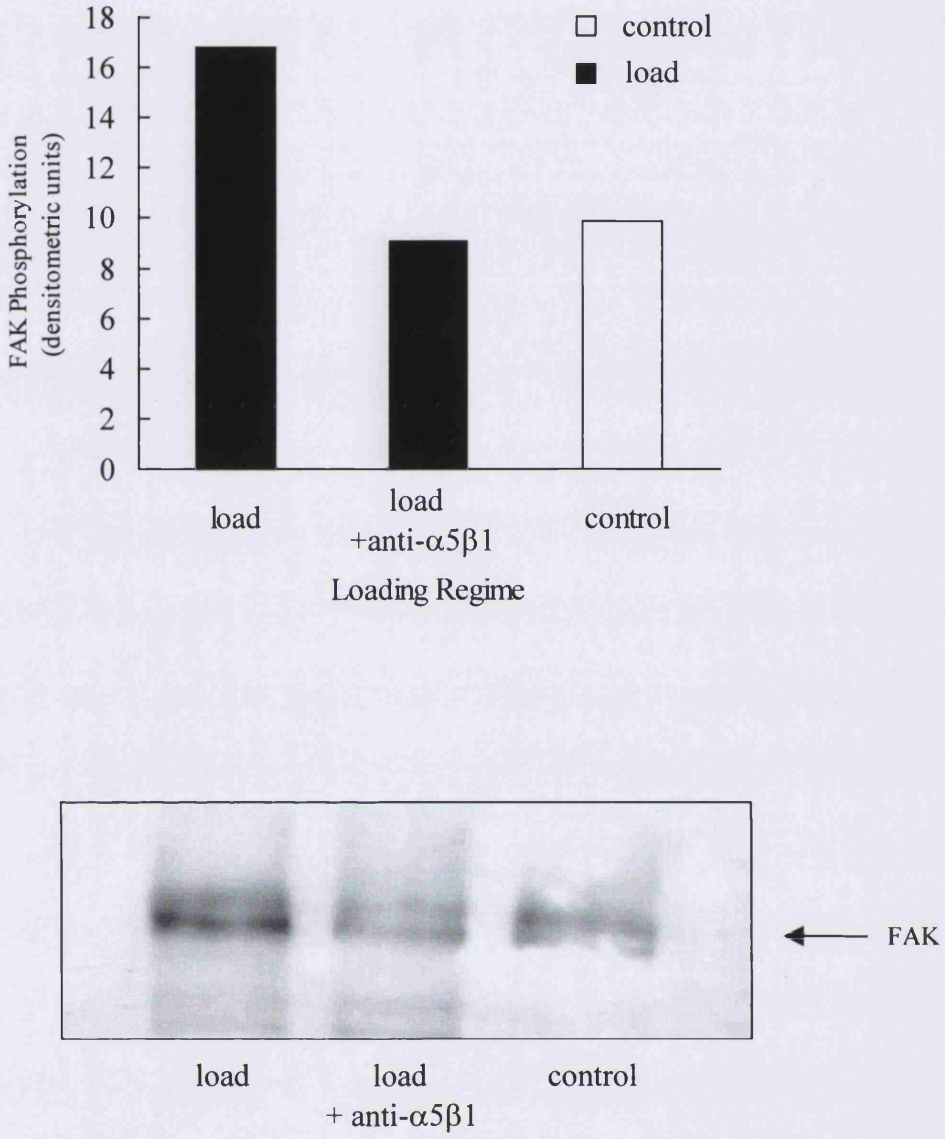


Figure 5.7. The effect of anti-α5β1 integrin blocking antibody on FAK phosphorylation. Fibroblasts were mechanically loaded for 5 min. Graph represents data from blot shown above. Blot shown is representative of 2 individual experiments. Values are expressed as FAK phosphorylation (densitometric units).

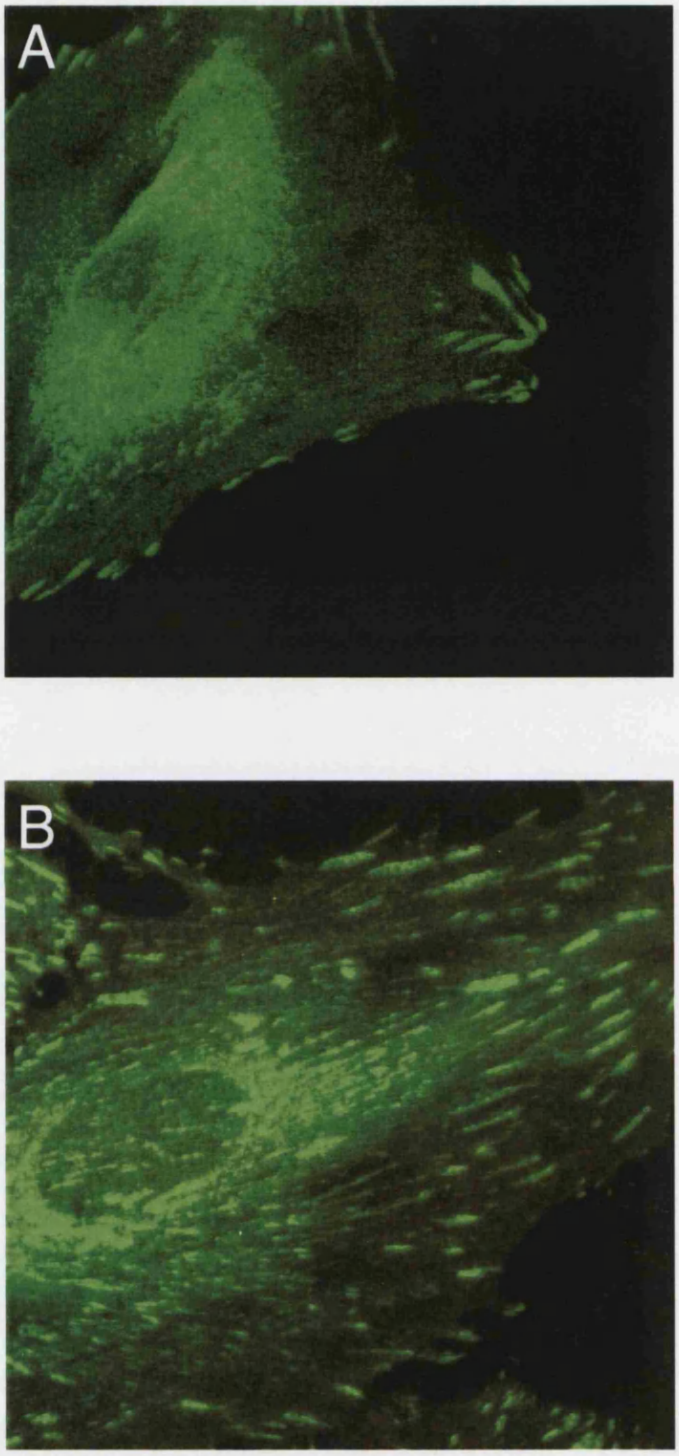


Figure 5.8. The effect of mechanical load on paxillin staining in focal adhesions.

Fibroblasts were either left as rigid controls (A) or mechanically loaded (B).

Magnification x64.

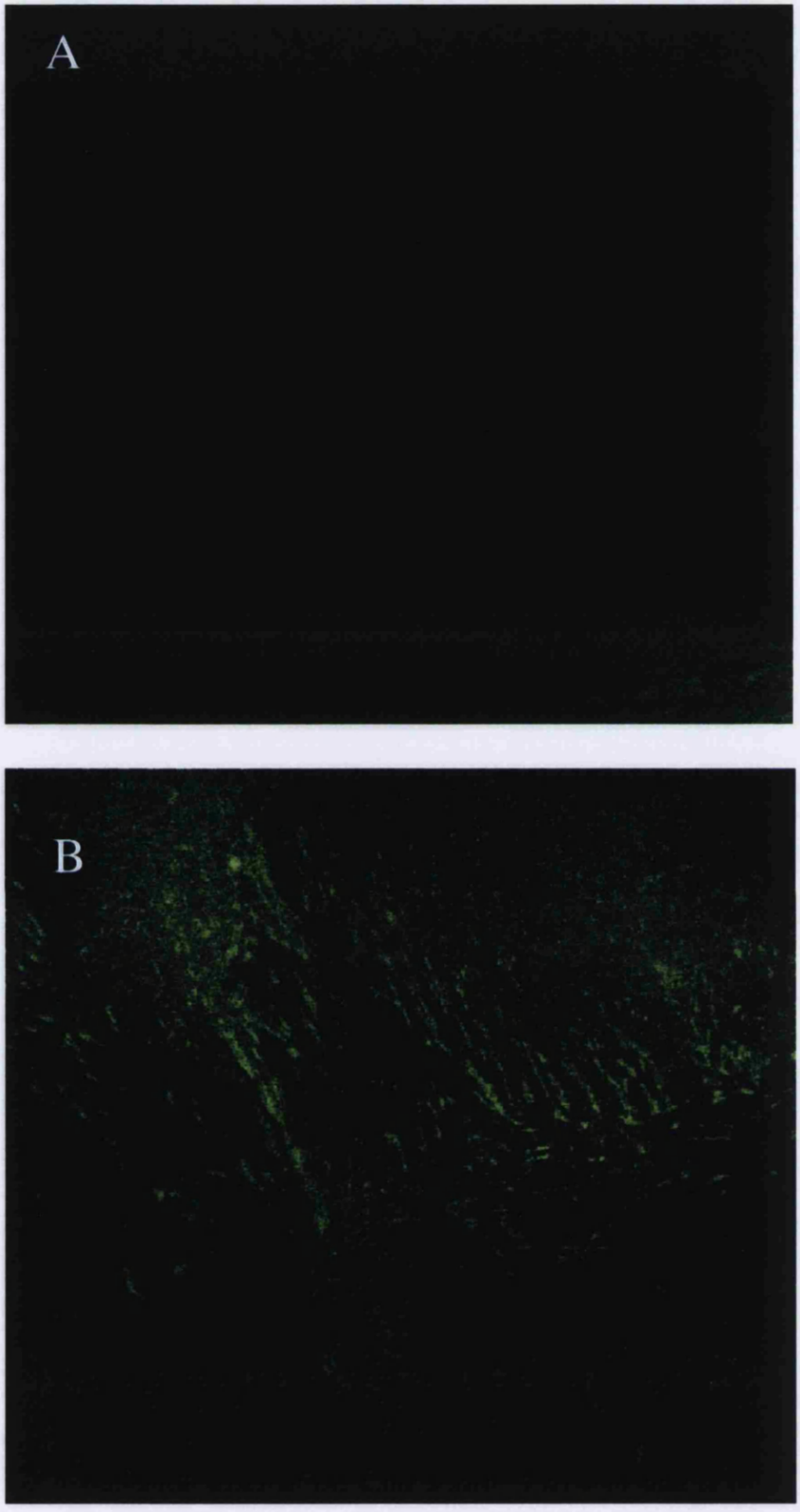


Figure 5.9 Vinculin staining in response to mechanical load. Fibroblasts were left as rigid controls (A) or mechanically loaded (B) in the absence of serum for 1 hr. Magnification x64.

after mechanical loading, whereas in the rigid controls they were localised at the cell periphery. This was particularly noticeable with paxillin. Mechanical load had no visible effect on α-actinin staining after the application of mechanical load (Figure 5.10). Actin stress fibres aligned and increased in number in response to mechanical load. In the rigid control cells the stress fibres had an unordered distribution, with fibres aligning in different directions (Figure 5.11). Immunofluorescent staining for FAK and $p130^{Cas}$ stained negative in all samples examined. Co-localisation of paxillin with the actin cytoskeleton was determined by dual labelling with the paxillin antibody and the rhodamine - phalloidin antibody. Co-localisation is seen as yellow staining (indicated with the arrow). Paxillin was shown to co-localise with the actin cytoskeleton (Figure 5.12).

5.1.6 Effect of tyrphostin 25 on collagen synthesis

To determine the involvement of FAK in a signalling pathway activating procollagen synthesis in response to mechanical load, tyrphostin 25 was added to cultures at doses of 5μM, 10μM and 20μM (Figure 5.13). These doses have been previously reported to inhibit FAK phosphorylation (Yano *et al*, 199). Procollagen synthesis levels were increased 2 fold above control levels in response to mechanical load (2.63±0.30 vs. 1.21±0.04 nmols OHpro/10^5 cells). The stimulation of procollagen synthesis by mechanical load was blocked in a dose dependent manner by tyrphostin 25. All concentrations of tyrphostin 25 blocked the mechanical load induced increase in procollagen synthesis with all loaded samples + tyrphostin 25 being significantly lower than the loaded samples without tyrphostin 25. In the presence of tyrphostin 25, there was no significant difference between rigid and loaded samples.

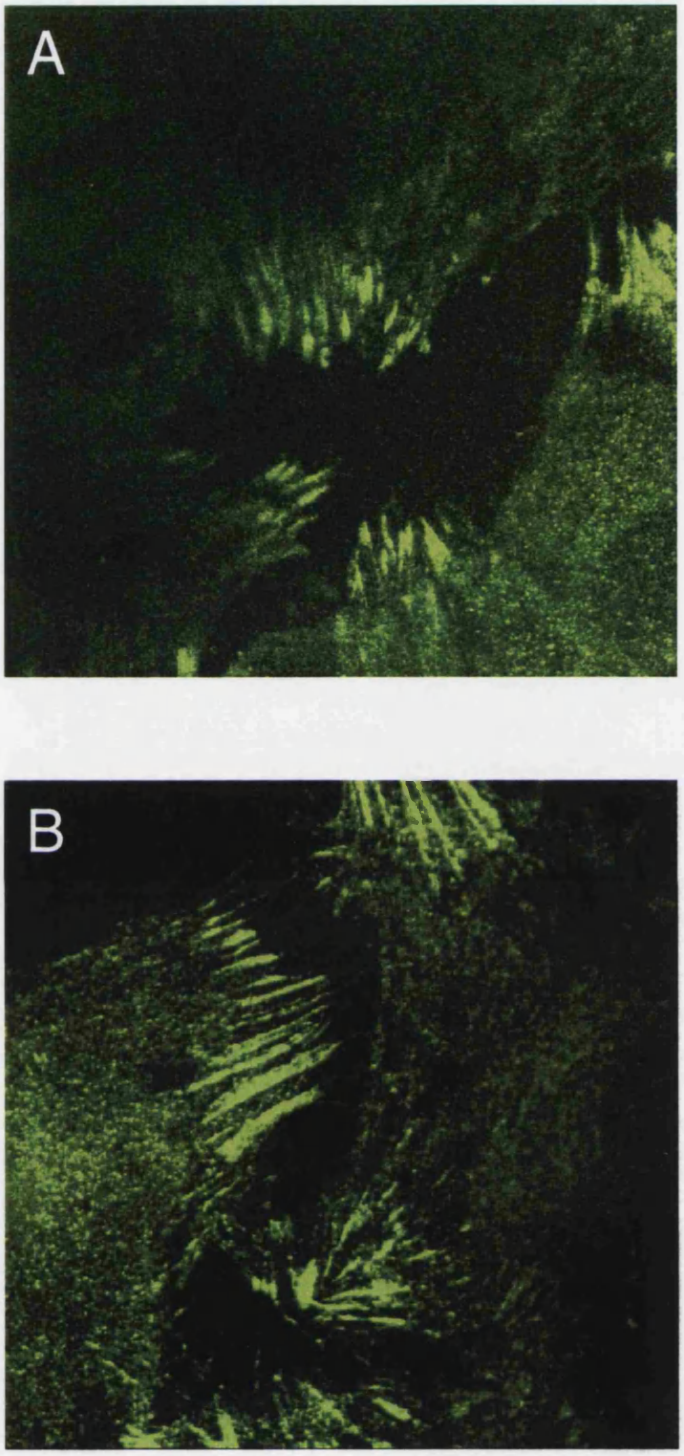


Figure 5.10. The effect of mechanical load on α-actinin staining.

Fibroblasts were either left as rigid controls (A) or mechanically loaded (B).

Magnification x64.

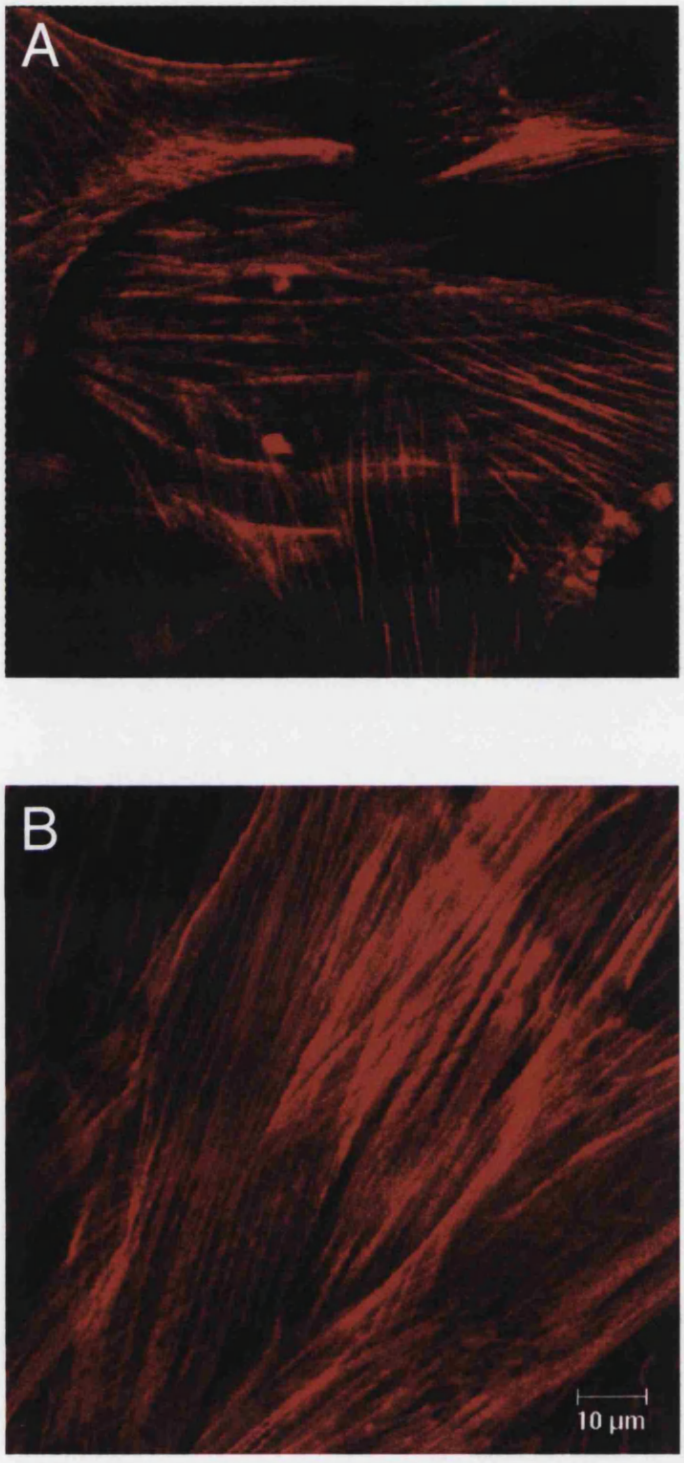
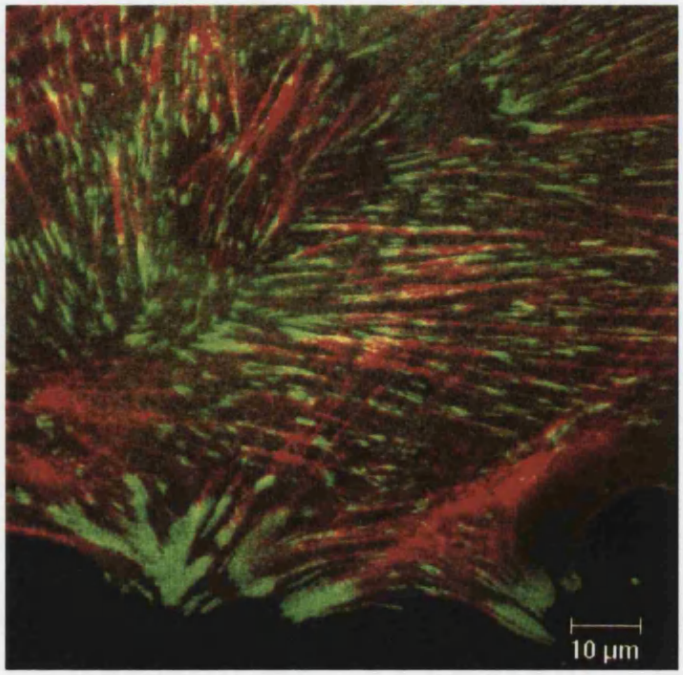


Figure 5.11. The effect of mechanical load on actin stress fibre formation.

Fibroblasts were either left as rigid controls (A) or mechanically loaded (B).

Actin stress fibres were stained with a rhodamine-phalloidin conjugated antibody.

Magnification x64.



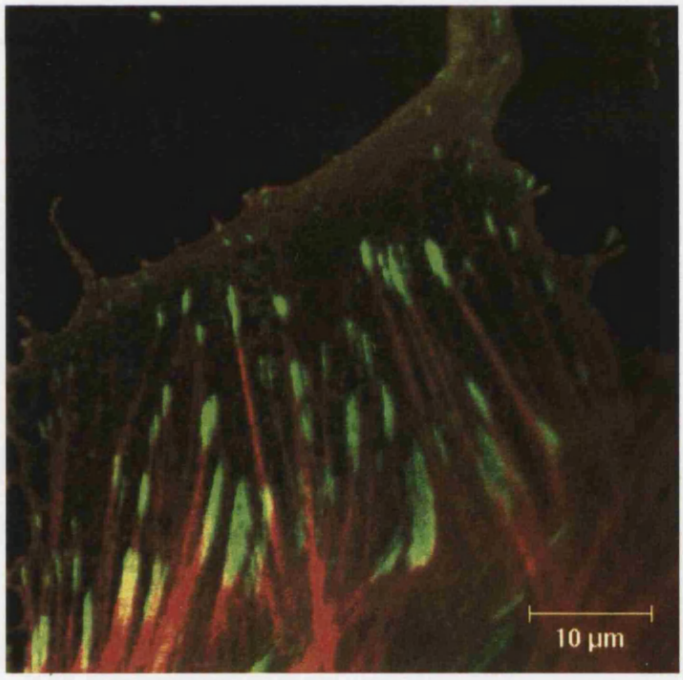


Figure 5.12. Co-localisation of paxillin with the actin cytoskeleton.

Co-localisation is seen as the yellow staining. Magnification x100 (zoom 2)

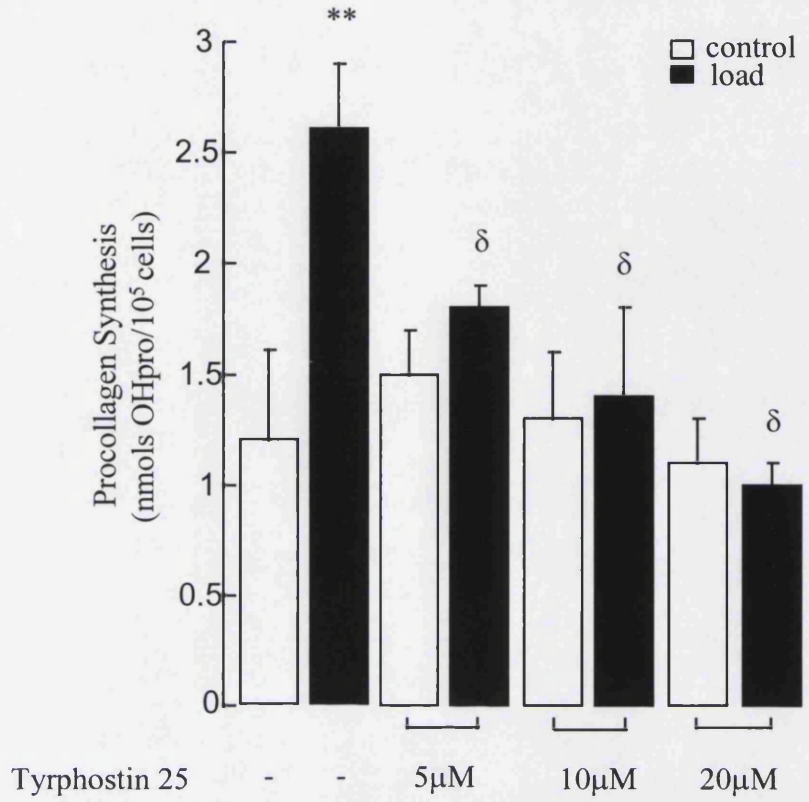


Figure 5.13. The effect of tyrphostin 25 on procollagen synthesis. Procollagen synthesis was measured after 24 hrs of mechanical load. Values expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **$P<0.01$ is the level of significance of the difference between loaded and rigid control samples. $^{\delta}P<0.01$ is the level of significance of the difference between loaded and loaded + tyrphostin 25 samples. Graph shown is representative of 3 individual experiments.

control procollagen synthesis levels were not affected by any concentration of tyrphostin 25. Table 5.4 shows the reproducibility of the experiment. Cell number was affected by addition of tyrphostin 25 in one out of four experiments performed (Table 5.5).

5.1.7 Effect of tyrphostin 25 on cell viability

Due to the potentially toxic nature of tyrphostin 25 and DMSO (used as vehicle), an LDH cell viability assay was performed, to determine the effect of the compound on human cardiac fibroblast viability (Figure 5.14). The highest dose of tyrphostin 25 was used (20μM) for the assay. Cell viability was not affected by the compound, compared to the positive control (supplied in the assay), in either rigid or mechanically loaded cells. DMSO also appeared to have no effect on fibroblast viability compared to the positive control.

5.1.8 Effect of the tyrosine kinase inhibitor tyrphostin 25 on FAK phosphorylation

To determine the effect of tyrphostin 25 on FAK phosphorylation in response to mechanical load, the compound was added to cell cultures at the same doses used to inhibit mechanical load induced procollagen synthesis (5 μM - 20 μM). In response to 5 mins of mechanical load, FAK tyrosine phosphorylation levels increased 58 % above rigid control levels (Figure 5.15). Addition of the inhibitor at all three doses prevented FAK tyrosine phosphorylation in response to load, to below rigid control phosphorylation levels. Table 5.6 shows the reproducibility of the experiments.

Experiment	#1	#2	#3	#4
24 hr rigid	2.99±0.66	1.21±0.04	1.82±0.12	2.84 ± 0.15
24 hr stretch	7.75±0.80**	2.63±0.30**	3.63±0.32**	5.65 ± 0.32**
5μM rigid	3.56±0.26	1.55±0.27	2.30±0.41	3.12 ± 0.41
5μM stretch	3.37±0.32$^{\delta}$	1.84±0.16$^{\delta}$	2.38±0.27$^{\delta}$	3.48 ± 0.06$^{\delta}$
10μM rigid	2.88±0.23	1.39±0.32	2.65±0.40	2.97 ± 0.14
10μM stretch	2.59±0.50$^{\delta}$	1.42±0.40$^{\delta}$	1.30±0.12$^{\delta}$	2.15 ± 0.18$^{\delta}$
20μM rigid	2.74±0.25	1.13±0.21	1.36±0.08	2.78 ± 0.65
20μM stretch	2.38±0.36$^{\delta}$	1.0±0.16$^{\delta}$	0.80±0.12$^{\delta}$	1.99 ± 0.11$^{\delta}$

Table 5.4. The effect of tyrphostin 25 on load induced procollagen synthesis from 4 individual experiments. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples, $^{\delta}$P<0.01 is the level of significance of the difference between loaded and loaded + tyrphostin 25 samples.

Experiment	#1	#2	#3	#4
Rigid	1.93±0.17	2.61±0.11	2.17±0.15	3.20±0.20
Stretch	1.69±0.12	2.84±0.12	2.82±0.18	3.40±0.22
Rigid + 5μM	1.54±0.08	2.74±0.12	2.36±0.38	3.42±0.22
Stretch + 5μM	1.51±0.13	2.68±0.16	3.21±0.17	3.76±0.24
Rigid + 10μM	1.67±0.21	2.42±0.34	2.00±0.16	3.38±0.18
Stretch +10μM	1.35±0.05*	2.75±0.15	3.17±0.18	3.12±0.12
Rigid + 20μM	1.56±0.23	2.50±0.16	2.18±0.16	3.73±0.23
Stretch +20μM	1.43±0.12*	2.46±0.03	3.29±0.14	3.45±0.14

Table 5.5. The effect of tyrphostin 25 on cell number from 4 individual experiments. Values are expressed as cell number (x 10^5 cells). Values represent mean ± SEM. *$P<0.05$ is the level of significance of the difference between loaded and loaded + tyrphostin 25 samples.

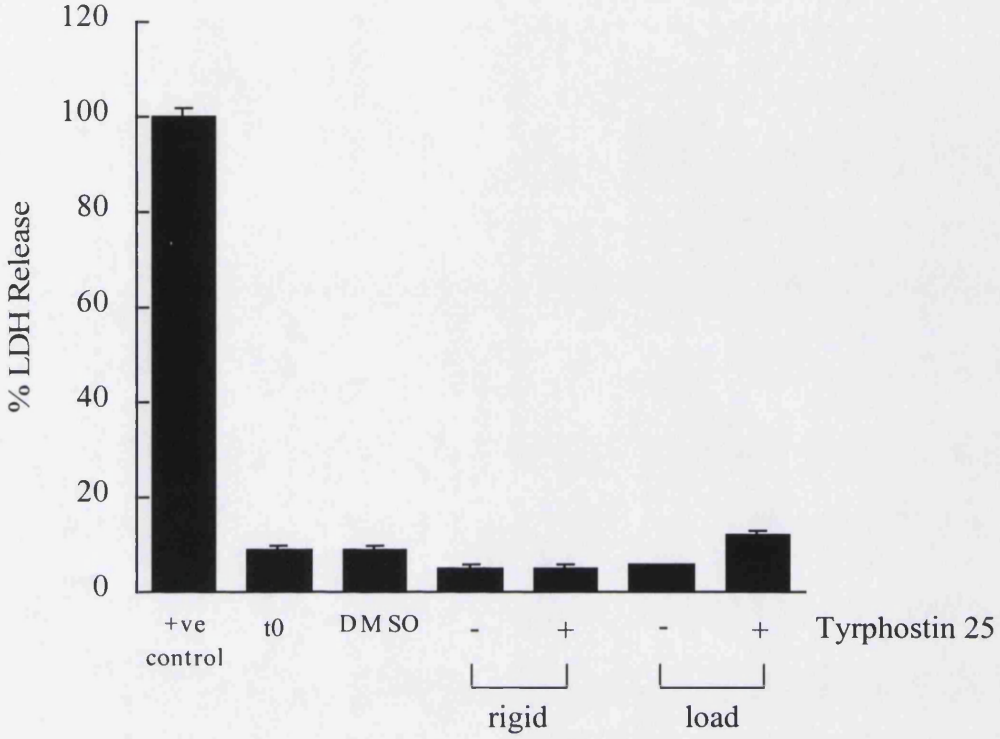


Figure 5.14. The effect of tyrphostin 25 (20μM) on fibroblast viability. LDH released by fibroblast cultures was measured after 24 hrs of mechanical load. Values are expressed as % LDH released. Values represent mean ± SEM (n=3). Graph shown is representative of 2 individual experiments.

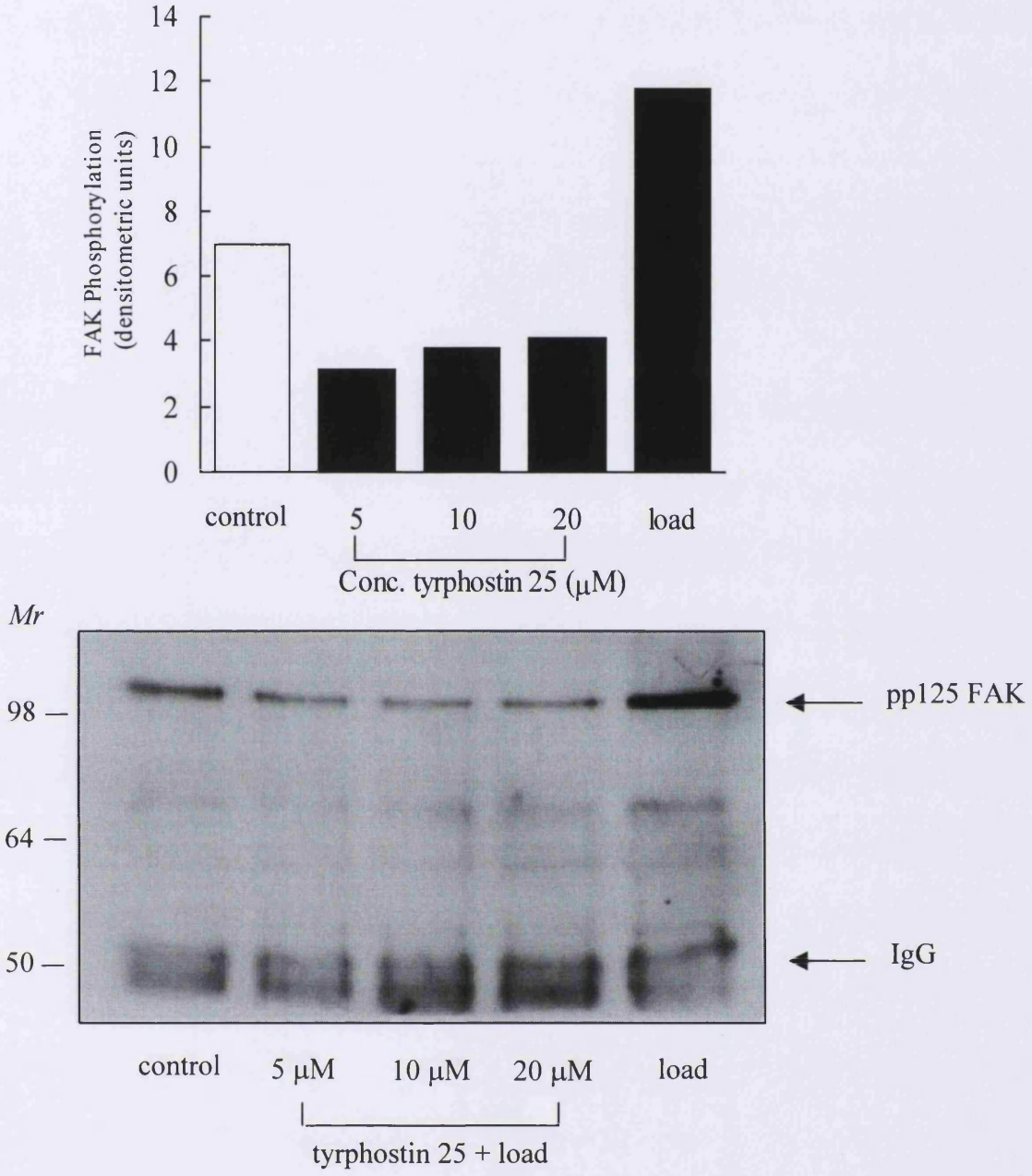


Figure 5.15. The effect of tyrphostin 25 on FAK phosphorylation. Fibroblasts were mechanically loaded for 5 min. Graph represents data from blot shown. Values are expressed as FAK phosphorylation (densitometric units). Blot shown is representative of 2 individual experiments. Molecular weights (*Mr*) are indicated down the left-hand side of the gel.

Effect of tyrphostin 25 on FAK phosphorylation

	#1	#2
control	7.00	4.51
load	12.00	8.22
5 μM tyrphostin+ load	3.35	3.65
10 μM tyrphostin+ load	4.10	2.81
20 μM tyrphostin+ load	4.47	2.06

Table 5.6. The effect of tyrphostin 25 on FAK phosphorylation from 2 individual experiments Values are expressed as densitometric units (arbitrary).

5.1.9 Effect of cytochalasin D on procollagen synthesis

Having determined the involvement of FAK in mechanical load induced procollagen synthesis, the importance of the cytoskeleton (to which paxillin physically attaches) in mechano-signal transduction was examined. This was carried out using the compound cytochalasin D. This compound is used to disrupt the actin cytoskeleton and has been previously shown to inhibit paxillin phosphorylation (Sadoshima & Izumo, 1997). Mechanical load induced procollagen synthesis levels were measured (Figure 5.16) in the presence and absence of cytochalasin D at various concentrations. Mechanical load lead to an increase in procollagen synthesis levels (1.67 ± 0.09 vs. 1.06 ± 0.16). All doses of cytochalasin D inhibited mechanical load induced procollagen synthesis. However, at 4μM the basal levels of procollagen synthesis were also affected. Table 5.7 shows the reproducibility of the experiment. Cell number did not change significantly in response to mechanical load or addition of cytochalasin D (Table 5.8).

5.1.10 Effect of cytochalasin D and mechanical load on cell viability

Cell viability was also determined by measuring LDH release in the presence and absence of cytochalasin D, at the highest dose used, with control and mechanically loaded fibroblasts. Cytochalasin D had no significant effect on fibroblast viability, compared to the positive control (Figure 5.17) in response to mechanical load. There was no significant difference in LDH release between loaded cells in the presence or absence of cytochalasin D. DMSO (vehicle) had no effect on cell viability compared to the positive control.

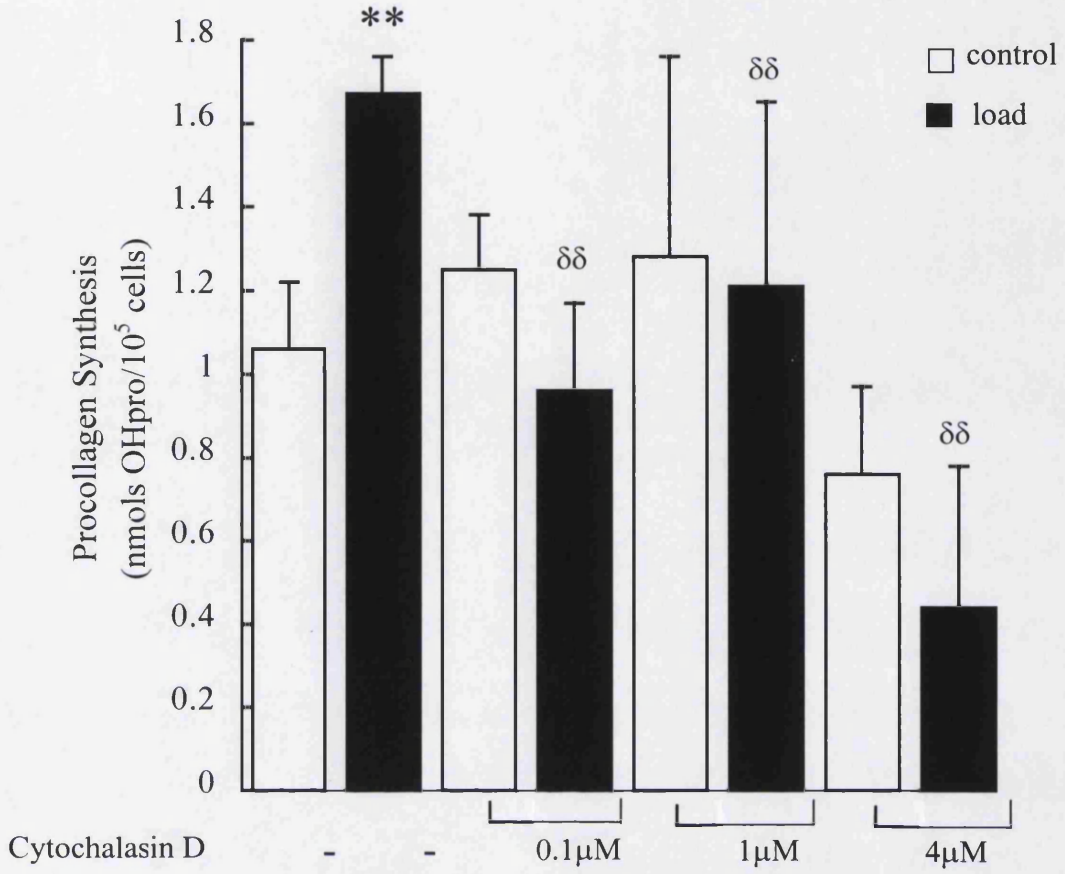


Figure 5.16. The effect of cytochalasin D on mechanical load induced procollagen synthesis. Procollagen synthesis was measured in response to 0.1, 1 and 4 μM cytochalasin D. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples, $^{\delta\delta}$P<0.01 is the level of significance of the difference between loaded and loaded + cytochalasin D samples. Graph shown is representative of 3 individual experiments.

	#1	#2	#3
24 hrs rigid	1.06 ± 0.16	1.15 ± 0.10	1.80 ± 0.09
24 hrs stretch	1.67 ± 0.09**	2.20 ± 0.20**	2.50 ± 0.09**
0.1μM cyto rigid	1.25 ± 0.13	1.54 ± 0.07	1.60 ± 0.09
0.1μM cyto stretch	0.96 ± 0.21$^{\delta\delta}$	1.01 ± 0.23$^{\delta\delta}$	1.30 ± 0.04$^{\delta\delta}$
1μM cyto rigid	1.28 ± 0.48	1.37 ± 0.18	1.80 ± 0.14
1μM cyto stretch	1.21 ± 0.44$^{\delta\delta}$	1.03 ± 0.31$^{\delta\delta}$	1.50 ± 0.15$^{\delta\delta}$
4μM cyto rigid	0.76 ± 0.21	1.41 ± 0.12	0.80 ± 0.08
4μM cyto stretch	0.44 ± 0.34$^{\delta\delta}$	1.31 ± 0.09$^{\delta\delta}$	1.21 ± 0.10$^{\delta\delta}$

Table 5.7. The effect of cytochalasin D on procollagen synthesis after 24 hrs mechanical load from 3 individual experiments. Values are expressed as nmols OHpro/10^5 cells. Values represent mean±SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples. $^{\delta\delta}$P<0.01 is the level of significance of the difference between loaded and loaded + cytochalasin D.

Experiment	#1	#2	#3
24 hrs rigid	2.80 ± 0.22	2.80 ± 0.31	3.38 ± 0.28
24 hrs stretch	3.02 ± 0.07	3.10 ± 0.21	4.12 ± 0.54
0.1μM cyto rigid	2.75 ± 0.10	2.88 ± 0.10	3.10 ± 0.34
0.1μM cyto stretch	2.72 ± 0.11	2.85 ± 0.17	3.50 ± 0.37
1μM cyto rigid	2.43 ± 0.14	2.54 ± 0.11	2.94 ± 0.06
1μM cyto stretch	2 .38 ± 0.09	2.43 ± 0.27	3.02 ± 0.11
4μM cyto rigid	2.54 ± 0.13	2.54 ± 0.07	2.74 ± 0.24
4μM cyto stretch	2.62 ± 0.15	2.84 ± 0.29	2.86 ± 0.26

Table 5.8. The effect of cytochalasin D and mechanical load on cell number from 3 individual experiments. Values are expressed as cell number ($x10^5$). Values represent mean ± SEM (n=6). (Cyto=cytochalasin D).

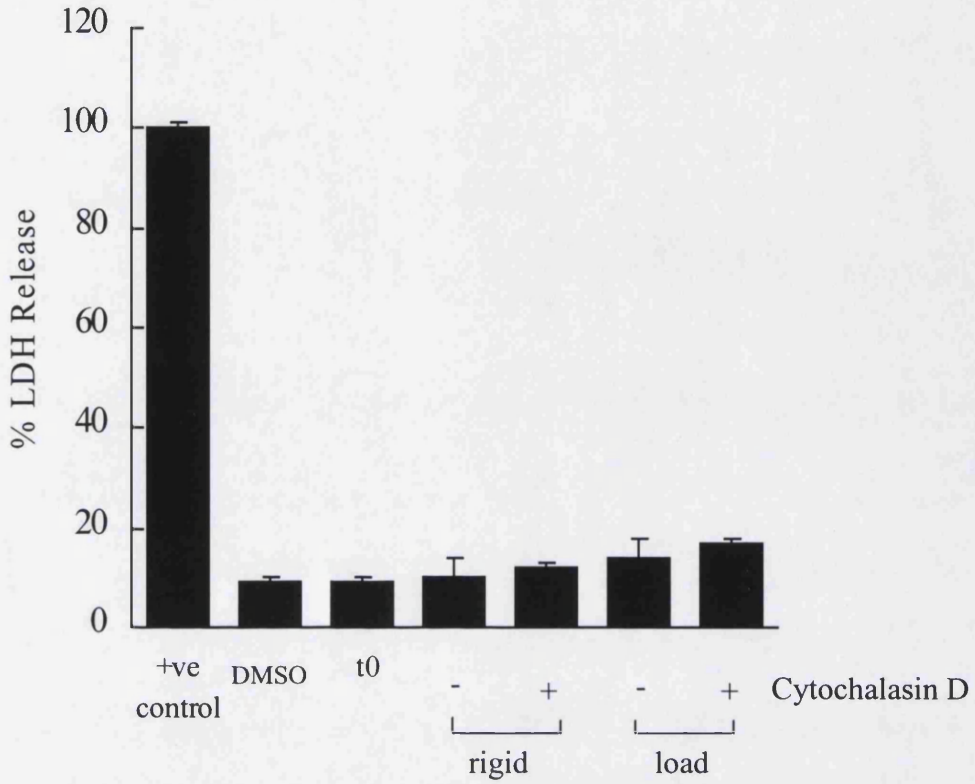


Figure 5.17. The effect of cytochalasin D (4 μM) on cell viability. LDH release was measured in fibroblast cultures in response to 24 hrs mechanical load. Values are expressed as % LDH released. Values represent mean ± SEM (n=6). Graph shown is representative of 2 individual experiments.

5.1.11 Effect of cytochalasin D on paxillin phosphorylation

Mechanical load induced a 60 % increase in paxillin phosphorylation levels compared to rigid control (Figure 5.18). At all doses of cytochalasin D used, paxillin phosphorylation in response to mechanical load was prevented. In fact the levels of phosphorylation dropped below control. Table 5.9 shows the reproducibility of the results.

5.1.12 Effect of cytochalasin D on actin cytoskeleton disruption and focal adhesion formation

The effect of cytochalasin D on the actin cytoskeleton and hence the formation of focal adhesions was analysed by confocal microscopy of FITC-labelled paxillin and rhodamine-phalloidin staining for the actin cytoskeleton. In the absence of cytochalasin D, paxillin staining at focal adhesions was minimal in control cells (Figure 5.19a) but increased in response to mechanical load (Figure 5.19b). Addition of cytochalasin D at a concentration of 1μM completely disrupted the actin stress fibre network, with only very minimal staining (Figure 5.19c). Addition of cytochalasin D to mechanically loaded cells prevented focal adhesion formation (Figure 5.19d), although not all cells appeared to be affected as some still stained for paxillin and had the appearance of focal adhesions(Figure 5.19d).

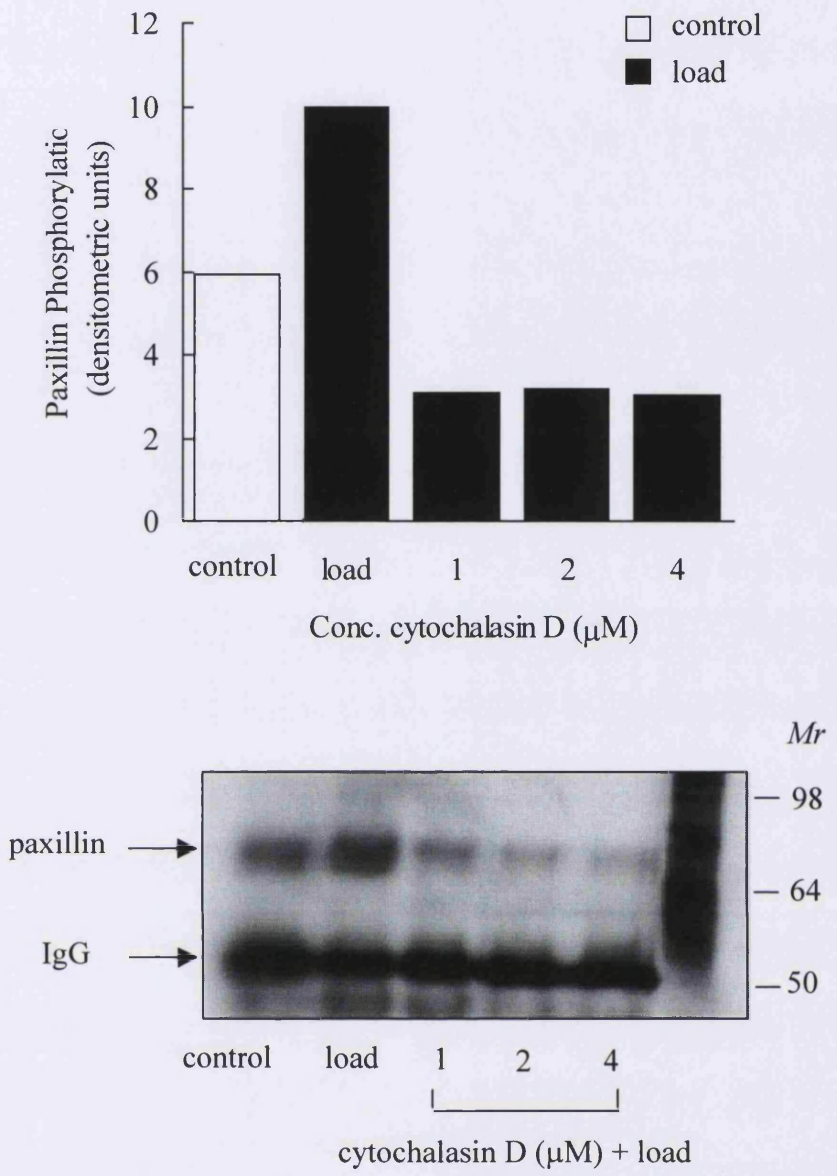


Figure 5.18. The effect of cytochalasin D on mechanical load induced paxillin phosphorylation. Graph represents data from blot shown above. Values are expressed as densitometric units (arbitrary). Blot shown is representative of 2 individual experiments. Molecular weights (*Mr*) are indicated down the right-hand side of the gel.

	#1	#2	#3
control	6.00	6.00	5.40
load	7.00	10.00	9.21
1 μM + load	2.90	3.20	3.10
2 μM + load	2.80	3.30	3.00
4 μM + load	2.50	2.97	3.44

Table 5.9. The effect of cytochalasin D on mechanical load induced paxillin phosphorylation from 3 individual experiments. Values are expressed as densitometric units (arbitrary).

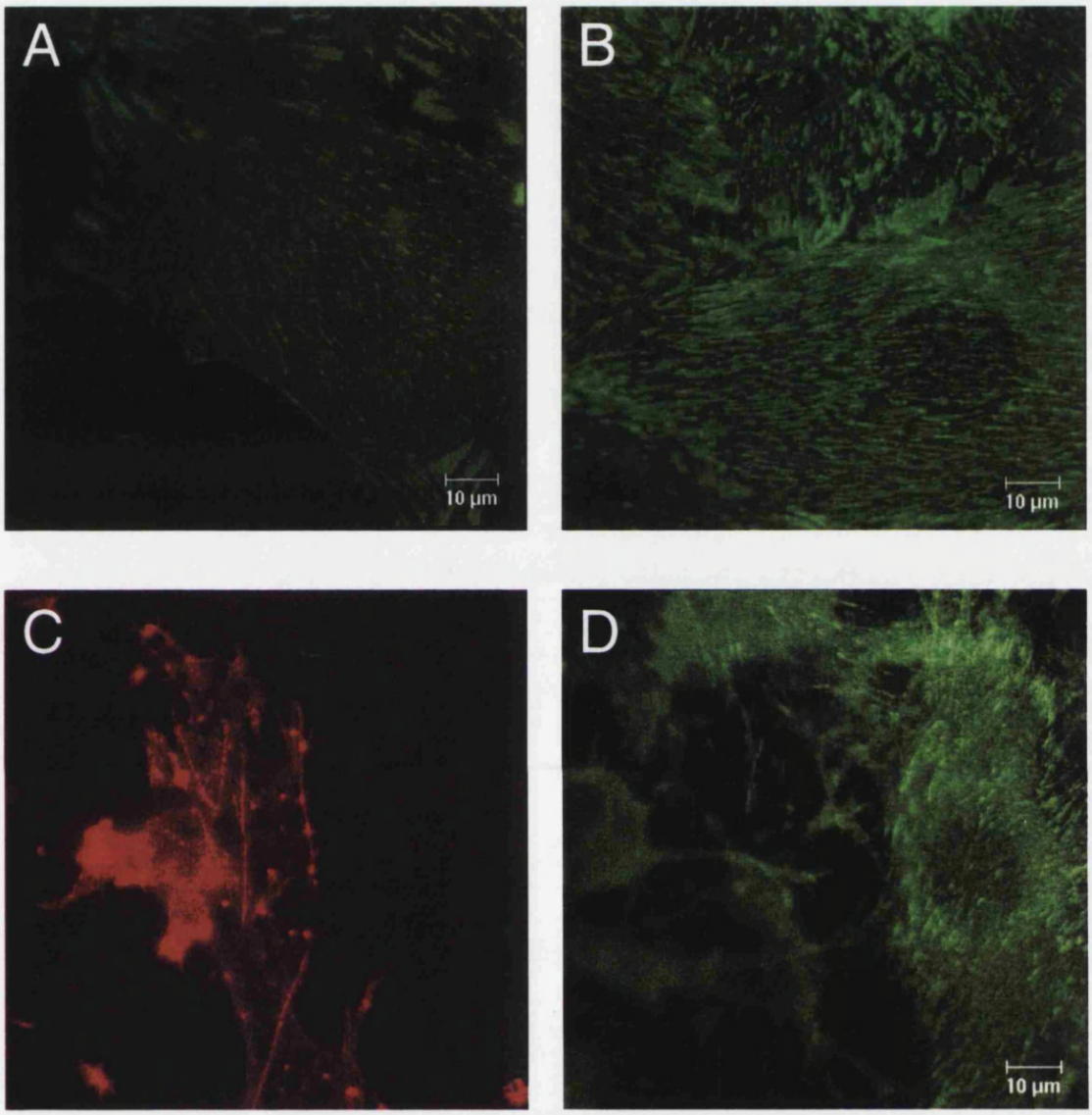


Figure 5.19. The effect of cytochalasin D on paxillin and actin

Fibroblasts were either left as rigid controls (A) or mechanically loaded (B). Addition of 1µM cytochalasin D reduced actin staining (C), however some fibroblasts did show some staining for paxillin (D).

5.1.13 Effect of static load on tyrosine phosphorylation of FAK, paxillin and p130^{Cas}

The previous data suggested a cyclical pattern of phosphorylation of FAK, paxillin and especially p130^{Cas}, which may be due, in part to the cyclical nature of the mechanical load. Therefore, the experiments were repeated using a static load, with a maximum and minimum stretch resulting in a 20 % elongation of the membrane.

Static loading lead to a greater than 100 % increase in tyrosine phosphorylation levels of FAK after 1 min (Figure 5.20) compared to rigid control. This level of tyrosine phosphorylation was almost maintained throughout 120 min of static load.

A similar observation was obtained with paxillin tyrosine phosphorylation levels, although the increase in phosphorylation levels was not as great as for FAK (Figure 5.21). There was an increase in paxillin phosphorylation levels after 1 min of mechanical load (47 % compared to rigid control). Similarly, levels were maintained at the same level for up to 120 mins.

Static mechanical load did not appear to have the same effect on p130^{Cas} as observed for FAK and paxillin although the repeated rise and fall in phosphorylation was diminished. There was an initial increase in response to load after 1 min (100 % above rigid control) (Figure 5.22). Phosphorylation levels fell back to rigid control levels then increased again after 20 min of mechanical load (150 %). At this time phosphorylation levels fell until they reached rigid control levels at 120 min.

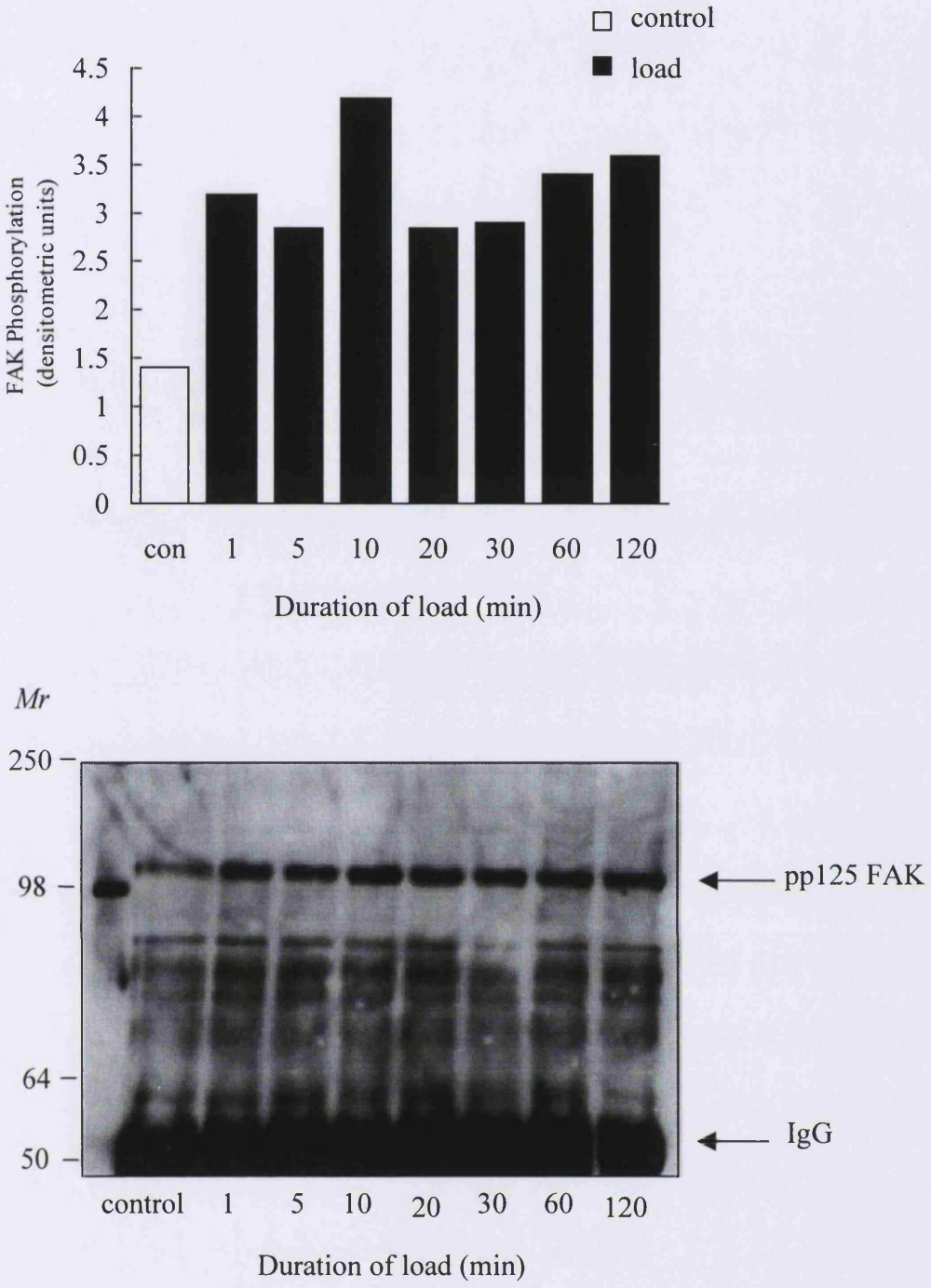


Figure 5.20. The effect of static mechanical load on FAK phosphorylation. Graph represents data from blot shown above. Values are expressed as FAK phosphorylation (densitometric units). Blot shown is representative of 3 individual experiments. Molecular weights (*Mr*) are indicated down the left-hand side of gel.

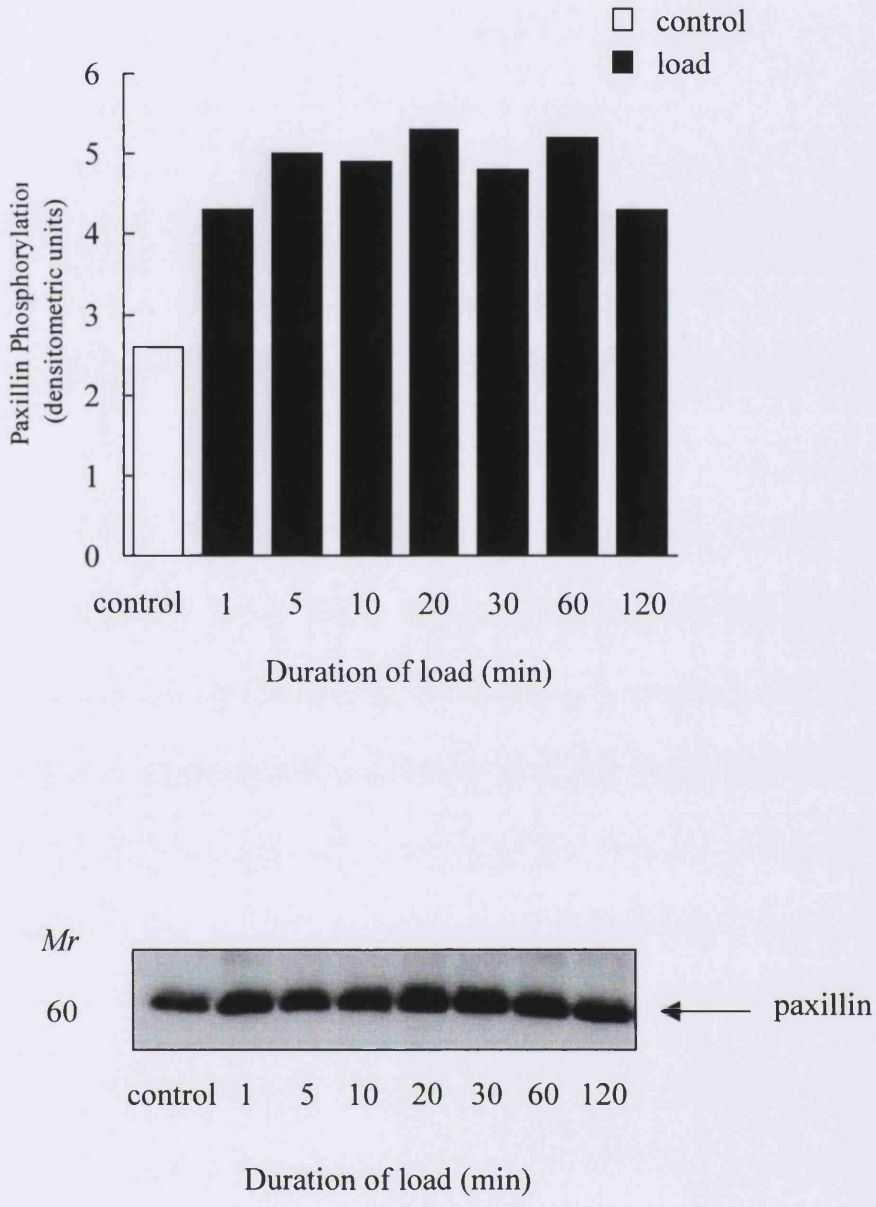


Figure 5.21. The effect of static mechanical load on paxillin phosphorylation. Graph represents data from blot shown above. Values are expressed as paxillin phosphorylation (densitometric units). Blot shown is representative of 3 individual experiments. Molecular weights (*Mr*) are indicated down the left-hand side of the gel.

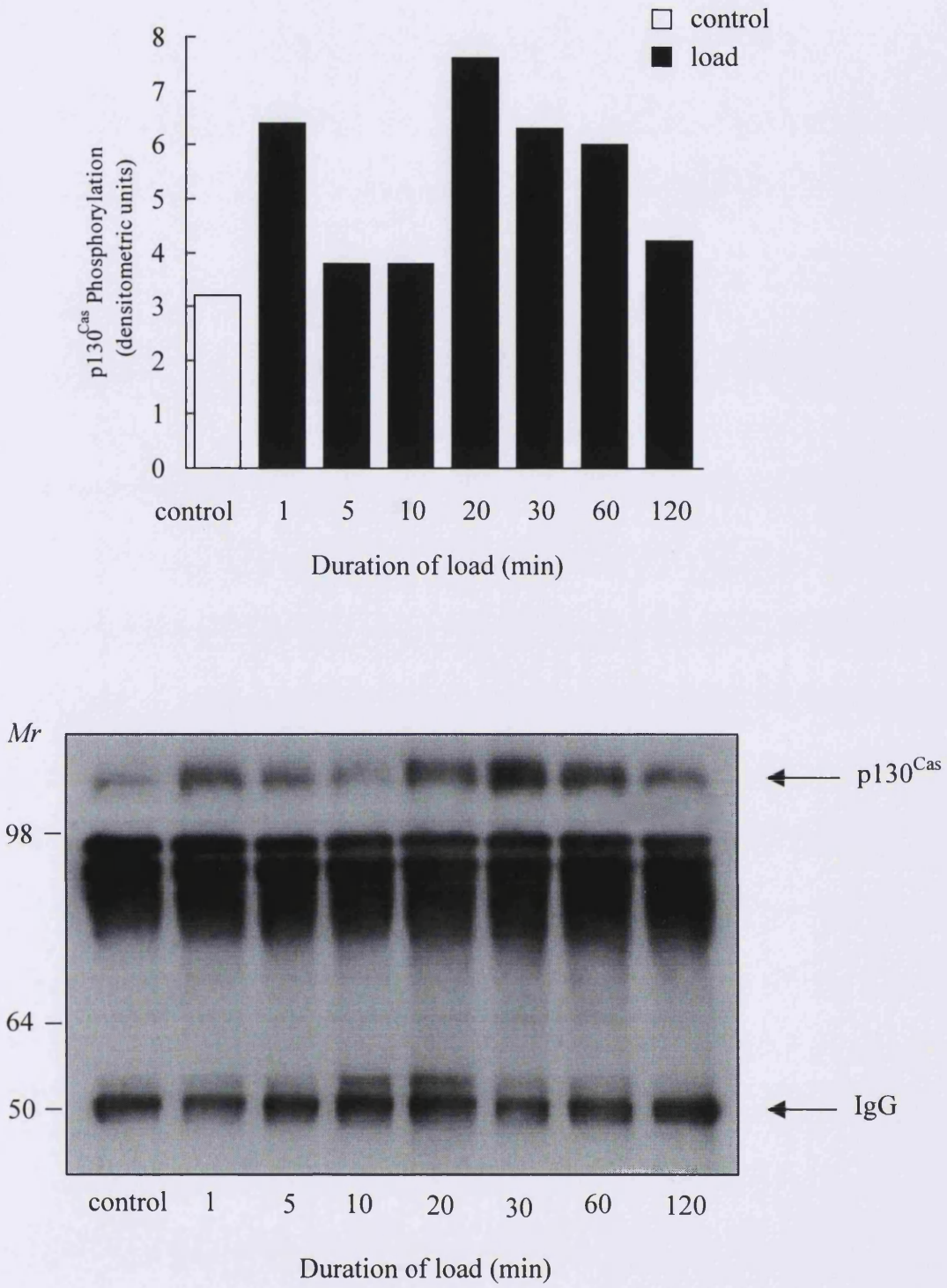


Figure 5.22. The effect of static mechanical load on p130^{Cas} phosphorylation. Graph represents data from blot shown above. Values are expressed as p130^{Cas} phosphorylation (densitometric units). Blot shown is representative of 3 individual experiments. Molecular weights (*Mr*) are indicated down the left-hand side of gel.

5.1.14 Summary

Results obtained from this chapter show that components of the focal adhesion complex are involved in mechanical load induced signal transduction. The results suggest that:

a) Tyrosine phosphorylation of FAK, paxillin and $p130^{Cas}$ increase in response to mechanical load. Addition of inhibitors can block mechanical load induced tyrosine phosphorylation.

b) FAK phosphorylation by mechanical load requires the activation of the integrin $\alpha5\beta1$. FAK phosphorylation can be decreased by the presence of an anti-$\alpha5\beta1$ integrin blocking antibody.

c) Inhibitors of FAK and paxillin phosphorylation block mechanical load-induced procollagen synthesis, suggesting an important role in the signalling pathway.

d) Confocal microscopy shows an increase in focal adhesions in response to mechanical load.

e) Use of cytochalasin D prevented the load induced stimulation of procollagen synthesis suggesting an important role for the cytoskeleton in mechano-signal transduction.

f) Static mechanical load resulted in a change in the pattern of phosphorylation of

FAK and paxillin, compared to cyclic mechanical load, suggesting that the cells not only respond to load but can distinguish between the type of load applied.

CHAPTER 6

POTENTIAL DOWNSTREAM SIGNALLING PATHWAYS ACTIVATED BY THE FOCAL ADHESION COMPLEX AND MECHANICAL LOAD

6.1 Downstream signalling pathways

Some of the proteins that make up the focal adhesion complex have the capacity to activate downstream signalling pathways. For example, FAK has a binding site for the enzyme PI 3-kinase (Chen & Guan, 1994). This enzyme phosphorylates members of the phospholipid pathway, components of which are elevated in the hypertrophied heart (von Harsdorf *et al*, 1989). Binding of adaptor proteins such as Grb 2 and Sos to FAK and paxillin can activate the MAP kinase pathway (Schlaepfer *et al*, 1998), via activation of Ras (Egan *et al*, 1993), thus this pathway may also be important. This chapter investigates the potential signalling pathways that may be activated in response to mechanical load acting downstream of the focal adhesion complex.

6.1.1 Role of the phospholipid pathway in mechanical load induced procollagen synthesis: Activation of inositol phosphates in response to mechanical load

The phospholipid pathway was examined in this study to determine its role in mechanical load induced procollagen synthesis. In *in vivo* models of cardiac hypertrophy, inositol metabolism is increased compared to controls (von Harsdorf *et al*, 1989, Kawaguchi *et al*, 1993) and *in vitro*, mechanical loading of rat calvarial bone cells stimulates proliferation via activation of the inositol phosphate pathway (Brighton *et al*, 1992). Therefore there is evidence to suggest the phospholipid pathway is activated in response to mechanical load *in vitro*, and in models of cardiac hypertrophy *in vivo*.

Total inositol and inositol phosphate levels were examined in response to mechanical load by measuring the incorporation of radiolabelled ^{3}[H]inositol. In response to 1 min of mechanical load, there was a significant increase in total inositol levels above the rigid control (20.0 % ± 1.5 % increase above rigid). A similar increase was observed after 5 min of mechanical load (24.0 % ± 4.5% increase above rigid control) (Figure 6.1). Analysis of the three inositol phosphates, IP_1, IP_2 and $IP_{3/4}$ suggested that after 1 min of mechanical load there was no significant increase (Figure 6.2). However, after 5 min of mechanical load a significant increase in all three inositol phosphates was observed compared to the rigid controls (IP_1: 43 % ± 9 %, IP_2: 27 % ± 8 %, $IP_{3/4}$: 100 % ± 15 %).

6.1.2 Effect of PI 3-kinase inhibitors on procollagen synthesis

A major component of the phospholipid pathway is the enzyme PI 3-kinase. This enzyme is required to phosphorylate phosphatidylinostol (PI), the first signalling step in the phospholipid pathway. By inhibiting PI 3-kinase the further downstream signalling pathway is prevented.

Two PI 3-kinase inhibitors wortmannin (1μM and 100nM) and the structurally different LY294002 compound (10μM) were added to the culture medium 2 hrs prior to the application of mechanical load. Mechanical load induced an almost 2 fold increase in procollagen synthesis compared to rigid control (2.85±0.01 vs. 1.78±0.21) (Figure 6.3). At the lowest dose of wortmannin used (100nM), load induced procollagen synthesis levels were not blocked, compared to mechanical load alone. LY294002 (10μM) also failed to inhibit load induced procollagen

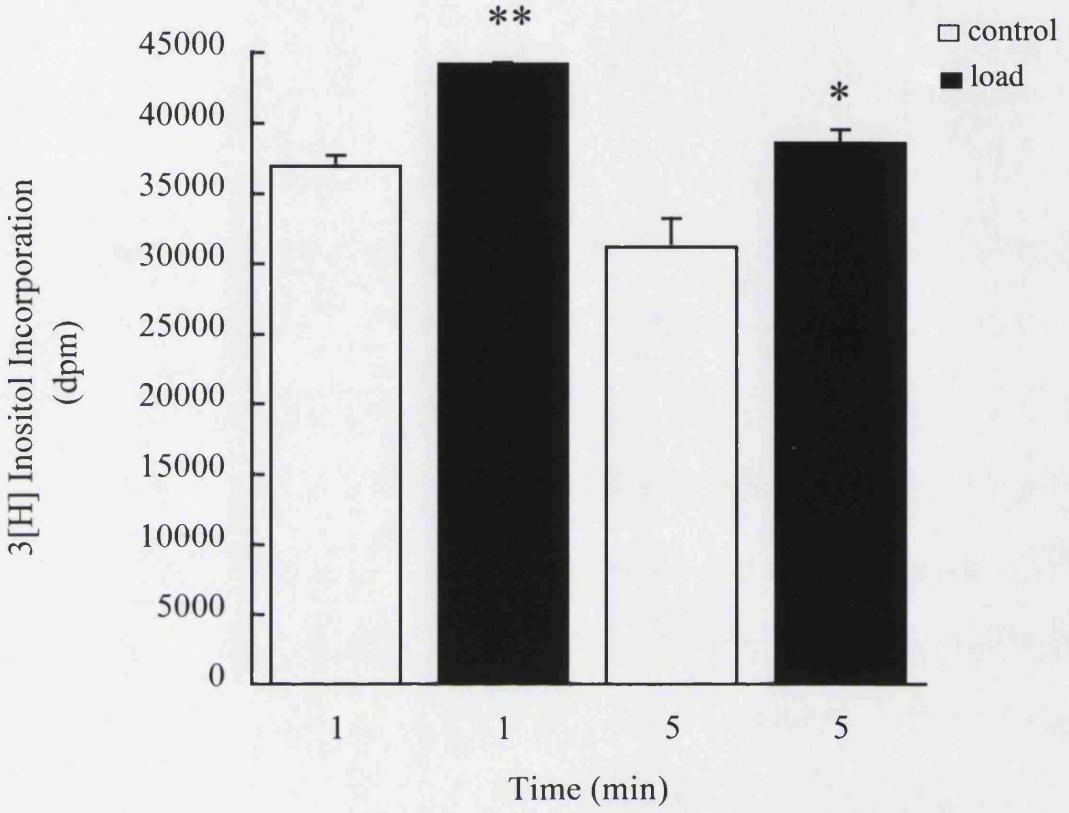


Figure 6.1 The effect of mechanical load on total inositol levels. Mechanical load for 1 and 5 min resulted in a significant increase in total inositol levels. Values are expressed as ^{3}[H] inositol incorporation (dpm). Values represent mean ± SEM (n=6). *P<0.005 is the level of significance of the difference between loaded and rigid control samples. **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

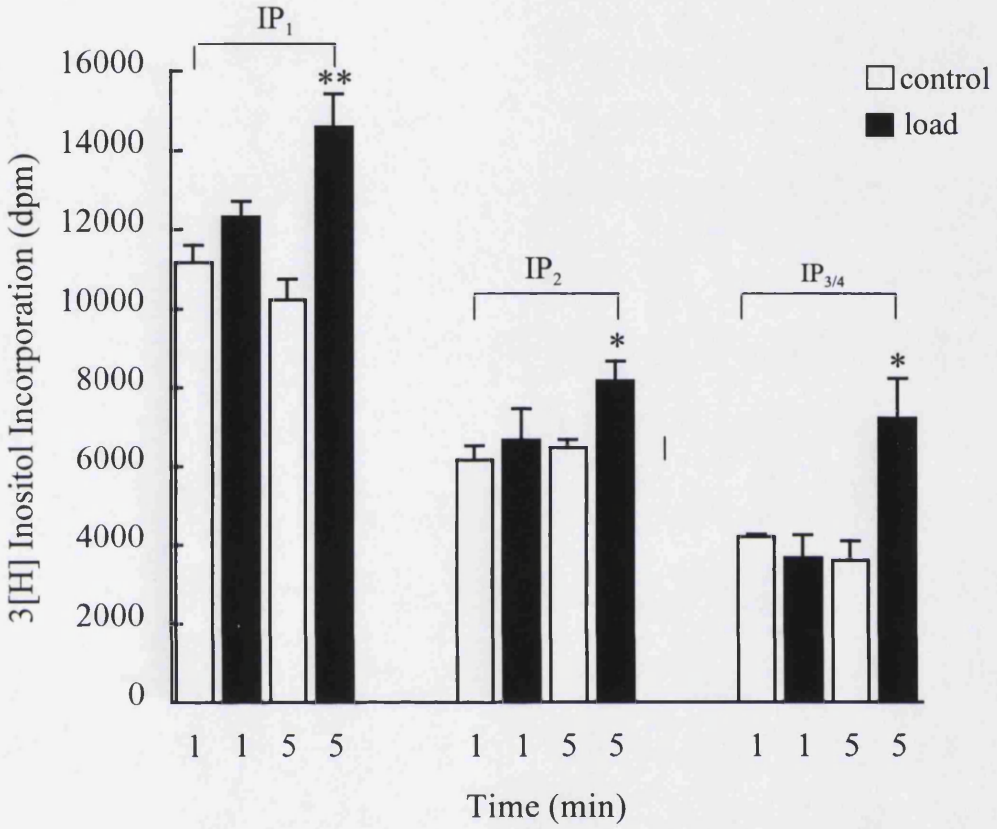


Figure 6.2. The effect of mechanical load on IP_1, IP_2 and $IP_{3/4}$ levels. Values are expressed as ^{3}[H] inositol incorporation (dpm). Values represent mean ± SEM. *P<0.05 represents the level of significance of the difference between loaded and rigid control samples. **P<0.01 represents the level of significance of the difference between loaded and rigid control samples. Graph shown is representative of 2 individual experiments.

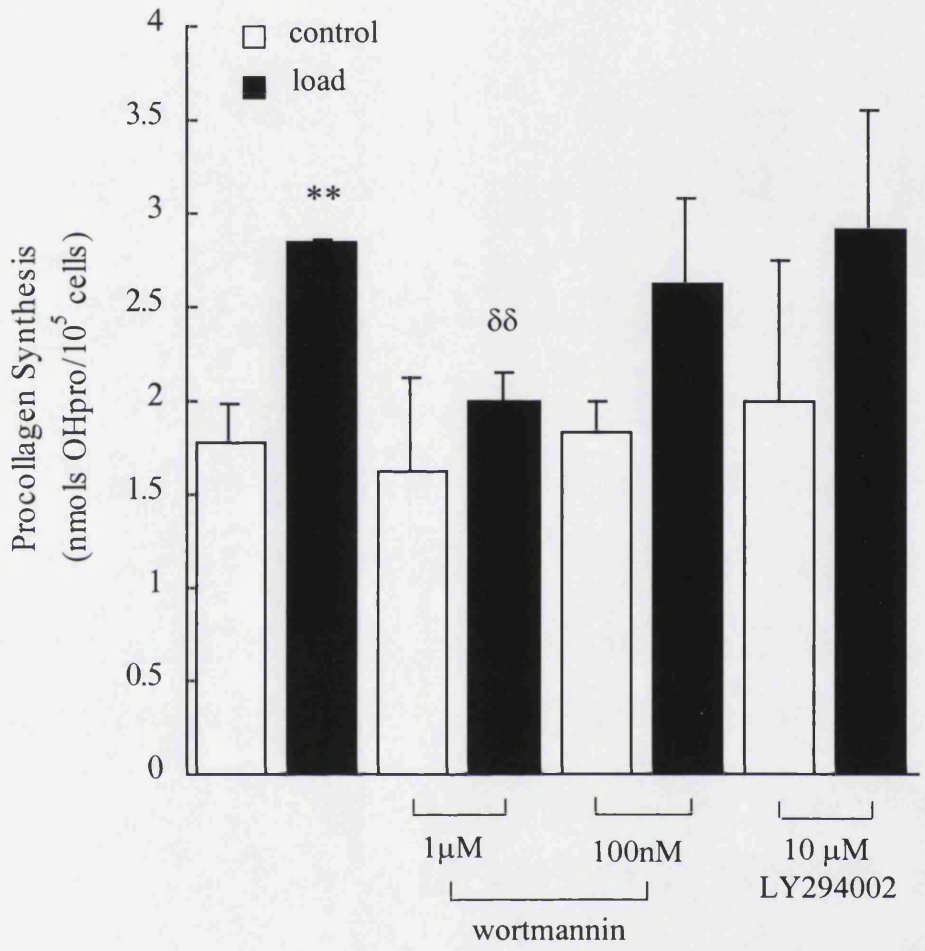


Figure 6.3. The effect of wortmannin (1μM and 100nM) and LY294002 (10μM) on procollagen synthesis. Procollagen synthesis was measured in cultured fibroblasts mechanically loaded for 24 hrs. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples. $^{\delta\delta}$P<0.01 is the level of significance of the difference between loaded and loaded + inhibitor samples.

At the highest dose of wortmannin, mechanical load induced procollagen synthesis was completely inhibited (2.85 ± 0.01 vs. 2.00 ± 0.15). Table 6.1 shows the reproducibility of the experiment. Cell numbers were not significantly increased or decreased in response to mechanical load in the presence or absence of inhibitors (table 6.2).

6.1.3 Effect of PI 3-kinase inhibitors on FAK phosphorylation

Wortmannin at concentrations of 1 μM and 100 nM was added to cell cultures to examine their effect on mechanical load induced FAK phosphorylation (Figure 6.4). Mechanical load caused a significant increase in FAK phosphorylation after 5 min (77 % increase above rigid control). Both concentrations of wortmannin lead to a decrease in FAK phosphorylation, below control levels. Table 6.3 shows the reproducibility of the experiment.

6.1.4 Effect of ethanol on procollagen synthesis

Ethanol is used as an inhibitor of the phospholipase D pathway, which has been shown to be activated in response to mechanical load in cardiac myocytes (Sadoshima & Izumo, 1993). Therefore, this pathway was examined to determine its role in mechanical load induced procollagen synthesis.

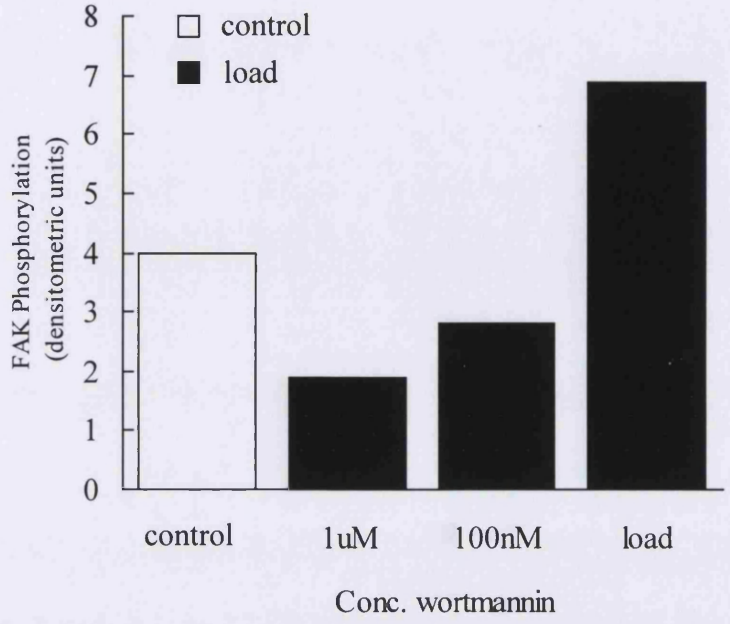
Ethanol, added to give a final concentration of 2 %, acts to inhibit formation of phosphatidic acid from phosphatidylcholine. This reaction is catalysed by phospholipase D, which can be activated by FAK, creating the by-product phosphatidylethanol, thus making it a potential downstream signalling

Experiment	#1	#2	#3
24 hrs rigid	1.15 ± 0.06	0.78 ± 0.15	1.78 ± 0.21
24 hrs stretch	2.31 ± 0.14**	1.35 ± 0.07**	2.85 ± 0.01**
1μM WT rigid	1.81 ± 0.59	0.62 ± 0.05	1.62 ± 0.50
1μM WT stretch	1.29 ± 0.11$^{\delta\delta}$	0.66 ± 0.05$^{\delta\delta}$	2.00 ± 0.15$^{\delta\delta}$
100nM WT rigid	2.38 ± 0.27$^{\phi}$	1.38 ± 0.10	1.84 ± 0.16
100nM WT stretch	2.04 ± 0.20	1.20 ± 0.09	2.62 ± 0.47
LY294002 rigid	1.99 ± 0.19$^{\phi}$	0.50 ± 0.03	2.00 ± 0.75
LY294002 stretch	2.18 ± 0.27	0.71 ± 0.12*	2.92 ± 0.64

Table 6.1. The reproducibility of three individual experiments examining the effect of PI 3-kinase inhibitors on procollagen synthesis. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples, *P<0.05 is the level of significance of the difference between loaded and loaded + inhibitor samples. $^{\delta\delta}$P<0.01 is the level of significance of the difference between loaded and loaded + inhibitor samples. $^{\phi}$P<0.05 is the level of significance of the difference between rigid and rigid + inhibitor samples. (WT=wortmannin).

Experiment	#1	#2	#3
24 hrs rigid	3.32 ± 0.36	2.00 ± 0.11	3.30 ± 0.28
24 hrs stretch	2.90 ± 0.35	2.20 ± 0.23	2.90 ± 0.11
1mM WT rigid	3.35 ± 0.21	2.43 ± 0.11	3.00 ± 0.28
1mM WT stretch	3.18 ± 0.25	2.04 ± 0.22	3.22 ± 0.06
100nM WT rigid	2.62 ± 0.17	2.30 ± 0.42	2.69 ± 0.17
100nM WT stretch	2.48 ± 0.20	1.56 ± 0.10	2.53 ± 0.14
LY294002 rigid	2.85 ± 0.25	2.98 ± 0.14	2.99 ± 0.13
LY294002 stretch	2.80 ± 0.26	2.60 ± 0.25	3.02 ± 0.08

Table 6.2. The effect of the wortmannin and LY294002 on cell number from 3 individual experiments. Values are expressed as cell number (x 10^5). Values represent mean ± SEM (n=6). (WT=wortmannin).



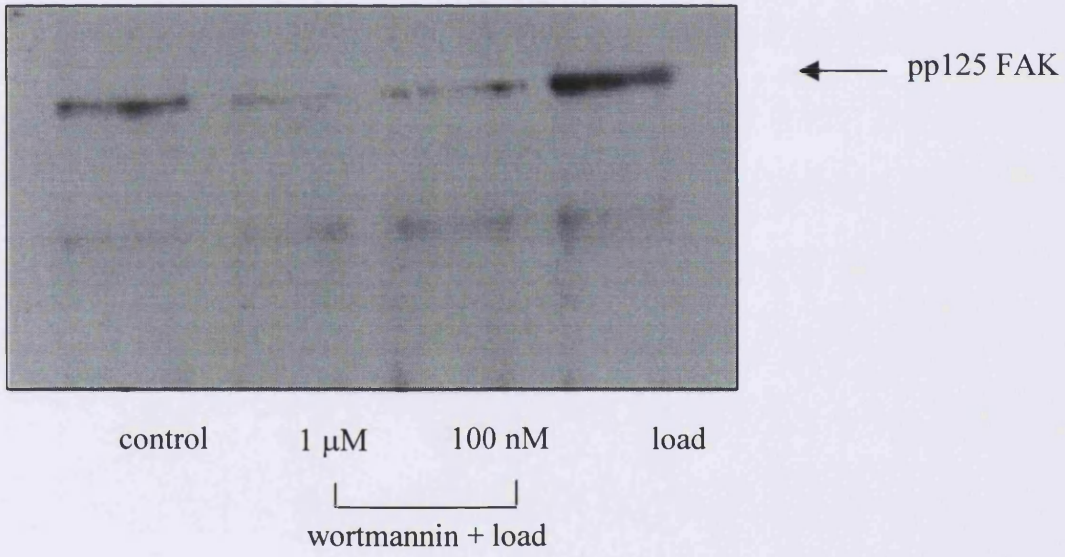


Figure 6.4. The effect of wortmannin on mechanical load induced FAK phosphorylation. Graph represents data from blot shown above. Values are expressed as FAK phosphorylation (densitometric units). Blot shown is representative of 2 individual experiments.

Effect of wortmannin on FAK phosphorylation

	#1	#2	#3
control	3.90	3.00	3.40
load	6.90	5.00	6.20
1 μM + load	1.95	1.86	1.88
100 nM + load	2.45	3.80	3.01

Table 6.3. The effect of wortmannin on FAK phosphorylation from 3 individual experiments. Values are expressed as densitometric units (arbitrary).

pathway of FAK. This pathway was therefore examined. In response to mechanical load, procollagen synthesis levels were increased 2 fold compared to the rigid control (1.15±0.06 vs. 2.31±0.14) (Figure 6.5). 2% ethanol had no effect on mechanical load induced procollagen synthesis levels (2.31±0.14 vs. 2.34±0.26). Procollagen synthesis in the rigid control was not affected by the addition of 2 % ethanol (1.15±0.06 vs. 1.41±0.03). Table 6.4 shows the reproducibility of the experiment. Cell number was not affected by the addition of ethanol (Table 6.5).

6.1.5 Effect of mechanical load on ERK 1/2 phosphorylation

To determine whether the MAP kinase pathway plays a role in the mechanical load signalling pathway, ERK 1/2 levels were examined in response to mechanical load. Mechanical load caused an increase in ERK 1/2 phosphorylation levels compared to the rigid control after 5 min (~200% increase vs. rigid control). This was maintained at 10 min then levels returned to basal levels by 60 min (Figure 6.6). The role of MAP kinases is currently under investigation in our laboratory in the PhD thesis of Jenny Papakrivopoulou.

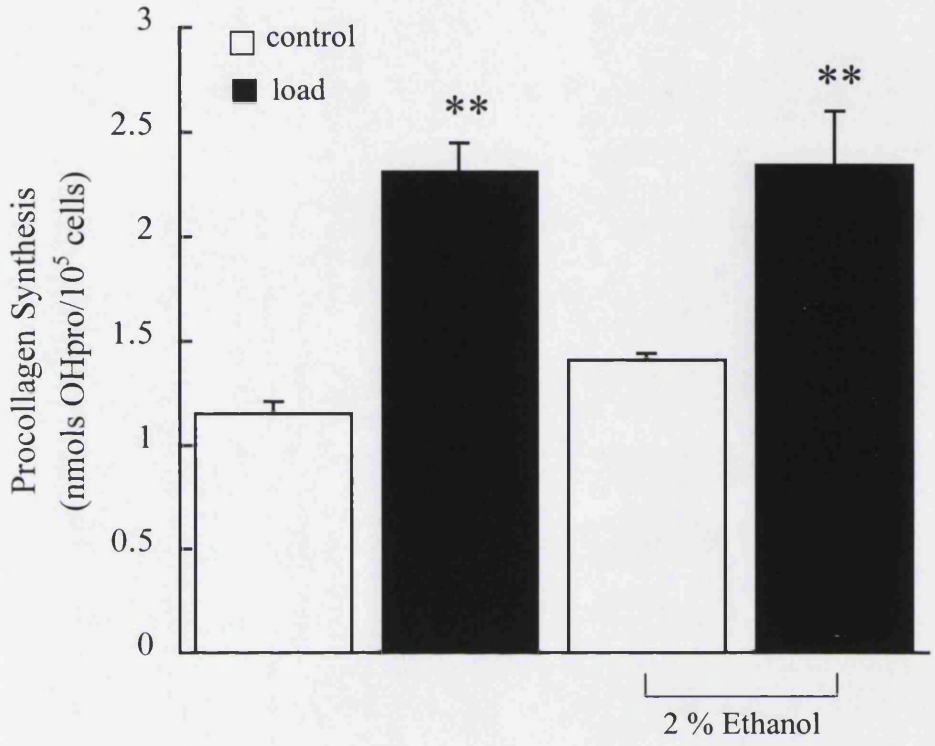


Figure 6.5. The effect of 2% ethanol on procollagen synthesis. Procollagen synthesis was measured in fibroblast cultures mechanically loaded for 24 hrs. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6). **P<0.01 is the level of significance of the difference between loaded and rigid control samples.

Effect of EtOH on procollagen synthesis

	#1	#2	#3
24 hr rigid	1.15 ± 0.06	0.78 ± 0.15	1.78 ± 0.21
24 hr stretch	2.31 ± 0.14**	1.35 ± 0.07**	2.85 ±0.01**
2% EtOH rigid	1.41 ± 0.03	0.48 ± 0.07	1.69 ± 0.05
2% EtOH stretch	2.34 ± 0.26**	0.10 ± 0.04**	3.00 ± 0.11**

Table 6.4. The effect of ethanol on procollagen synthesis from 3 individual experiments. Values are expressed as nmols OHpro/10^5 cells. Values represent mean ± SEM (n=6).

Effect of EtOH on cell number

	#1	#2	#3
24 hr rigid	3.32 ± 0.36	2.64 ± 0.51	3.45 ± 0.13
24 hr stretch	2.90 ± 0.35	2.87 ± 0.14	2.81 ± 0.32
2% EtOH rigid	3.08 ± 0.20	2.43 ± 0.09	3.16 ± 0.14
2% EtOH stretch	2.35 ± 0.28	2.52 ± 0.10	3.00 ±0.50

Table 6.5. The effect of 2% ethanol on cell number from 3 individual experiments. Values are expressed as cell number (x 10^5). Values represent mean ± SEM (n=6).

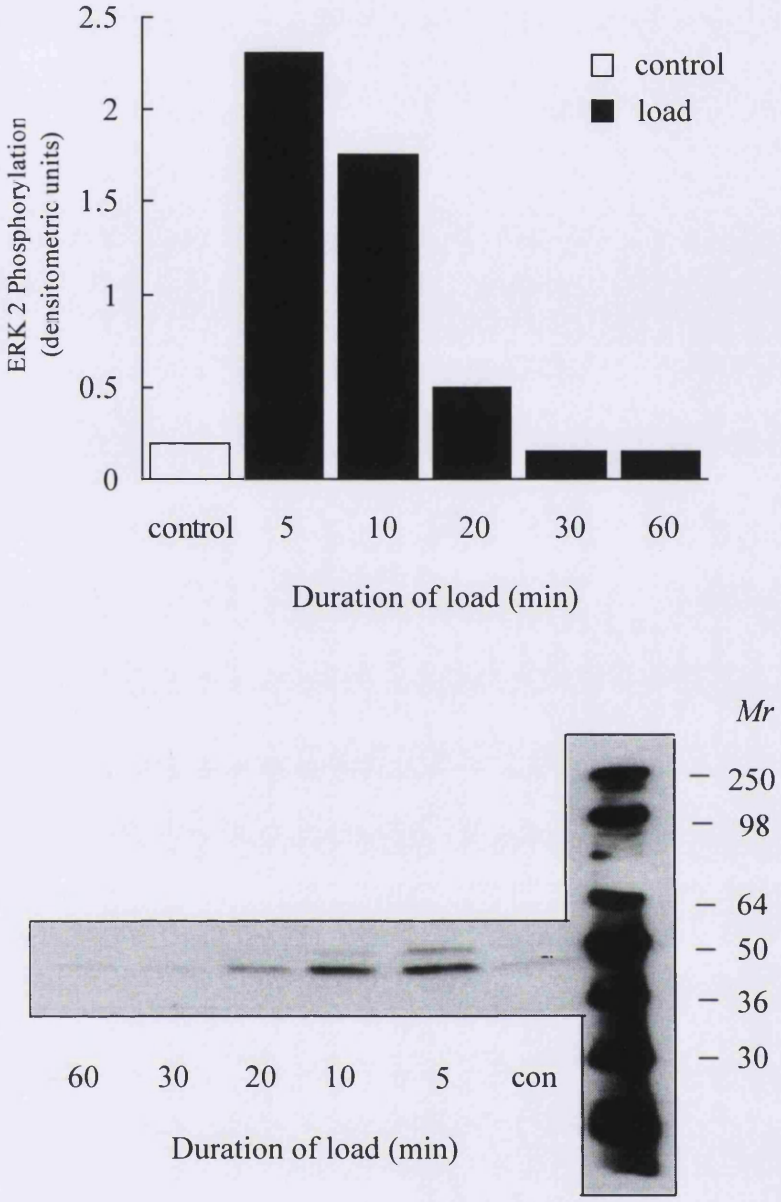


Figure 6.6. The effect of mechanical load on ERK 1/2 phosphorylation. Blot shown is representative of 2 individual experiments. Graph represents data from blot shown. Values are expressed as densitometric units (arbitrary).

6.1.6 Summary

The data in this final Results chapter suggest that mechanical load may activate more than one signalling pathway. These results have shown that:

a) Mechanical load increases total levels of inositol phosphates, after both 1 and 5 min. Levels of IP, IP_2 and IP_3 increase in response to 5 min of mechanical load.

b) PI 3-kinase is a substrate for FAK, suggesting that mechanical load induced FAK phosphorylation involves PI 3-kinase. However PI 3-kinase is not involved in mechanical load induced procollagen synthesis.

c) ERK 1/2 is activated in response to mechanical load, therefore the MAP kinase pathway may also be activated in response to mechanical load.

d) The phospholipase D pathway does not appear to be involved in the procollagen synthesis signalling pathway. 2 % ethanol had no effect on mechanical load induced procollagen synthesis levels.

CHAPTER 7: DISCUSSION

Foreward

The heart is continually subjected to mechanical forces due to changes in blood volume and pressure. As the physical properties of the heart are so important to its function, so the ECM is fundamental in maintaining the structure and function of the myocardium. The fibrillar collagens, produced by the cardiac fibroblasts, are the most abundant component of the ECM in the heart, and contribute significantly to the physical properties of the tissue. During pathophysiological conditions such as hypertension, the mechanical environment of the heart changes, resulting in tissue remodelling in an effort to adapt to these changes. *In vivo*, this remodelling includes enlargement of myocytes, proliferation of fibroblasts and increased procollagen synthesis. *In vitro*, we have previously shown that mechanical load stimulates cardiac fibroblast procollagen synthesis and gene expression. However, although the effects of mechanical load on fibroblast function are becoming increasingly documented, the mechanism of detection of mechanical forces and the signal transduction pathways that mediate these responses are less well understood. The aim of this study was to investigate the importance of the cell:matrix interaction and thus the role of integrins as mechanotransducers in response to mechanical load. Secondly, the signalling pathways mediating mechanical load induced procollagen synthesis were investigated.

7.1 CHARACTERISATION OF PRIMARY CELLS

Primary cell cultures of human cardiac fibroblasts were isolated and used in this study. The collagenase method of fibroblast isolation was used instead of explant culture as it results in the isolation of a homogeneous population of cells, i.e. fibroblasts. Explant culture results in the isolation of only migrating fibroblasts, resulting in the isolation of a specific subculture of fibroblasts, whereas collagenase digests ensures that all fibroblasts present in the tissue are isolated. Cells were grown in conditions to optimise the isolation of fibroblast cultures (Carver *et al*, 1991, Chua *et al*, 1991). Fibroblasts outgrow other cell types in culture, thereby giving a monolayer in which the majority of cells are fibroblastic. Neither endothelial cells nor myocytes were likely to have grown in the culture conditions used, as endothelial cells require specific culture media supplements and myocytes are unlikely to survive the isolation procedure or to adhere to the plasticware used.

The tissue samples obtained, and hence fibroblasts isolated, in this study are recognised to be primarily "diseased" myocardium. The use of diseased tissue compared to normal tissue may potentially result in a phenotypic change in the fibroblasts compared to those isolated from normal tissue, for example, the presence of a small population of myofibroblasts within the culture. These cells may be more active and therefore synthesise more collagen and other ECM proteins than normal fibroblasts. However, one could also argue that since the initial stimulation for the disease in these tissue samples would have occurred some time ago, the activity of these cells may be back to normal levels. Cells from these hearts may also have adapted to exposure to higher mechanical loads, however,

throughout all the experiments described in this thesis, stimulation of procollagen synthesis by mechanical load was observed repeatedly.

The staining pattern exhibited by the primary cells was compared with that obtained in various cell lines employed as controls: SMCs (A10s) and epithelial cells (MDCKs). Control cells also served to confirm antibody specificity. Control cells demonstrated the expected staining patterns. Thus, A10s stained positively for the muscle cell markers, α-SMA and myosin and negatively for vimentin and von Willebrand Factor and cytokeratin. The latter are mesenchymal and endothelial cell markers, respectively. MDCKs stained positively for vimentin and cytokeratin. Primary cardiac fibroblasts in culture stained positive for vimentin and negative for desmin, myosin, von Willebrand Factor and cytokeratin. In addition, some cells stained positively for α-SMA, indicating that some cells were of the myofibroblast phenotype. Cell cultures of human cardiac fibroblasts were therefore assumed to be basically free of contaminating cell types, including smooth muscle and endothelial cells. Fibroblasts of different passage number were stained in order to determine maintenance of phenotypic characteristics. Primary cells stained at early and late passages displayed identical patterns of staining.

7.2 PROCOLLAGEN SYNTHESIS IN HUMAN CARDIAC FIBROBLASTS: EFFECT OF MATRIX AND MECHANICAL LOAD

Increased mechanical load is a powerful stimulant for increasing collagen deposition. *In vitro*, rat cardiac fibroblasts show increased procollagen synthesis levels in response to mechanical load (Butt & Bishop, 1997). *In vivo*, in models of pressure and volume

overload cardiac hypertrophy, fractional procollagen synthesis rates are significantly increased (Turner & Laurent, 1986, Bishop *et al*, 1994, Bishop *et al*, 1990). Enhanced numbers of fibroblasts and myofibroblasts may represent a mechanism for increasing collagen deposition (Grove *et al*, 1969, Leslie *et al*, 1991), as well increased procollagen synthesis rates per cell. In this study it was demonstrated that both mechanisms may occur in response to mechanical load.

The mechanical forces to which fibroblasts and other cells of the cardiovascular system are exposed, which can lead to cardiac hypertrophy and fibrosis, *in vivo,* were mimicked here *in vitro* to determine the mechanisms underlying the stimulation of procollagen synthesis in response to mechanical load in human cardiac fibroblasts. This was carried out by stretching fibroblasts cultured on flexible-bottomed plates coated with different matrix proteins, collagen, elastin or fibronectin. Upon reaching confluency, each plate was placed in a manifold, linked to the Flexercell Strain Unit™ (FX-3000) which applies a defined mechanical load to each well (~ 20 % elongation of the membrane at a frequency of 1.5Hz).

7.2.1 METHOD VALIDATION

Mechanical loading of human cardiac fibroblasts

As described above, the Flexercell Strain Unit™ allows cells to be cyclically mechanically loaded on different matrices at a set frequency, mimicking the *in vivo* environment of the heart. The advantages of using this system include: use of a high number of replicates for each experiment; the comparison of different conditions in one experiment; and the ability

for the system to mimic the *in vivo* stretch conditions. This also allows for experiments to be repeated knowing that conditions will be the same as in previous experiments. All experiments were carried out using a defined loading regimen. The elastic membrane allows the cells to become well attached, and is highly elastic allowing it to return to its starting position after many cycles. Upon exposure to mechanical load, fibroblasts become elongated and align perpendicular to the load. Because of the translucent nature of the elastic membrane, this can be observed under a microscope. Disadvantages of this system include the differences in the gradient of strain that is set up when loading. Cells attached to different areas of the membrane are exposed to a different level of strain. Cells located around the edge of the membrane are exposed to greater strain than cells located in the centre (Banes *et al*, 1990). However, an average strain of ~ 10 % across the membrane is measured for each experiment. A further disadvantage of this system is that it is 2 D, with cells *in vivo* being stretched in a 3 D environment.

Measurement of OHPro

In this study, fibroblast procollagen synthesis was assessed by measuring OHpro in hydrolysed protein harvested from cultured fibroblasts (Campa *et al*, 1990, McAnulty *et al*, 1991). Reverse-phase-HPLC was used to assess rates of procollagen synthesis from cultures of human cardiac fibroblasts under basal conditions, in response to mechanical load, in the presence or absence of 10 % FCS, and in response to the ECM proteins collagen, elastin or fibronectin to which the cells were adhered.

There are other methods which have been employed to measure collagen production. These include measuring the incorporation of radiolabelled proline into procollagen

(Hildebran *et al*, 1981). This method is technically difficult to perform and has limitations due to precursor pool problems linked to potential effects of cell culture conditions. Collagen levels have also been measured directly using an enzyme-linked immunosorbent assay (ELISA) (Breul *et al*, 1980). However, only large increases in collagen production can be detected using this method. Compared with the various methods available to assess procollagen production, the one used in this thesis is very sensitive: it can detect 50 pmols OHpro; it can detect small changes in procollagen production; avoids the requirement for large numbers of cells and the use of radioactivity. Previous studies from this laboratory have demonstrated that the results obtained with this method are highly reproducible, with a coefficient of variation of less than 2 % for OHpro measured in the same sample hydrolysate assayed on different days, and less than 5 % for procollagen production rates obtained from replicate cultures (Chambers, PhD Thesis, 1995).

Cell culture conditions were optimised for procollagen synthesis, as changes in culture conditions have been shown to exert considerable influence on procollagen synthesis rates by cultured cells. To achieve this, cells were grown to visual confluence since procollagen production has been reported to be optimal at this stage (Steinberg, 1978, Booth *et al*, 1980). This also allows the effects of procollagen production to be uncomplicated by cell proliferation. Further, the cell culture medium used in these experiments contains various elements which are required for optimal procollagen production. These include ascorbic acid which is an essential co-factor for prolyl hydroxylase. The concentration of ascorbic acid (50 μg/ml) in these experiments was chosen on the basis that this concentration had previously been shown to maximise hydroxylation of newly synthesised procollagen

(Booth *et al*, 1980) and was sufficient to allow maximum activity even after 24 hrs (Monboisse *et al*, 1989). Finally, in all experiments performed, cells were pre-incubated with medium supplemented with ascorbic acid to ensure that all underhydroxylated procollagen, accumulated prior to the onset of the experiment, was hydroxylated and secreted (Pacifici and Iozzo, 1988). Proline which is the precursor amino acid for hydroxyproline, was also added to the medium. as was iron which also acts as a co-factor for prolyl hydroxylase. In these conditions the cells are not maximally stimulated but have all the right elements so that should the fibroblasts be exposed to a stimulus there are no rate limiting factors in terms of synthesis of procollagen

Glutamine is a major energy source for cultured fibroblasts (Sumbilla *et al*, 1981) and is normally present in DMEM. However, this amino acid is very unstable and degrades rapidly on storage, even at 4°C. In order to ensure an adequate supply, fresh L-glutamine was added to both pre-incubation and incubation media.

7.2.2 Effect of mechanical load and matrix composition on procollagen synthesis in human cardiac fibroblasts

The effect of mechanical load and 10 % FCS on procollagen synthesis was investigated, with fibroblasts grown on a collagen, elastin or fibronectin matrix. An increase in procollagen synthesis levels was observed after 48 hrs on all matrices compared to the rigid control. The data for the collagen and elastin matrices is consistent with previous data obtained in our laboratory using rat cardiac fibroblasts, whereby an increase in procollagen $\alpha_{(I)}$ I mRNA and protein synthesis was observed only after 48 hrs (Butt &

Bishop, 1997). In contrast, after 24 hrs of mechanical load only those fibroblasts adhering to a fibronectin matrix showed a significant increase in procollagen synthesis levels compared to the rigid controls. In the absence of serum, fibroblasts adhering to the collagen and elastin matrices showed no increase in procollagen synthesis levels after 24 or 48 hrs, whereas procollagen synthesis levels were increased at both times by cells grown on the fibronectin matrix. These data suggest that adhesion to a fibronectin matrix may be an important factor in the ability of fibroblasts to perceive mechanical load and translate this stimulus into procollagen synthesis. Mechanical load induced procollagen synthesis only occurs on collagen and elastin in the presence of serum, suggesting that the serum provides the cells with a specific stimulus that is already present when the cells are plated on fibronectin (discussed further below).

Other studies have observed differences in response to a given mechanical stimulus with cells grown on different matrices. Wilson *et al* (1995) observed that the composition of the ECM affects VSMC mitogenic response to mechanical strain, with cells grown on fibronectin and collagen responding to load with dramatic increases in thymidine incorporation compared to laminin and elastin. McKenna *et al* (1998) observed that the signalling components ERK 1/2 and JNK are differentially activated depending on the matrix to which the cells are attached. From their studies it was concluded that cells "sense" mechanical stimuli through interaction of integrin receptors expressed on the cells surface with specific matrix sites fixed to the moving substrate. Specific integrins may be acting as mechanotransducers depending on the matrix to which cells attach. Their observations and the results obtained in this thesis imply that binding of human cardiac

fibroblasts to a fibronectin matrix utilises specific integrins which can act as mechanotransducers, allowing fibroblasts to respond to mechanical load at 24 hrs.

The observed early response to mechanical load, resulting in increased procollagen synthesis on fibronectin has not been previously documented. This response may involve the expression of specific integrins which fibroblasts express when bound to fibronectin, but not collagen or elastin, and which act as mechanotransducers. The integrin of importance is postulated to be $\alpha5\beta1$, a specific fibronectin integrin. Interactions of $\alpha5\beta1$ with the fibronectin ECM, induces fibronectin matrix assembly (Hynes, 1992). $\alpha5\beta1$ may mediate the interaction of fibroblasts with fibronectin and this interaction may play a key role in mechanical load induced procollagen synthesis at 24 hrs. It may be postulated that the expression of the $\alpha5\beta1$ integrin at the onset of cell plating and cell culture increases the fibroblasts ability to incorporate soluble fibronectin (present in serum) into an insoluble matrix. It has been well documented that $\alpha5\beta1$ functions in an early and essential step in fibronectin matrix assembly (Wu *et al*, 1993), as cells deficient in the $\alpha5$ integrin cannot assemble a fibronectin matrix (Wu *et al*, 1993). The mechanisms of fibronectin matrix assembly involves assembly of cell-or plasma-derived soluble fibronectin into insoluble fibrils (Mosher *et al*, 1992), and has been shown to occur *in vitro* with cultured human fibroblasts (McKeown-Longo & Mosher, 1983). The first step in matrix assembly requires the interaction of the amino-terminal end of soluble fibronectin with cell-associated matrix assembly sites (Quade & McDonald, 1988, McDonald *et al*, 1987, Sottile & Wiley *et al*, 1994, Hocking *et al*, 1994), which are composed of $\alpha5\beta1$ integrins. Subsequent polymerisation of soluble fibronectin into fibrils is thought to occur via

disulphide cross-linking and results in an anti-parallel arrangement of fibronectin monomers. The use of blocking α5β1 antibodies and antibodies to the RGD-containing site inhibits fibronectin matrix assembly by fibroblasts (McDonald *et al*, 1987, Roman *et al,* 1989, Fogerty *et al*, 1990.). The insoluble fibronectin fibrils formed by cells grown on fibronectin are known to interrelate with the actin cytoskeleton, as there is frequently co-distribution of extracellular fibronectin fibres and intracellular actin filaments (Tomasek & Haaksma, 1991). Indeed, fibronectin matrix assembly on the cell membrane occurs synchronously with F-actin bundles in wounds *in vivo* (Welch *et al*, 1990), and interaction of fibronectin with the cells surface promotes the formation of stress fibres. This association between the fibronectin matrix and the actin cytoskeleton is called a fibronexus (Tomasek & Haaksma, 1991). These structures may serve as a link that permits transmission of cell generated contractile forces across the surrounding matrix, resulting in tissue contraction. As cells grown on fibronectin have the ability to incorporate more fibronectin matrix, more connections between the fibronectin matrix and the cytoskeleton may occur. Therefore, in the present system, fibroblasts grown on fibronectin may produce many fibronexi, making cells grown on fibronectin more sensitive to deformation of the membrane, allowing the transmission of the mechanical stimulus into intracellular signalling pathways. As the collagen and elastin matrix do not support incorporation of soluble fibronectin into an insoluble matrix, due to the lack of expression of α5β1, it is hypothesised there are fewer matrix/cytoskeleton interactions. As a result, the mechanical load has less of a deforming effect on the cells attached to collagen and elastin compared to fibronectin and consequently the mechano-transduction signal is weaker and insufficient to lead to an increase in procollagen synthesis after 24 hrs.

Procollagen synthesis in response to mechanical load was only achieved on the collagen and elastin matrix after 48 hrs in the presence of serum. The delay in the increase in procollagen synthesis in the presence of serum may involve the requirement of serum to stimulate fibronectin production by fibroblasts grown on collagen or elastin or the requirement of serum to cause $\alpha 5\beta 1$ expression.

Cells grown on collagen and elastin may also require expression of growth factor receptors, as well as integrin expression to respond to mechanical load. Cross-talk between integrins and growth factor receptors is not a novel idea. Both integrin activation and growth factor receptors are needed for FAK activation in human foreskin fibroblasts, and ERK activation (Miyamoto *et al*, 1996). It is postulated that integrin occupancy and aggregation results in a small stimulation of downstream signalling components, and the presence of growth factors, which bind to their respective receptors, results in a synergistic response. This leads to a sustained and greater stimulation of signalling proteins. This may explain why mechanical load induced procollagen synthesis only occurs on collagen and elastin plates in the presence of serum. Fibroblasts grown on these matrices need a combination of growth factors and integrin signalling to produce an increase in procollagen synthesis in response to mechanical load. Fibroblasts grown on fibronectin do not require this added stimulus.

7.2.3 Replication of fibroblasts: effect of mechanical load and ECM.

A common *in vivo* response to increased mechanical tension by vascular cells and mesenchymal cells of the myocardium, is cell proliferation. In order to determine if

mechanical load has a direct effect on cardiac fibroblast proliferation *in vitro*, a radioactive method of thymidine incorporation was performed with subconfluent fibroblasts, loaded on the Flexercell Strain Unit™. Unlike the data obtained from the procollagen synthesis studies, ECM composition appeared to have no significant effect on fibroblast replication in response to mechanical load at any time point examined. Replication was increased in response to mechanical load on all matrices, but only in the presence of serum. Whereas the integrin:matrix interaction appears to play a critical role in mechanical load induced procollagen synthesis, it would appear that cardiac fibroblast replication in response to mechanical load is not dependent on a specific integrin:matrix interaction. One possible explanation may be that the stimulation of replication involves a different signalling mechanism to that of procollagen synthesis. For the human cardiac fibroblasts used in this study, cell replication, unlike procollagen synthesis, may not require the binding of specific integrins to the ECM in order for the cells to transduce the mechanical load.

It is hypothesised that replication in response to mechanical load in human cardiac fibroblasts involves stretch activated ion channels (SACs) rather than integrins. SACs have been implicated as mechanotransducers in some cell types, including muscle and bone cells (Ingber, 1991, Vandenburgh, 1992, Watson, 1991). The earliest event in mechanotransduction appears to be ion influx through the opening of SACs. SACs are distinct from ion channels that respond to voltage changes or ligand-receptor interactions. The energy required to open these channels is provided by membrane strain rather than metabolic stimulation. These channels may then be kept open by energy derived from the flux of ions (Sheetz & Diaz, 1996, Morris, 1990). SACs are mostly cation channels such

as K^+, Na^+, or Ca^{2+} (Hagiwara *et al*, 1992). Of particular interest is the Ca^{2+} channel which, from patch-clamp recordings, increases its opening frequency in response to exposure to mechanical stretch in aortic endothelial cell membranes (Lansman *et al*, 1987). Furthermore, this particular ion channel has been shown to participate in the regulation of several aspects of cell division. Addition of Ca^{2+} (given as $CaCl_2$) to Balb/c 3T3 fibroblasts induces cell replication (Dulbecco & Elkington, 1975). PDGF stimulated mouse fibroblasts require a Ca^{2+} influx for proliferation to occur (Wang *et al*, 1993). *In vivo*, genetically hypertensive rats show increased mechanosensitivity of stretch-activated ion channels, including Ca^{2+}, implicating this ion channel is experimental hypertension (Kohler *et al*, 1999). Mechanical stretch of both human jejunal smooth muscle cells (Farruga *et al*, 1999) and barareceptor neurons (Sullivan *et al*, 1997) resulted in an increase in Ca^{2+} influx (which could be blocked by addition of gadolinium) concluding that Ca^{2+} SACs are mechanotransducers in these cell types. In human cardiac fibroblasts used in this thesis, mechanical load may activate Ca^{2+} channels resulting in a flux of ions. The presence of these ions may lead to an increase in DNA synthesis in response to mechanical load.

Contrary to this, other studies have shown that ECM composition does affect the mitogenic response to mechanical load. Such studies include those carried out by Wilson *et al* (1995), who were able to demonstrate that VSMCs grown on fibronectin and collagen, compared to laminin and elastin, responded to load with dramatic increases in thymidine incorporation. In a similar study, where VSMCs were subjected to mechanical strain and AII, adhesion to fibronectin and collagen lead to an increase in replication of 53 fold whereas on plastic there was no response (Sudhir *et al*, 1993). These data suggest

that the response to mechanical load and the role of the underlying matrix is cell-type specific and may depend on the cell's ability to produce autocrine mitogens in response to load.

7.2.4 Synergy between growth factors and mechanical load

It has been suggested that growth factors may act synergistically with mechanical load to stimulate the changes in fibroblast activity that occur during cardiac hypertrophy and that load alone is unable to stimulate procollagen synthesis (Butt, PhD Thesis, 1997, Butt & Bishop, 1997). Rat cardiac fibroblasts require the presence of high levels of serum or growth factors in order to increase production of collagen in response to mechanical load (Butt & Bishop, 1997). However, in these experiments fibroblasts were grown on elastin Flex I® plates, and from studies carried out in our laboratory it has been shown that human cardiac fibroblasts do not produce growth factors that stimulate procollagen synthesis. The response of rat fibroblasts to load may be specific to rat cardiovascular fibroblasts since Wilson *et al* (1995) showed that vascular smooth muscle cell replication is enhanced by load in the absence of any growth factors. However these cells produce growth factors such as PDGF in response to load. Therefore it appears that a link between mechanical load and growth factors exists. However, depending on the cell type, it may be that autocrine factors are produced in response to load or that they are required from a paracrine source to act in conjunction with mechanical load to enhance cellular activity.

Much work has been carried out on the role of autocrine growth factors in myocytes, in response to mechanical stimuli resulting in a hypertrophic response. Presence of

autocrine/paracrine growth factors has been proposed as one of the important mechanisms in the pathogenesis of stretch-induced organ hypertrophy (Czerwinski *et al*, 1993). In many cell systems, it is thought that mechanical stretch stimulates production and/or secretion of growth factors and that the growth factors secreted in response to mechanical stretch mediate mechanical-stretch induced cell growth responses. Such growth factors include basic fibroblast growth factor (bFGF), released in response to mechanical stress by cardiac myocytes (Kaye *et al*, 1996). TGF-β expression is increased in response to hypertrophic stimuli in rat cardiac myocytes, it is released from these cells and acts in an autocrine/paracrine manner and is thought to play a role in myocardial remodelling by hypertrophic stimuli (Takahashi *et al*, 1994). Other growth factors which are released in response to mechanical stimuli are AII, which itself stimulates the autocrine production of TGF-β in rat cardiac fibroblasts (Lee *et al*, 1995). In VSMCs, mechanical strain increases the secretory function of these cells, including PDGF (Wilson *et al*, 1995), bFGF, TGF-β and VEGF. As previously discussed, human cardiac fibroblasts may not produce their own growth factors in response to load, therefore in the absence of serum cells grown on collagen and elastin do not respond. They only respond to mechanical load in the presence of serum. Serum provides the growth factors needed to bind to growth factor receptors which are required along with integrin activation for these fibroblasts to respond to mechanical load, resulting in enhanced procollagen synthesis at 48 hrs. It may also explain the observation that fibroblast replication in response to mechanical load only occurs in the presence of serum. Again, growth factors present in the serum are required for these fibroblasts to respond to load.

Summary

Fibroblasts grown on fibronectin, but not collagen or elastin can respond to mechanical load with enhanced procollagen synthesis at 24 hrs. This may be due to the ability of fibroblasts grown on fibronectin to synthesise their own matrix through the presence of $\alpha5\beta1$ integrins, allowing them to be more sensitive to load. Fibroblasts grown on collagen and elastin do not express $\alpha5\beta1$, and therefore cannot synthesise their own matrix. In turn, they are less sensitive to load. For fibroblasts grown on collagen and elastin to respond to load, they require the presence of growth factors.

Fibroblast procollagen synthesis and replication in response to mechanical load occur by distinct mechano-signal transduction mechanisms in human cardiac fibroblasts. Mechano-signal transduction occurs via integrins in the procollagen synthesis response to load, whereas it is postulated that Ca^{2+} ion channels may be involved in mechanical load induced replication, since specific integrin-matrix interactions do not appear to be involved. Serum is also required by fibroblasts grown on all matrices to respond to mechanical load, resulting in fibroblast replication.

7.3 EFFECT OF INTEGRIN BLOCKING ANTIBODIES ON PROCOLLAGEN METABOLISM

Integrins have been implicated as mechanotransducers in many cell types including osteoblasts (Pavalko *et al*, 1998), rat cardiac fibroblasts (MacKenna *et al*, 1998) and VSMCs (Wilson *et al*, 1995). However, to date studies have not examined human cardiac

fibroblasts and the role of integrins as mechanotransducers, particularly not the role of integrins in the load-induced stimulation of procollagen synthesis.

Soluble fibronectin was used initially to determine the importance of the fibronectin matrix:cell interaction involved in mechanical load induced procollagen synthesis. Soluble fibronectin was added to the culture media of fibroblasts grown on all three matrices. Soluble fibronectin was used in this instant as an antagonist to fibronectin receptors present on the cell surface. Soluble fibronectin has previously been used by Wilson and co-workers (1995) to block the mitogenic response to mechanical load, which was thought to be occurring through specific integrin:matrix interactions. In the present study, fibroblasts mechanically loaded for 48 hrs (in the presence of serum) all showed an increase in procollagen synthesis compared to rigid controls. Addition of soluble fibronectin inhibited this stimulation, suggesting that the fibronectin integrins are acting as mechanotransducers on all the matrices tested-fibronectin, collagen and elastin. This would indicate that on collagen and elastin the key cell:matrix interactions are through association with matrix synthesised by the cells rather than interactions with pre-coated matrix. This result supports the earlier hypothesis that fibroblasts grown on collagen and elastin need serum present to synthesise their own fibronectin matrix, allowing them to respond to mechanical load.

To further determine the role of integrins as mechanotransducers, function blocking antibodies to specific integrins were added to cell culture medium prior to mechanical load. These antibodies are thought to act by binding to specific integrins on the surface of

cells, preventing binding to the ECM, although there was no change in cell number observed in the presence of the blocking antibodies. This may be due to integrin compensation, i.e. by blocking particular integrins, preventing them from functioning, other integrins may be expressed by the cell to compensate for the loss of another integrin. This phenomenon has been observed in α5 knock out studies, where α5 deficient cells can still bind to a fibronectin matrix and spread normally. When comparing wild type cells with the α5 knockout cells there was an increase in the recruitment of αv integrins in focal contacts in the α5-null cells. Therefore αv may replace the role of α5β1 in fibronectin matrix assembly and attachment *in vitro* (Yang & Hynes, 1996). A similar form of compensation in human cardiac fibroblasts may be occurring whereby blocking α5β1 inhibits mechanical load induced procollagen synthesis, because these integrins act as mechanotransducers. However there is no cell loss as other integrins compensate for the inhibition of α5β1, although these compensating integrins do not act as mechanotransducers.

Addition of the integrin blocking antibodies to α5β1 inhibited procollagen synthesised by fibroblasts cultured on fibronectin coated plates. This blocking antibody also inhibited mechanical load induced procollagen synthesised by fibroblasts cultured on collagen coated plates. The collagen integrin blocking antibody against α2β1 had no effect on mechanical load induced procollagen synthesis on either the fibronectin or collagen coated plates. The results of this study strongly implicate the fibronectin integrin α5β1 (and αvβ3) as specifically acting as mechanotransducers on human cardiac fibroblasts and suggest the integrin that acts as the mechanotransducer is not determined by the matrix to

which the cell adheres. This observation may also explain the initial observations of the early response to mechanical load on fibronectin, since these fibroblasts already express $\alpha5\beta1$ (required for cell binding to the fibronectin matrix) whereas those on collagen and elastin do not. What was suprising was that both anti-fibronectin integrin antibodies completely inhibited the mechanical load induced procollagen response. If both integrins are acting as mechanotransducers, blocking each one individually would only result in partial inhibition of the load response. However, both $\alpha5\beta1$ and $\alpha v\beta3$ are known to cross link with each other, thus addition of either of the blocking antibodies would block both the integrins leading to a complete inhibition of the load response (personal communication-Dr. Hodivala-Dilke).

The cellular mechanisms of mechanically induced signal transduction are largely unknown. During the course of these studies an increasing number of research papers have confirmed that integrins may act as mechanotransducers (McKenna *et al*, 1998).

To date, the role of integrins as mechanotransducers in human cardiac fibroblasts has not been investigated, nor their importance in mechanical load induced procollagen synthesis. Many studies have concentrated on examining endothelial cells and osteoblasts in response to mechanical stress, as both these cell types are continually exposed to mechanical forces (Yano *et al*, 1995, Pavalko *et al*, 1998). The integrins $\alpha v\beta3$, $\beta1$ and $\beta5$ function as mechanotransducers in response to shear strain in endothelial cells (Chen *et al*, 1999). In HUVECs the integrins $\alpha5\beta1$ and $\alpha2\beta1$ also transduce external mechanical forces into biochemical signals (Yano *et al*, 1995). $\alpha5\beta1$ acts as a mechanotransducer in

cultured human chondrocytes (Wright *et al*, 1997). The mechanisms of mechanical load activation of integrins is discussed below.

7.3.1 Integrin activation by mechanical load

This thesis has demonstrated that mechanical load activates integrin signalling to a greater extent than integrin clustering alone, supporting the idea that integrins function as mechanotransducers. Mechanical load is thought to trigger tyrosine phosphorylation of signalling proteins, that in turn may have consequences for gene expression (Schmidt *et al*, 1998). One of the initial effects of a mechanical stress to an integrin is the induction of the cytoskeletal anchorage. This has been demonstrated in migrating cells by integrin-crosslinking or mechanical twisting of the integrins (Wang *et al*, 1993, Nebe *et al*, 1996, Schmidt *et al*, 1993). Induction of cytoskeletal anchorage promotes anchorage of tyrosine phosphorylated proteins to the cytoskeleton, which is increased with mechanical load. This theory also implicates the cytoskeleton as being critical in upregulation of signalling pathways in response to mechanical load. Subsequently, this thesis has shown an important role for the cytoskeleton in mechanical load induced procollagen synthesis, as disruption of the cytoskeleton resulted in a complete inhibition of procollagen synthesis in response to mechanical load (discussed in more detail later). The cytoskeleton may therefore also represent a key site where physical forces are transformed into biochemical signals. In human cardiac fibroblasts, application of mechanical load results in an increase in cytoskeletal anchorage. This allows more phosphorylated signalling proteins to attach resulting in enhanced activation of downstream signalling pathways. Another study has suggested that mechanical stress (in particular shear stress) induces a conformational

change in the integrins which facilitates their association with signalling molecules (Shc was examined as part of the study by Chen *et al*, 1999).

7.3.2 Integrin expression by mechanical load

Since much of the explanation given above for the significance of the fibronectin matrix is based on α5β1 expression, the effect of cyclic mechanical stretch on the expression of the α5β1 and α2β1-as a control- were investigated. The level of expression of integrin β1 protein was increased in response to mechanical load after 24 hrs, by cells grown on collagen and fibronectin. α2 integrin expression was increased in response to load on the collagen and fibronectin matrix. α5 integrin expression did not change in response to mechanical load on either matrix.

Other studies examining the effect of mechanical stimulation on integrin expression have observed similar results, although the same integrins were not used as in this study. Shear stress responses in endothelial cells involves the β3 integrin subunit, with expression levels of β3 mRNA and protein increased in response to stretch (Suzuki *et al*, 1997). In TE85 human osteosarcoma cells β1 integrin synthesis is upregulated in response to mechanical strain. However, analysis of αv integrin subunit mRNA showed no change with strain. In a similar study using HUVECs, the involvement of different integrins in signalling induced by cyclic stretchwas examined. β1 integrin, α5 and α2 reorganised in a linear pattern parallel with the long axis of the elongated cells on both collagen and fibronectin plates after exposure to cyclic strain. β3 integrin however, did not redistribute. However, in contrast to the other described studies, the expression of integrins α2, α5, and β1 did not

change in response to cyclic strain (Yano *et al*, 1997). Although cyclic stretchdid not change expression levels of α5 integrin, HUVECs on fibronectin expressed more α5 than those on collagen. Other groups have observed the same phenomenon and speculate that since integrin metabolism involves the continuous synthesis and transport to the cell surface with subsequent internalisation/degradation, integrin binding to ECM proteins (fibronectin in this case) interrupts the cycle by blocking the internalisation/degradation stage and permits the retention of integrins at the cell surface (Dalton *et al*, 1995). It has been proposed that spatial redistribution of integrins, especially β1, and not expression, plays an important role in early signalling events such as tyrosine phosphorylation and cytoskeletal reorganisation.

From the results obtained in this study it may be hypothesised that integrin expression in response to mechanical load is not necessarily related to the role of that integrin in mechanotransduction. For example, an increase in α2 integrin expression in response to mechanical load was observed in this study, but from earlier experiments this integrin was found not to be involved in mechanotransduction. Therefore, the increase observed may be related to the increase in collagen production in response to mechanical load.

This study has shown for the first time a specific role for the α5β1 integrin in mechanotransduction resulting in enhanced procollagen synthesis, in human cardiac fibroblasts. To my knowledge there is very little evidence for the role of specific integrins in any signalling.

Summary

This study has shown that the integrins α5β1 and αvβ3 act as mechanotransducers in human cardiac fibroblasts, resulting in an increase in procollagen synthesis, making fibronectin the most effective matrix on which cells may perceive load. These integrins act as mechanotransducers irrespective of the nature of the underlying matrix. However, the ECM composition does not appear to affect fibroblast replication, suggesting that a different mechanism of mechanotransduction and signalling may operate for this response.

7.4 PHOSPHORYLATION OF FOCAL ADHESION COMPLEX PROTEINS IN HUMAN CARDIAC FIBROBLASTS

The focal adhesion complex is made up of various proteins required for the activation of cell signalling pathways (Burridge *et al*, 1988). Many proteins make up the complex including those that bind to the cell cytoskeleton and adaptor proteins that link the integrins to downstream pathways. In this study, phosphorylation levels of some of the focal adhesion proteins that constitute the focal adhesion complex, were measured in response to exposure to mechanical strain. This was to determine whether focal adhesion complex proteins were involved in the mechanical load, integrin mediated procollagen synthesis signalling pathway. These proteins examined were FAK, paxillin and $p130^{Cas}$.

7.4.1 The effect of mechanical load on FAK, paxillin and $p130^{Cas}$ phosphorylation

Application of mechanical load for up to 1 hr resulted in an increase in tyrosine phosphorylation levels of all three proteins examined. Phosphorylation of FAK, paxillin and $p130^{Cas}$ followed similar trends in response to mechanical load, with a significant

increase in phosphorylation at early time points, followed by a cyclical pattern of phosphorylation over time.

FAK phosphorylation levels of human cardiac fibroblasts increased after 1 min or 5 min of mechanical load. In agreement with the results of this study, several studies have reported increases in tyrosine phosphorylation levels of focal adhesion proteins in response to stretching of cells (Yano *et al*, 1996, Hamasaki *et al*, 1995). Mechanical loading (average strain of 10 %, using the Flexercell machine) of endothelial cells (EC) isolated from bovine thoracic aortas lead to an increase in both FAK and paxillin phosphorylation after 30 min (Yano *et al*, 1996). Rat mesangial cells subjected to mechanical load (average strain of 5 %) resulted in stimulation of FAK phosphorylation after 10 min (Hamasaki *et al*, 1995). In response to chemical stimuli, including vasopressin, bombesin (Zachary *et al*, 1995), and thrombin (Lipfert *et al*, 1992), FAK and paxillin phosphorylation is observed after 1 min. The discrepancy in the time course of tyrosine phosphorylation of FAK observed in these studies may be due to activation of different signalling mechanisms by different stimuli.

FAK is a critical mediator of integrin signalling. Although much is known about the role of FAK in integrin mediated signalling, the mechanism of FAK activation by integrins is not clear, although it is thought to be specifically dependent on the integrin β subunit (Akiyamay *et al*, 1994, Guan & Shalloway, 1992). Other evidence suggests that direct binding of FAK to the β subunit is insufficient for FAK activation. Another model proposes an indirect association of FAK with the β subunit via talin (Horwitz *et al*, 1986,

Tapley *et al*, 1989), which had been demonstrated *in vitro* (Chen *et al*, 1995). This model of FAK activation is believed to be the most likely based on several observations: that FAK activation is dependent on an intact cytoskeleton (Burridge *et al*, 1992, Sinnett-Smith *et al*, 1993), and the binding site on the β integrin overlaps with the site required for FAK activation (Chen *et al*, 1995). Studies in this thesis using the α5β1 blocking antibody resulted in the inhibition of mechanical load induced FAK phosphorylation, suggesting that in human cardiac fibroblasts FAK may bind directly to α5β1 leading to the activation of downstream signalling pathways.

Investigations into integrin signal transduction pathways mediated by FAK show that FAK is involved in several different integrin-mediated cellular events, including the promotion of cell migration (Romer *et al*, 1994), proliferation and spreading (Ilic *et al*, 1995), and the prevention of cell apoptosis (Hungerford *et al*, 1996). FAK has not however, been linked to activation of gene expression, either in the presence or absence of mechanical load, thus the data presented here is novel in that it suggests FAK is involved in mechanical load induced signalling pathway resulting in procollagen synthesis.

In accordance with many previous studies, paxillin tyrosine phosphorylation was increased in response to mechanical load in this investigation. This has been reported in airway smooth muscle cells (Smith *et al*, 1998), BAEC's (Yano *et al*, 1997) and HUVECs (Yano *et al*, 1996). Examination of the subcellular distribution of paxillin showed a dramatic increase in staining in response to mechanical load, compared to the control, a similar result to that observed in other studies (Joachim *et al*, 1998). Staining with the paxillin

antibody also revealed that paxillin localised to the ends of actin fibres, confirming its role in the focal adhesion complex. Stimulation of paxillin phosphorylation has not yet been reported in human cardiac fibroblasts in response to mechanical load, this is the first time it has been observed and reported. This data suggests that in human cardiac fibroblasts paxillin becomes localised to the focal adhesion to a greater extent in response to mechanical load and may therefore play an important role in linking integrins and the focal adhesion complex to the cytoskeleton, and is therefore involved in mechano-signal transduction in human cardiac fibroblasts.

Paxillin is thought to play a role in integrin-mediated signal transduction and/or cytoskeletal organisation, based on its localisation to focal adhesions, binding to vinculin, and tyrosine phosphorylation concomitantly with FAK upon integrin activation (Turner *et al*, 1990, Turner *et al*, 1994). The latter result also suggests that paxillin may be a substrate of FAK or a FAK-associated kinase. In various *in vitro* and *in vivo* binding assays, paxillin binds vinculin (Turner *et al*, 1990, Turner *et al*, 1994), integrin β subunits (Tanaka *et al*, 1996), Src family kinases (Weng *et al*, 1993), FAK (Turner *et al*, 1994, Hildebrand *et al*, 1995, Tachibana *et al*, 1995) and other proteins. Because of its lack of enzymatic activity, paxillin is generally believed to act as a scaffolding protein in focal adhesions by mediating interaction with other signalling and/or cytoskeletal proteins. Other than being involved in integrin signal transduction, a functional role for paxillin is thought to be in the localisation of FAK to focal adhesions, and may play additional roles downstream of FAK in the regulation of cell spreading and other events.

To date there has only been one report of an increase in phosphorylation of $p130^{Cas}$, in response to shear stress (Okuda *et al*, 1999), although it has been well documented to be phosphorylated by growth factors (Zachary *et al*, 1997). In our study, mechanical load resulted in an increase in tyrosine phosphorylation levels of $p130^{Cas}$ after 1 min. This suggests it plays a role in mechanical load signalling pathways in human cardiac fibroblasts. This correlates well with current evidence that $p130^{Cas}$ has an important role in integrin signal transduction pathways. It is localised to focal adhesions in rat fibroblasts (Petch *et al*, 1995, Harte *et al*, 1996) as well as in COS cells plated on fibronectin (Nakamoto *et al*, 1997). Further evidence for its role in integrin signal transduction comes from the demonstration that $p130^{Cas}$ binds FAK directly through it SH3 domain (Burnham *et al*, 1996, Harte *et al*, 1996, Polte *et al*, 1995). The functional role of $p130^{Cas}$ binding to FAK is in the regulation of cell motility, as demonstrated in several ways in CHO cell overexpressing FAK (Cary *et al*, 1998). In our system, it can be hypothesised that $p130^{Cas}$ is important in integrin-mediated mechano-signal transduction. To confirm the importance of $p130^{Cas}$ in mechanical load induced procollagen synthesis a specific inhibitor would have to be used. Unfortunately at the present time there are no $p130^{Cas}$ specific inhibitors.

This study has shown for the first time that $p130^{Cas}$ is activated by a mechanical stimulus in human cardiac fibroblasts. These observations collectively suggest that FAK, $p130^{Cas}$ and paxillin phosphorylation in response to mechanical load may lead to the formation of a focal adhesion complex in human cardiac fibroblasts (along with other proteins not examined in this study). The focal adhesion then permits mechanical signals that are transduced by specific integrins, which in human cardiac fibroblasts is the $\alpha5\beta1$ integrin,

to be converted to biochemical signals at the focal adhesion site. In turn, downstream signalling pathways may then be activated leading to procollagen synthesis.

Observed increases in FAK, paxillin and $p130^{Cas}$ phosphorylation in response to mechanical load in this thesis, can be explained by the anchorage of these proteins to the integrin-cytoskeleton linkage, which increases in strength in response to load (discussed earlier in "Integrin activation in response to mechanical load"). The increase in response to mechanical load provokes an increase in anchorage of tyrosine phosphorylated proteins to the linkage. An intact cytoskeleton is required for this to occur, otherwise tyrosine phosphorylated signalling proteins are unable to associate with the integrin-cytoskeleton site.

7.4.2 Effect of static mechanical load on FAK, paxillin and $p130^{Cas}$ phosphorylation

Static mechanical loading of human cardiac fibroblasts resulted in a change in the pattern of phosphorylation, compared to cyclical stretching. For all protein examined, mechanical load increased tyrosine phosphorylation after 1 min. This level of phosphorylation was maintained up to 1 hr. These results suggest that human cardiac fibroblasts are able to detect different types of mechanical load. Other cells may also respond differently to different types of mechanical forces.

Evidence for this comes from quantitative measurements of tyrosine phosphorylation, showing that phosphorylation is influenced by the mode of receptor stressing. For example, intermittent forces are more important than permanent forces in mechanical stressing of bones (Lanyon, 1984). Intermittent stretch is more effective in stimulating

growth than permanent strain. In this case, the intermittent cyclic strain provides a cellular signalling resulting in bone growth. When comparing static mechanical load with cyclical mechanical load in this thesis, human cardiac fibroblasts respond with a difference in the pattern of tyrosine phosphorylation of the focal adhesion proteins FAK, paxillin and $p130^{Cas}$. A cyclical load resulted in a more or less cyclical pattern of phosphorylation of these particular proteins. Static load lead to an increase in tyrosine phosphorylation compared to the rigid control, the level of which was maintained throughout the static load. These results suggest that the nature of the stretch does play a significant role in the pattern of tyrosine phosphorylation. Reasons for this difference in the pattern of phosphorylation may be due to the cyclical movement of the membrane to which the fibroblasts are attached. Cyclical loading may lead to constant association and disassociation of integrins with the ECM. This may lead to changes in the dynamics of focal adhesion assembly/disassembly, which consequently results in changes in tyrosine phosphorylation levels of focal adhesion proteins. Carver *et al* (1991), showed that protein synthesis levels were increased to a lesser extent by cells exposed to static load compared a cyclical load.

Although the effects of mechanical load on fibroblast function are becoming increasingly documented, the signal transduction pathways that mediate these responses are less well understood. Most information on the signal transduction pathways stimulated by mechanical load has been gained from the study of myocytes (Sadoshima & Izumo, 1997). None of these studies have, however linked any signalling pathways to activation of collagen gene expression. Therefore, having shown an involvement of FAK, paxillin and $p130^{Cas}$ in human cardiac fibroblasts response to mechanical load, it was essential to

investigate their role in mechanical load induced procollagen synthesis. This was performed using the tyrosine kinase inhibitor, tyrphostin 25 and an inhibitor of paxillin and the actin cytoskeleton, cytochalasin D.

7.4.3 Effect of focal adhesion complex inhibitors on mechanical load induced procollagen synthesis

The effect of various inhibitors on tyrosine phosphorylation and fibroblast procollagen synthesis were examined. The tyrosine kinase inhibitor tyrphostin 25 and cytochalasin D, which has been well documented to inhibit the actin cytoskeleton, were used to inhibit FAK phosphorylation and paxillin phosphorylation and cause disruption of the cytoskeleton, respectively.

Effect of tyrphostin 25 on procollagen metabolism

Tyrphostin 25 appeared to inhibit both FAK phosphorylation and mechanical load induced procollagen synthesis in a dose dependent manner. Results showing an inhibition of FAK phosphorylation by tyrphostin 25 are in agreement with other similar studies (Yano *et al*, 1996). To my knowledge, no studies have investigated the effect of tyrphostin 25 on procollagen synthesis levels. These data suggest a role for FAK in the mechanical load induced signalling pathway of procollagen synthesis. Tyrphostin 25 is a tyrosine kinase inhibitor that inhibits the phosphorylation of tyrosine residues on signalling proteins. In this thesis, tyrphostin 25 has been used to inhibit FAK phosphorylation, because as yet there are no specific inhibitors of FAK phosphorylation. Tyrphostin 25 is not specific for FAK, although has been used previously to inhibit FAK phosphorylation. Therefore, observations made in this thesis using this inhibitor are speculative. In previous studies

tyrphostin 25 inhibits EGF-stimulated growth and EGF receptor tyrosine autophosphorylation (Peterson & Barnes, 1993) and paxillin phosphorylation (Yano *et al*, 1996). The effect of tyrphostin 25 on paxillin phosphorylation may imply that paxillin is an important protein in mechanical load induced signalling. To confirm the important role of FAK in mechanical load induced procolllagen signalling, FAK knockout cells, which have been used in previous studies to examine the role of FAK in cell migration (Sieg *et al*, 1999) could be utilised.

It has been demonstrated that mechanical load causes integrin activation clustering and association with the cytoskeleton, which in turn causes localisation of focal adhesion proteins to the intracellular domain of the β1 subunit of the integrin and the cytoskeleton. Autophosphorylation, and hence activation of FAK occurs. This then permits recruitment and phosphorylation of other signalling and adaptor proteins including paxillin and $p130^{Cas}$. A similar mechanism may be occurring in human cardiac fibroblasts in response to mechanical load.

Inhibition of FAK phosphorylation by tyrphostin 25 prevent phosphorylation on tyrosine residues of FAK. This would prevent other signalling proteins including paxillin from binding to FAK, preventing its phosphorylation. As a result, adaptor proteins which have been shown to bind to phosphorylated paxillin, including Grb2 and Sos, would not be able to bind, therefore there would be no activation of downstream signalling pathways, mechanical load induced procollagen gene expression would not occur.

Effect of cytochalasin D on procollagen metabolism

Addition of cytochalasin D to human cardiac fibroblasts blocked both paxillin phosphorylation and mechanical load induced procollagen synthesis. Staining for the actin cytoskeleton and paxillin, followed by analysis using confocal microscopy confirmed that the actin cytoskeleton had been disrupted and paxillin staining was significantly reduced. These results not only suggest that paxilllin may be involved in the procollagen synthesis signalling pathway activated by mechanical load, but that the actin cytoskeleton plays a very important role in mechano-signal transduction.

Evidence from the literature has suggested that cell shape is an important determinant of cellular activity. The cell attaches to the ECM via cell surface receptors which changes the cell's shape, forming an elongated and flattened cell, creating tension within the cell. The cytoskeleton therefore ensures a continuity of information from the extracellular environment to the nucleus in the form of structural forces. Stimulation of actin polymerisation and the development of stress fibres occurs in response to mechanical load. mRNA, polysomes and rough endoplasmic reticulum (rER) are all attached to the actin cytomatrix during active translation of protein. Bissell & Barcellos-Hoff (1987) postulated that such interactions stabilise mRNA and increase the efficiency of protein synthesis. *In vivo*, pressure overload leads to an increased efficiency of protein synthesis which might support such a hypothesis. Thus in addition to its role in the formation of focal adhesions, mechanical deformation of the cytoskeleton may directly influence protein synthesis.

The cytoskeleton is linked to integrins and stretch activated ion channel activation and may directly transmit forces applied to the cells, into changes in ion channel activation by the conversion of physical energy into chemical energy (see earlier discussion for the role of ion channels in mechanotransduction). As previously mentioned, focal contacts are specialised sites where cells attach to the ECM (Burridge & Chrzanowska-Wodnika, 1996, Jockush *et al*, 1995). At these sites, clusters of integrins bind externally to the ECM and internally to several cytoplasmic proteins that in turn bind to actin filaments. The key cytoskeletal components of focal contacts include talin and α-actinin, which in turn bind to other structural proteins including vinculin, paxillin and tensin, ultimately leading to the recruitment of actin filaments. Focal adhesions require days to mature into clusters but can reorganise within seconds to minutes when a cell respond to shear stress or tension (Burridge *et al*, 1988, Davies *et al*, 1994). Thus the cytoskeleton may play an important role in mechano-sensing in cells. Schmidt *et al* (1998) observed that application of physical forces to integrins with magnetic beads lead to both an increase in tyrosine phosphorylation of proteins, but also an increased physical anchorage of tyrosine phosphorylated proteins to the cytoskeleton. This suggests that the cytoskeleton may serve as a physical structure where mechanical signals can be converted into chemical-signalling pathways. Indeed, one of the initial effects of a mechanical stress to integrins is the induction of the cytoskeletal anchorage. This has been demonstrated in migrating cells by integrin cross-linking or mechanical twisting of the integrins (Wang *et al*, 1993, Nebe *et al*, 1996, Schmidt *et al*, 1993). In these experiments, it is suggested that mechanical forces induce increased strength of the integrin/cytoskeletal linkage. Activation of MAP kinase by integrins, in response to mechanical stress, depends on an intact cytoskeleton

(Morino *et al*, 1995, Chen *et al*, 1994), as addition of cytochalasin D resulted in complete blocking of MAP kinase phosphorylation. MAP kinase phosphorylation is abolished because cytochalasin disrupts the integrin/cytoskeletal linkage, preventing an accumulation of signalling proteins to this site, which in turn results in no activation of downstream signalling pathways. This highlights the significance of a controlled cytoskeletal anchorage of tyrosine phosphorylated proteins for consequences in cell behaviour.

Using a feline model of right ventricular pressure overload, Kuppuswamy *et al* (1997) showed cytoskeletal association of c-src (a non-receptor tyrosine kinase), FAK and β3 integrin after 4 hr pressure overloading. They also observed tyrosine phosphorylation of several cytoskeleton-associated proteins including c-*src* and movement of c-*src* to the cytoskeleton in cardiac tissue.

In contrast, other data suggests that the cytoskeleton is not involved in the mechano-signal transduction process. Sadoshima *et al* (1992) examined whether the cytoskeleton acts as a mechanotransducer in mediating stretch-induced activation of immediate early (IE) genes c-*fos*, c-*jun*, and c-*myc* and hypertrophy using an *in vitro* model of load-induced cardiac hypertrophy. Transcriptional activation of IE genes is one of the earliest nuclear responses to a variety of external stimuli in muscle and other cell types. Pre-treatment of neonatal rat cardiac myocytes with colchine (binds to tubulin thus inhibiting its assembly to form microtubules) and cytochalasin D followed by a 30 min stretch did not significantly affect the stretch-induced expression of the c-*fos* gene or other IE genes.

Two other focal adhesion proteins, vinculin and α-actinin were examined, using confocal microscopy. Vinculin is a cytoskeletal linker protein and a major protein of focal adhesion complexes, linking the actin cytoskeleton to the integrin adhesion proteins of focal contacts. α-actinin is an actin linker protein. Subcellular distribution of vinculin showed an increase in staining in response to mechanical load in these studies. Results from studies using cytochalasin D to disrupt the cytoskeleton suggest that the cytoskeleton is essential for mechanical load induced procollagen synthesis, and because of vinculin's role as a linker protein it would suggest that vinculin is important in mechano-signal transduction in our system. Actin stress fibres increased in number and became highly organised in response to mechanical load in human cardiac fibroblasts. α-actinin showed no significant changes in staining intensity in response to mechanical load, compared to the rigid controls. To date there have been no studies involving mechanical load and its effect on α-actinin.

Another study has suggested that vinculin is not involved in mechanical transduction, in primary bovine osteoblasts (Meyer *et al*, 1997). The study determined that application of mechanical load resulted in disassembly of vinculin from focal contacts, and that after loading its distribution in focal adhesions was fully restored. However, studies of mechanical stressing of osteoblasts reported an increase in vinculin protein upon application of stress (Meazzini *et al*, 1998).

Summary

This study has shown that the focal adhesion proteins FAK, paxillin and $p130^{Cas}$ become tyrosine phosphorylated in response to mechanical load. Subcellular distribution of paxillin and another focal adhesion protein, vinculin showed an increase in focal adhesion formation after exposure to mechanical load, suggesting that mechanical loading of human cardiac fibroblasts leads to a co-ordinated change in the cytoskeleton. These proteins appear to play an important role in mechanical load signalling events. Further investigations were able to show that both FAK and paxillin (via the cytoskeleton) were involved in the mechanical load induced procollagen synthesis signalling pathway, as inhibitors to these proteins prevented the mechanical-load induced stimulation of procollagen synthesis.

These data taken together, implicate the focal adhesion complex as being an important part of the process of mechano-signalling leading to increased procollagen synthesis. To date, the signalling pathway(s) which leads to the activation of procollagen synthesis has yet to be elucidated, some progress was made in this thesis in advancing our understanding of this process.

7.5 POTENTIAL DOWNSTREAM SIGNALLING PATHWAYS ACTIVATED BY MECHANICAL LOAD

Mechanical stretching has been shown to stimulate a variety of signal transduction pathways including the MAPK family member ERK 1/2 and its upstream activators MEK 1 and Raf-1 (Yamazaki *et al*, 1996). c-Jun NH2 - terminal kinase (JNK), another MAPK family member, has also been shown to be activated by stretch in cardiac myocytes (Liang

et al, 1997) and by flow in endothelial cells (Ishida *et al*, 1995). Activation of these signalling pathways by mechanical load, as well as activation of protein kinase C (Yazaki *et al*, 1993) and Rsk (Baliga *et al*, 1999), have each been implicated in signalling one or more components of the hypertrophic phenotype. In fibroblasts very little is known about the signalling pathways stimulated by mechanical load and the biological events that they mediate. Elucidation of the signalling pathways involved may be important in designing therapeutic agents for blocking the development of cardiac fibrosis during cardiac hypertrophy.

Signalling downstream of the focal adhesion complex may activate one or multiple signalling pathways. It is known that FAK has a binding site for the p85 subunit of PI 3-kinase. This enzyme is an essential component of the phospholipid signalling pathway where it is required to phosphorylate phosphatidylinositol lipids (see figure 1.4 in introduction). PI 3-kinase has been shown to lead to the downstream activation of the MAPK signalling pathway (King *et al*, 1997). The role of these pathways in mechanical load induced procollagen synthesis was therefore investigated.

7.5.1 Role of the phospholipid pathway in mechanical load induced procollagen synthesis

The selective and potent PI 3-kinase inhibitor, wortmannin blocked both FAK phosphorylation and mechanical load induced procollagen synthesis at the highest dose used (1μM). Procollagen synthesis was not affected by the lower dose of 100 nM, but FAK phosphorylation was inhibited. LY294002, which also inhibits PI 3-kinase, also had

no effect on procollagen synthesis. Evidence in the literature has shown that a dose of 100nM, wortmannin acts as a specific inhibitor of PI 3-kinase activity. This dose has no effect on PI 4-kinase, PKC, c-src or PLC activity (Powis *et al*, 1994). In the same study, LY294002 was also found to be specific for PI 3-kinase, but with decreased potency compared to wortmannin. The high dose of wortmannin used (1μM) specifically inhibits PI 4-kinase (Powis *et al*, 1994, Meyers & Cantley, 1997, Balla *et al*, 1997).).

Although PI 3-kinase has recently been demonstrated to play a role in integrin signal transduction (Chen *et al*, 1996, Chen *et al*, 1994) and signalling by mechanical load (Go *et al*, 1998), results obtained in this thesis suggest that PI 3-kinase is involved in mechanical load induced FAK phosphorylation but not procollagen synthesis (which appears to involve PI 4-kinase). This has never been reported, with very little information available on PI 4-kinase and its role in signalling. In one particular study, neither PI 3-kinase nor PI 4-kinase, but PI 5-kinase was found to accumulate in response to integrin ligation. PI 5-kinase in this situation was thought to function in the formation of focal adhesions and actin stress fibres in fibroblasts (McNamee *et al*, 1993). From this evidence it may be possible that in human cardiac fibroblasts PI 4-kinase as well as PI 3-kinase accumulates and is activated in response to mechanical load.

Although no studies have implicated PI 4-kinase in mechanical load activated signalling pathways in any cell type, expression patterns of PI 4-kinase have been found in high levels in the human heart, brain and placenta (Nakagawe *et al*, 1996, Balla *et al*, 1997). Epidermal growth factor (EGF) stimulation of epithelial and fibroblast cells stimulated

PIP_2 hydrolysis with a concomitant increase in PI 4-kinase activity (Pike & Eakes, 1987, Payrastre *et al*, 1990, Cochet *et al*, 1991).

Wortmannin was also used in this thesis in an attempt to inhibit FAK phosphorylation. This was carried out to examine the role of PI 3-kinase in the phosphorylation of FAK induced by mechanical load. At the low dose of wortmannin, tyrosine phosphorylation of FAK stimulated by mechanical load was inhibited, as well as at the high dose used. These results suggest that mechanical load stimulates FAK phosphorylation through a PI 3-kinase dependent pathway in human cardiac fibroblasts, but that procollagen synthesis induced by mechanical load occurs through a PI 4-kinase pathway. Both PI 3-kinase and PI 4-kinase are essential enzymes required for the activation of the phospholipid signalling pathway. Both enzymes phosphorylate phosphatidylinositol (PI) to produce phosphatidylinositol monophosphate (PIP). PIP is phosphorylated producing phosphatidylinositol bisphosphate (PIP_2). The enzymes only differ in the position at which they phosphorylate PIP on the inositol ring. By the action of PLC, PIP_2 is broken down to produce inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Figure 7.1). Therefore activation of either enzyme results in the formation of the same downstream products-with the only differences being on their position of phosphorylation.

The phospholipid pathway is known to be activated *in vivo* during cardiac hypertrophy, as well as in *vitro* in rat cardiac myocytes (Komuro *et al*, 1991). The activation of this pathway in response to mechanical load was therefore examined in human cardiac fibroblasts to determine its role in mechanical load induced procollagen synthesis.

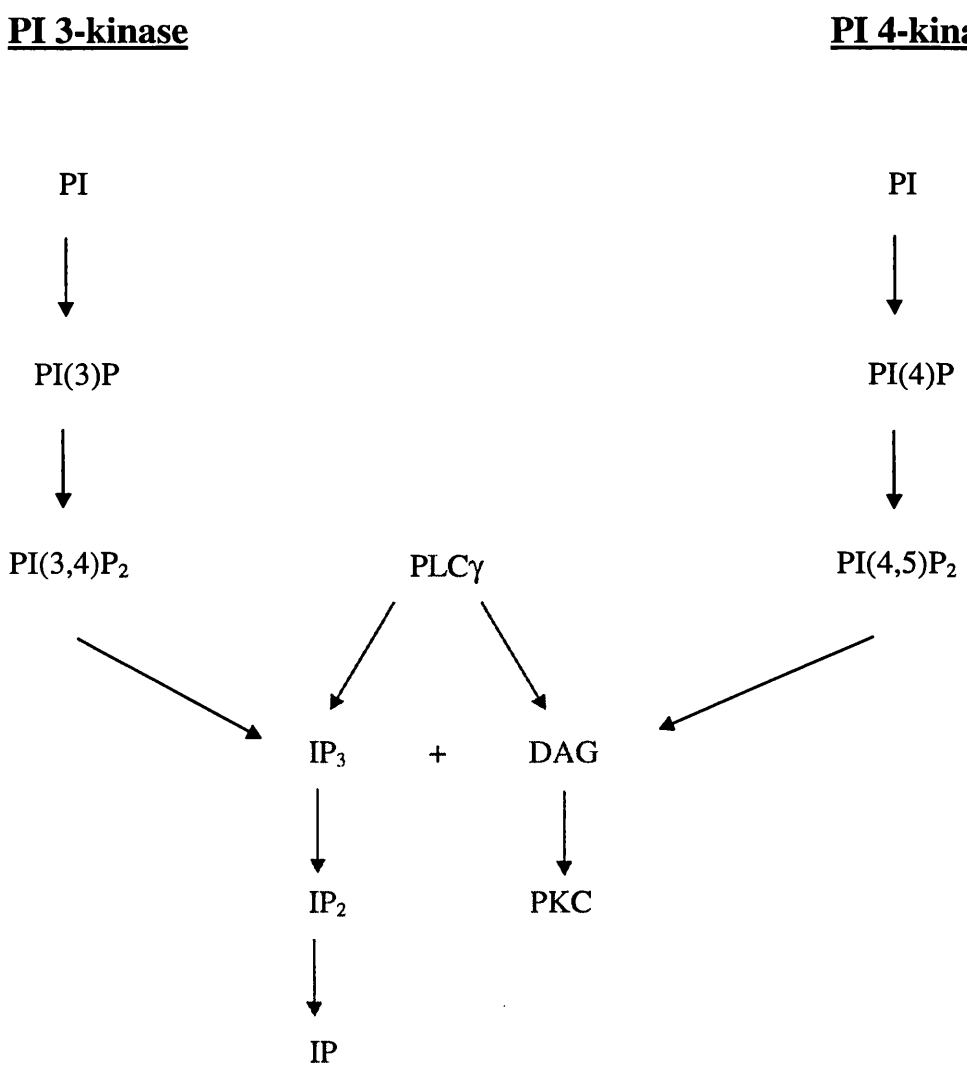


Figure 7.1 Diagram showing the pathways stimulated by PI 3-kinase and PI 4-kinase. PI 4-kinase phosphorylates phoshatidylinositol (PI) at a different position on the inositol ring. The pathway in grey indicates a common pathway for both enzymes.

Measurements of phosphatidylinositol turnover resulted in an increase in IP_1, IP_2 and IP_3 levels after 1 and 5 min of mechanical load. In agreement with the results of this study, numerous other studies have reported similar results. Immediately after stretching, the activation of phosphatidylinositol turnover is observed in rat cardiac myocytes. One minute after stretching, IP_1 and IP_2 significantly increased and reached about two-fold levels of control after 5 min (Yazaki *et al*, 1993). New-born rat calvarial bone cells subjected to a cyclical biaxial mechanical strain resulted in inositol metabolism. IP, IP_2 and IP_4 all reached peak accumulation after 20 s mechanical strain (Brighton *et al*, 1992). Exposure of endothelial cells to cyclic strain induced rapid generation of IP_3, 10 secs after cyclic deformation (Rosales *et al*, 1997), accompanied by an increase in intracellular Ca^{2+} concentration.

Although such studies have been performed and increases in inositol observed, the role of phosphatidylinositols in the heart is not well understood. Little is known about their effect in the hypertrophied heart. The increase in phosphatidylinositols has been observed on numerous occasions in both *in vivo* and *in vitro* studies, resulting from mechanical stimulation or chemical stimulation (von Harsdorf *et al*, 1989). In a comparison between cardiomyopathic hamster heart cells and control cells (Kawaguchi *et al*, 1993), an increase in phosphatidylinositol metabolism was observed in the cardiomyopathic heart. Release of IP_3 and DAG followed. Both these proteins act as second messengers activating other signalling molecules, including increasing levels of intracellular Ca^{2+} and PKC which in turn, have been implicated in mechanical stretch induced signalling (Sadoshima & Izumo, 1996).

Enhanced PIP_2, IP_3 and Ca^{2+} or DAG and PKC may increase protein synthesis in the heart and contribute to the development of pressure overload cardiac hypertrophy. Komuro and co-workers (1991) also observed an increase in PI turnover after mechanical stimuli in rat cardiac myocytes. They suggested that mechanical stimuli might directly induce cardiac hypertrophy and specific gene expression via PKC activation, with PKC activation occurring in response to PI metabolism. Activation of a similar pathway may occur in our fibroblasts.

The phospholipase D pathway provides an alternative source of DAG, through the production of phosphatidic acid (PA), which in turn activates isoforms of PKC. The PLD pathway can be the major pathway for the activation of PKC in some systems (Exton *et al*, 1990). PKC isozymes are responsible for the phosphorylation of specific cardiac substrate proteins that may be involved in the regulation of cardiac contractility, hypertrophic growth and gene expression. The membrane phospholipid phosphatidylcholine (PC) is hydrolysed by PLD (as well as PLC and PLA_2) in response to various growth stimuli, and their breakdown products, including PA, act as second messengers (Exton *et al*, 1990). Therefore, the role of the PLD pathway in mechanical load induced procollagen synthesis was examined. Ethanol acts as an alternate substrate in place of water for PLD producing phosphatidylethanol (PEt) instead of phosphatidic acid (PA), thus blocking the PLD pathway. The presence of ethanol did not have any effect on mechanical load induced procollagen synthesis levels. This data suggests there is no involvement of PLD in the regulation of mechanical load induced procollagen synthesis. These observations are in contrast with many previous studies, whereby PA has been observed in the hypertrophied

heart, and been implicated in the development of cardiac hypertrophy (Sadoshima & Izumo, 1993).

In rat cardiac myocytes, a 30 min stretch resulted in an almost 3 fold increase in PA (Sadoshima & Izumo, 1993), suggesting that mechanical stretch of cardiac myocytes activates the PLD pathway. Addition of 10 mM PA to adult rat cardiac myocytes results in the stimulation of both MAP kinase and protein synthesis (Xu *et al*, 1996), suggesting that PA may play an important role in the development of cardiac hypertrophy. PLD has been implicated, with other signalling pathways to converge into activation of the $p67^{SRF}$-$p62^{TCF}$ complex via the serum response element, causing induction of c-*fos* (Sadoshima & Izumo, 1993) in cardiac myocytes, and activating gene expression. PA increases IP_3 levels, and levels of PA are found to be elevated in the hypertrophied heart. It has also been demonstrated to increase Ca^{2+} concentrations in freshly isolated adult rat cardiac myocytes (Xu *et al*, 1996).

To my knowledge there have been no investigations as to the role of PLD and its downstream signalling components in mechanical load induced procollagen synthesis in human cardiac fibroblasts. In human cardiac fibroblasts the PLD pathway may not be a dominant pathway activated by mechanical load (Sadoshima & Izumo, 1996).

This thesis has shown that neither PI 3-kinase nor the PLD pathway is not involved in the procollagen synthesis signalling pathway. This implies that other signalling pathways may be activated in response to mechanical load in human cardiac fibroblasts. These may include the MAP kinase pathways, which are activated in response to mechanical load and

have recently been linked to mechanical load induced procollagen gene expression (Papakrivopoulou, PhD Thesis, 1999).

7.5.2 Role of the MAP kinase pathway in mechanical load induced procollagen synthesis

A time course of ERK 1/2 phosphorylation showed an increase in ERK 1/2 phosphorylation in response to mechanical load after 5 min. This preliminary data suggests that the ERK 1/2 MAPK pathway may be involved in the mechanical load induced procollagen synthesis signalling pathway. Activation of ERK has previously been demonstrated in various cell types, in response to mechanical stimuli (Sadoshima *et al*, 1996, Yazaki *et al*, 1993).

MacKenna and co-workers (1998) reported that rat cardiac fibroblasts responded to biaxial stretch by transiently activating both ERK and JNK1 cascades. These pathways were activated as a direct result of the stretch, which appeared to be mediated by integrins. Although no downstream gene expression/protein synthesis was examined, it was proposed that activation of both ERK and JNK through specific integrins may lead to remodelling of the ECM in response to stretch, because JNK activation leads to the phosphorylation of c-*jun*, which is a component of the transcription factor AP-1. This transcription factor can activate many genes, including collagenase, that may aid remodelling of the matrix (Vincenti *et al*, 1996). AP-1 is also present on the collagen promoter in the first intron. A pulsatile mechanical stretch of rat cardiac myocytes also resulted in an increase in ERK as well as the SAPK and JNK pathways (Seko *et al*, 1999). ERK was not directly activated by stretch but was mediated in part by VEGF (induced by

TGF-β). Pulsatile stretch was thought to induce myocardial hypoxia, as this state induces VEGF to adapt to the hypoxic condition. Human skin fibroblasts bound to fibronectin show an increase in ERK 1/2 phosphorylation. This activation requires a matrix:integrin interaction as addition of soluble anti-β1 integrin antibody inhibited phosphorylation (Morino *et al*, 1995). Finally, in a very recent study cyclic mechanical stress of serum starved VSMCs isolated from rat aorta induced phosphorylation of ERK, SAPK, JNK and p38 MAP kinase (Li *et al*, 1999).

Although the data for ERK phosphorylation in response to mechanical load is only preliminary, it implies that the ERK pathway may be involved in the mechanical load induced procollagen synthesis signalling pathway. Other studies in our laboratory have shown that inhibition of ERK 1/2 phosphorylation using a specific MEK inhibitor (PD98059) not only inhibited ERK 1/2 phosphorylation but also procollagen mRNA (Papakrivopoulou, PhD Thesis, 1999). Although no other studies have reported this to date, activation of ERK has previously been demonstrated in other cell types in response to mechanical stimuli (Sadoshima & Izumo, 1993, Komoru *et al*, 1996, Li *et al*, 1996, Yamazaki *et al*, 1993, Jo *et al*, 1997, Ishida *et al*, 1996).

Growth factor activation of MAP kinases is the best characterised pathway. Ligand binding to their receptors results in receptor dimerisation and autophosphorylation on tyrosine residues in the intracellular domains of the receptor. Coupling of these receptors to a MAP kinase pathway occurs via adaptor proteins, Grb2 and Sos (son of sevenless). binding of Grb2 to SH2 domains of growth factor receptors recruits Sos to the plasma

membrane (Egan *et al*, 1993). This brings it in close proximity with Ras, a small GTP-binding protein located at the cytoplasmic surface of the membrane (Margolis *et al*, 1994). Sos induces the dissociation of GDP from Ras, allowing the formation of an activated GTP-Ras complex (Bonfini *et al*, 1992). This in turn leads to ERK 1/2 activation. This pathway of MAP kinase activation also occurs upon integrin activation, which results in the formation of the focal adhesion complex. As this thesis has shown that focal adhesion proteins in tyrosine phosphorylation increases in response to load, which via their association with Src and Grb/Sos, activate ERK 1/2, the MAP kinase pathway may be the major pathway involved in mechanical load induced procollagen synthesis. Two components of the focal adhesion complex Grb2 and Sos, which act as adaptor proteins, become phosphorylated and in turn activate upstream signalling components on the MAP kinase pathway. This results in the activation of ERK, which translocates to the nucleus where it activates transcription factors by phosphorylation resulting in procollagen gene expression.

Mechanical load of cardiac myocytes has been shown to activate several components of the ERK 1/2 pathway (Yamazaki *et al*, 1995, Sadoshima *et al*, 1993), similar to that which occurs in response to growth factors. This is not surprising as growth factors such as ET-1 and AII are released by myocytes in response to mechanical load and mediate at least in part the activation of this pathway by mechanical load (Yazaki *et al*, 1995, Sadoshima *et al*, 1997). From previous studies and results obtained in this thesis, it can be hypothesised that mechanical load activation of MAP kinase occurs through integrin activation, via tyrosine phosphorylation of focal adhesion proteins and adaptor proteins, which activate the MAP kinase pathway.

Taken together, data from this thesis suggest that mechanical load activates both the phospholipid and the ERK MAP kinase pathway which may play important role in stimulating procollagen synthesis in the hypertrophic heart.

Summary

In summary, these experiments suggest that two signalling pathways are activated in response to mechanical load. The first pathway is the phospholipid pathway, and in response to mechanical load, the enzyme PI 4-kinase becomes activated. This results in phosphorylation of PIP leading in turn to phosphorylation of phospholipids downstream of this molecule. Through the action of PLC, PIP_2 is broken down to IP_3 and DAG which activate intracellular Ca^{2+} and PKC which may act as second messengers involved in procollagen synthesis. The ERK 1/2 MAP kinase pathway may play an important role in mechanical load induced procollagen synthesis, although further studies are needed to determine this.

7.6 SUMMARY, IMPLICATIONS AND SUGGESTIONS FOR FUTURE STUDIES

In summary, this study has demonstrated that human cardiac fibroblasts respond to mechanical load by increasing procollagen synthesis levels. This effect occurs after 24 hrs when fibroblasts are cultured and stretched on a fibronectin matrix. The collagen and elastin matrix increase mechanical load induced procollagen synthesis levels only after 48 hrs of mechanical load and in the presence of serum. Together these results suggest that increased collagen synthesis in response to mechanical load is dependent on the ECM composition.

Integrins appear to be acting as mechanotransducers in human cardiac fibroblasts, allowing the transmission of an extracellular mechanical signal into an intracellular signal of gene regulation. For human cardiac fibroblasts the integrins $\alpha5\beta1$ and $\alpha v\beta3$ carry out this process. The cytoskeleton also appears to play an important role in mechano-signal transduction, possibly through linkage to these integrins which in turn results in the anchorage of signalling proteins including those proteins that compose the focal adhesion complex.

Examination of the signalling pathways activated in response to mechanical load lead to the observation that the focal adhesion complex plays a major role in mechanical load induced signalling. FAK, paxillin and $p130^{Cas}$ all responded to exposure to mechanical load with an increase in tyrosine phosphorylation.

Preliminary data suggests that PI4 kinase is activated in response to mechanical load, activating the phospholipid pathway. This pathway in turn generates second messengers which are involved in the hypertrophic response in the heart. The ERK 1/2 MAP kinase pathway is also activated in response to mechanical load, and this pathway may also be involved in mechanical load induced procollagen synthesis. These two pathways may converge in response to mechanical load.

Results obtained from studies carried out in this thesis highlight three potential areas which would be of particular interest for further studies:

1) Further elucidation of the importance of the fibronectin matrix in mechanical load induced procollagen synthesis

Future studies should be carried out on the fibronectin matrix:fibroblast interaction in greater depth. Measurements of incorporation of soluble fibronectin into the ECM on the three matrices would help determine why cells on fibronectin respond to mechanical load at 24 hrs and in the absence of serum. This could be carried out by radiolabelling soluble fibronectin then measuring the levels that become incorporated into the matrix. Fluorescent staining of the fibronectin matrix after mechanical load on all three matrices would also determine whether there is more fibronectin produced when cells are grown on the fibronectin matrix.

2) Integrin signalling

Use of α5-null cells, αv-null cells and wild type cells would allow the comparison of signalling pathways in these different cells in response to load. This would determine whether signalling still occurs in the absence of the integrin, or if its presence is necessary for procollagen synthesis signalling.

3) Examination of signalling pathways involved in mechanical load induced procollagen synthesis

Full elucidation and characterisation of the signalling pathways activated by mechanical load and resulting in procollagen synthesis should be examined. There are clearly many processes activated by load, but the key experiments are to determine which are important in the stimulation of procollagen synthesis. At the moment such studies are hampered by the lack of sufficiently specific inhibitors of various pathways. This thesis has proposed two potential signalling pathways, but there is further evidence from our laboratory that other MAP kinase pathways are activated in response to mechanical load (Papakrivopoulou, PhD Thesis, 1999).

Use of specific inhibitors to these signalling molecules could potentially lead to the treatment of fibrotic disorders. This could lead to *in vivo* studies, examining the role of these inhibitors in reversing collagen synthesis stimulated by pressure overload.

REFERENCES

Abedi, H., and Zachary, I. 1997. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J. Biol. Chem.* 272: 15442-15451.

Abedi, H., Dawes, K., and Zachary, I. 1995. Differential effects of platelet-derived growth factor BB on p125 focal adhesion kinase and paxillin tyrosine phosphorylation and on cell migration in rabbit aortic vascular smooth muscle cells and Swiss 3T3 fibroblasts. *J. Biol. Chem.* 270: 11367-11376.

Abrahams, C., Janicki, J.S., and Weber, K.T. 1987. Myocardial hypertrophy in *Macaca fascicularis*. Structural remodelling of the collagen matrix. *Lab. Invest.* 56: 676-683.

Ahumada, G.G., and Saffitz, J.E. 1984. Fibronectin in rat heart: a link between cardiac myocytes and collagen. *J. Histochem. Cytochem.* 32: 383-388.

Akiyama, S.K., Yamada, S.S., Yamada, K.M., and La Flamme, S.E. 1994. Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. *J. Biol. Chem.* 269: 15961-15964.

Amenta, P.S., Gay, S., Vaheri, A., and Martinez-Hernandez, A. 1986. The extracellular matrix is an integrated unit: Ultrastructural localisation of collagen types I, III, IV, V, VI, fibronectin and laminin in human term placenta. *Collagen Rel. Res.* 6: 125-152.

Anderson, K.R., St John Sutton, M.G. and Lie, J.T. 1979. Histopathological types of cardiac fibrosis in myocardial disease. *J. Pathol.* 128: 79-85.

Anversa, P., Levicky, V., Beghi, C., McDonald, S.L., and Kikkawa, Y. 1983. Morphometry of exercise-induced right ventricular hypertrophy in the rat. *Circ. Res.* 52: 57-64.

Anversa, P., Olivetti, G., and Loud, V.A. 1980. Morphometric study of early postnatal development in the left and right ventricular myocardium of the rat. Hypertrophy, hyperplasia and binucleation of myocytes. *Circ. Res.* 46: 495-502.

Anversa, P., Palackal, T., Sonnenblick, E.H., Olivetti, G., and Capasso, J.M. 1990. Hypertensive cardiomyopathy. Myocyte nuclei hyperplasia in the mammalian rat heart. *J. Clin. Invest.* 85: 994-997.

Arroyo, A.G., Campanero, M.R., Sanchezmateos, P., Zapata, J.M., Ursa, M.A., Delpozo, M.A., and Sanchezmadrid, F. 1994. Induction of tyrosine phosphorylation during ICAM-3 and LFA-1- mediated intercellular-adhesion, and its regulation by the cd45 tyrosine phosphatase. *J. Cell Biol.* 126: 1277-1286.

Astier, A., Avraham, H., Manie, S.N., Groopman, J., Canty, T., Avraham, S., and Freedman, A.S. 1997. The related adhesion focal tyrosine kinase is tyrosine-

phosphorylated after β1-integrin stimulation in B cells and binds to $p130^{cas}$. *J. Biol. Chem.* 272(1): 228-232.

Baglia, R.R., Pimental, D.R., Zhao, Y.Y., Simmons, W.W., Marchionni, M.A., Sawyer, D.B., and Kelly, R.A. 1999. NRG-1 induced cardiomyocyte hypertrophy. Role of PI3-kinase, p70(S6K), and MEK-MAPK-RSK. *Am. J. Physiol.* 277: H2026-H2037.

Balla, T., Downing, G.J., Jaffe, H., Kim, S., Zolyomi, A., and Catt, K.J. 1997. Isolation and molecular cloning of wortmannin-sensitive bovine type III phosphotidylinositol 4-kinases. *J. Biol. Chem.* 272: 18353-18366.

Banes, A.J., Link, G.W.J., Gilbert, J.W., Tran Son Tay, R., and Monbureau, O. 1990. Culturing cells in a mechanically active environment. *Am. Biotechnol. Lab.* 8: 12-22.

Banes, A.J., Tsuzaki, M., Yamamoto, J., Fischer, T., Brigman, B., Brown, T., and Miller, L. 1995. Mechanoreception at the cellular level: The detection, interpretation and diversity of responses to mechanical signals. *Biochem. Cell Biol.* 73: 349-365.

Bashey, R.I., Cox, R., McCann, J., and Jiminez, S.A. 1989. Changes in collagen biosynthesis, types, and mechanisms of aorta in hypertensive rats. *J. Lab. Clin. Med.* 113: 604-611.

Bashey, R.I., Donnelly, M., Insinga, F. and Jiminez, S.A. 1992. Growth properties and biochemical characterization of collagens synthesised by adult rat heart fibroblasts in culture. *J. Mol. Cell. Cardiol.* 24: 691-700.

Baskin, L., Howard, P.S., and Macarak, E. 1993. Effect of mechanical forces on extracellular matrix synthesis by bovine urethral fibroblasts in vitro. *J. Urol.* 150: 637-641.

Bellis, S.L., Miller, J.T., and Turner, C.E. 1995. Characterisation of tyrosine phosphorylation of paxillin *in vitro* by focal adhesion kinase. *J. Biol. Chem.* 270: 17437-17441.

Berg, R.A., Schwartz, M.L, and Crystal, R.G. 1980. Regulation of the production of secretory proteins: Intracellular degradation of newly synthesised "defective" collagen. *Proc. Natl. Acad. Sci. USA.* 77: 4746-4750.

Bienkowski, R.S. 1984. Collagen degradation in human lung fibroblasts: Extent of degradation, role of lysosomal proteases, and evaluation of an alternative hypothesis. *J. Cell. Physiol.* 121: 152-158.

Birge, B.B., Fajardo, J.E., Reichman, C., Shoelson, S.E., Songyang, Z., Cantley, L.C., and Hanafusa, H. 1993. Identification and characterization of a high-affinity interaction between v-Crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. *Mol. Cell. Biol.* 13(8). 4648-4656.

Birk, D.E., and Trelstand, R.L. 1986. Extracellular compartments in tendon morphogenesis: Collagen fibril, bundle and microaggregate formation. *J. Cell. Biol.* 103: 231-240.

Birk, D.E., Fitch, J.M., Barbiarz, J.P., Doane, K.J. and Linsenmayer, T.F. 1990. Collagen fibrillogenesis *in vitro*: Interaction of types I and V collagen regulates fibril diameter. *J. Cell Sci.* 95: 649-657.

Bishop, J.E. and Laurent, G.J. 1989. Mediators affecting fibroblast function are present in hypertrophied rabbit right ventricles following the development of pulmonary hypertension. *Am. Rev. Respir. Dis.* 139: A171.

Bishop, J.E., Greenbaum, R., Gibson, D.G., Yacoub, M., and Laurent, G.J. 1990b. Enhanced deposition of predominantly type I collagen in myocardial disease. *J. Mol. Cell. Cardiol.* 22: 1157-1165.

Bishop, J.E., Guerreiro, D., and Laurent, G.J. 1990a. Changes in the composition and metabolism of arterial collagens during the development of pulmonary hypertension in rabbits. *Am. Rev. Respir. Dis.* 141: 450-455.

Bishop, J.E., Mitchell, J.J., Absher, P.M., Baldor, L., Geller, H.A., Woodcock-Mitchell, J., Hamblin, M.J., Vacek, P. and Low, R.B. 1993. Cyclic mechanical deformation

stimulates human lung fibroblast proliferation and autocrine growth factors. *Am. J. Respir. Cell. Mol. Biol.* 9: 126-133.

Bishop, J.E., Rhodes, S., Laurent, G.J., Low, R.B., and Stirewalt, W.S. 1994. Increased collagen synthesis and decreased collagen degradation in right ventricular hypertrophy induced by pressure overload. *Cardiovasc. Res.* 28: 1581-1585.

Bissell, M.J., and Barcellos-Hoff, M.H. 1987. The influence of extracellular matrix on gene expression: is structure the message? *J. Cell Sci. Suppl.* 8: 327-343.

Boerrigter, G., Mundhenke, M., Stark, P., Schulte, H.D., Strauer, B.E., and Schwartzkopff, B. 1998. Immunohistochemical video-microdensitometry of myocardial collagen type I and III. *Histochem. J.* 30: 783-791.

Bonfini, L., Karlovich, C.A., Dasgupta, C., and Banerjee, U. 1992. The Son of sevenless gene product: a putative activator of Ras. *Science.* 255: 603-606.

Booth, B.A., Polak, K.L., and Uitto, J. 1980. Collagen biosynthesis by human skin fibroblasts. I. Optimization of the culture conditions for synthesis of type I and type III procollagens. *Biochim. Biophys. Acta.* 607: 145-160.

Booz, G.W., and Baker, K.M. 1995. Molecular signalling mechanisms controlling growth and function of cardiac fibroblasts. *Cardiovasc. Res.* 30: 537-543.

Borg, T.K. and Cauldfield, J.B. 1981. The collagen matrix of the heart. *Fed. Proc.* 40: 2037-2041.

Borg, T.K., Sullivan, T., and Ivy, J. 1982. Functional arrangement of connective tissue in striated muscle with emphasis on cardiac muscle. *Scan. Electron. Microsc.* 4: 1775-1784.

Braga, V.M., Machesky, L.M., Hall, A., Hotchin, N.A. 1997. The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* 137: 1421-1431.

Brayden, J.E, Halpern, W., and Brann, L.R. 1982. Biochemical and mechanical properties of resistance arteries from normotensive and hypertensive rats. *Hypertension.* 5: 17-25.

Breul, S.D., Bradley, K.H., Hance, A.J., Schafer, M.P., Berg, R.A., Crystal, R.G. 1980. Control of collagen production by human diploid lung fibroblasts. *J. Biol. Chem.* 255: 5250-5260.

Brighton, C.T., Sennett, B.J., Farmer, J.C., Iannotti, J.P., Hansen, C.A., Williams, J.L., and Williamson, J. 1992. The inositol phosphate pathway as a mediator in the proliferative response to rat calvarial bone cells to cyclical biaxial mechanical strain. *J. Orthop. Res.* 10: 385-393.

Brilla, C.G., and Maisch, B. 1994. Regulation of the structural remodelling of the myocardium: from hypertrophy to heart failure. *Eur. Heart J.* 15 (Suppl D): 45-52.

Brody, J.S., Burki, R., and Kaplin, N. 1978. Deoxyribonucleic acid synthesis in lung cells during compensatory lung growth after pneumonectomy. *Am. Rev. Respir. Dis.* 117: 307-316.

Brooks, P.C., Montgomery, A.M., Rosenfeld, M., Reisfeld, R.A., Hu, T., Klier, G., and Cheresh, D.A. 1994. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell.* 79: 1157-1164.

Brown, M.C., Perrotta, J.A., and Turner, C.E. 1996. Idenification of LIM 3 as the principal determinant of paxillin focal adhesion localisation and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell. Biol.* 135: 1109-1123.

Brown, S.L., Lundgren, C.H., Nordt, T., and Fujii, S. 1994. Stimulation of migration of human aortic smooth muscle cells by vitronectin: implications for atherosclerosis. *Cardiovasc. Res.* 28: 1815-1820.

Buday, L., and Downward, J. 1993. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell.* 73: 611-620.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4: 487-525. Review.

Burridge, K., and Chrzanowska-Wodnicka, M. 1996. Focal adhesions, contractility, and signaling. ***Annu. Rev. Cell Dev. Biol.*** 12: 463-518.

Burridge, K., Turner, C.E., and Romer, L.H. 1992. Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. *J. Cell. Biol.* 119: 893-903.

Burnham, M.R., Harte, M.T., Richardson, A., Parsons, J.T., and Bouton, A.H. 1996. The identification of p130cas-binding proteins and their role in cellular transformation. *Oncogene.* 12: 2467-72.

Butt, R.P. and Bishop, J.E. 1997. Mechanical load enhances the stimulatory effect of serum growth factors on cardiac fibroblast procollagen synthesis. *J. Mol. Cell. Cardiol.* 29: 1141-1151.

Butt, R.P., Laurent, G.J., and Bishop, J.E. 1995. Mechanical load and polypeptide growth factors stimulate cardiac fibroblast activity. *Ann N Y Acad Sci.* 27: 387-393.

Cadre, B.M., Qi, M., Eble, D.M., Shannon, T.R., Bers, D.M., and Samarel, A.M. 1998. Cyclic stretch down-regulates calcium transporter gene expression in neonatal rat ventricular myocytes. *J. Mol. Cell. Cardiol.* 30: 2247-2259.

Campa, J.S., McAnulty, R.J., and Laurent, G.J. 1990. Application of high-pressure liquid chromatography to studies of collagen production by isolated cells in culture. *Anal. Biochem.* 186: 257-263.

Campbell, S.E., and Katwa, L.C. 1997. Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. *J. Mol. Cell. Cardiol.* 29: 1947-1958.

Carroll, E.P., Janicki, J.S., Pick, R., and Weber, K.T. 1989. Myocardial stiffness and reparative fibrosis following coronary embolism in the rat. *Cardiovasc. Res.* 23: 655-661.

Carvalho, R.S., Scott, J.E., and Yen, E.H.K. 1995. The effects of mechanical stimulation on the distribution of β1 integrin and the expression of β1-integrin mRNA in TE-85 human osteosarcoma cells. *Archs. Oral. Biol.* 40: 257-264.

Carver, W., Molano, I., Reaves, T.A., Borg, T.K., and Terracio, L. 1995. Role of the α1β1 integrin complex in collagen gel contraction *in vitro* by fibroblasts. *J. Cell Physiol.* 165: 425-437.

Carver, W., Nagpal, M.L., Nachtigal, M., Borg, T.K., and Terracio, L. 1991. Collagen expression in mechanically stimulated cardiac fibroblasts. *Circ. Res.* 69: 116-122.

Cary, L.A., Han, D.C., Polte, T.R., Hanks, S.K., and Guan, J.L. 1998. Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *J. Cell Biol.* 140: 211-21.

Casamassima, A., and Rozengurt, E. 1997. Tyrosine phosphorylation of $p130^{cas}$ by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor. Signaling pathways and formation of a $p130^{cas}$. Crk complex. *J. Biol. Chem.* 272: 9363-9370.

Casamassima, A., and Rozengurt, E. 1998. Insulin-like growth factor I stimulates tyrosine phosphorylation of $p130^{Cas}$, focal adhesion kinase, and paxillin: Role of phosphatidylinositol 3'-kinase and formation of a $p130^{Cas}$. Crk complex. *J. Biol. Chem.* 273: 26149-26156.

Casscells, W., Bazoberry, F., Speir, E. 1990. Transforming growth factor-β_1 in normal heart and in myocardial infarction. *Ann. NY. Acad. Sci.* 593: 148-160.

Chambers, R.C. 1995. PhD Thesis, London. The effect of cadmium on connective tissue protein metabolism.

Chapman, D., Weber, K.T., and Eghbali, M. 1990. Regulation of fibrillar collagen types I and III and basement membrane type IV collagen gene expression in pressure overloaded rat myocardium. *Circ. Res.* 67: 787-794.

Chardin, P., Camonis, J.H, Gale, N.W., van Aelst, L., Schlessinger, J., Wigler, M.H., and Bar-Sagi, D. 1993. Human Sos2: a guanine nucleotide exchange factor for Ras that binds to Grb2. *Science* 260: 1338-1343.

Chen, C.C., Chua, B.H., Zhao, Z.Y., Krebs, C., Diglio, C., and Perrin, E. 1991. Effect of growth factors on collagen metabolism in cultured human heart fibroblasts. *Connect. Tissue Res.* 26: 271-281.

Chen, H.C., Appeddu, P.A., Isoda, H., and Guan, J.L. 1996. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J. Biol. Chem.* 271: 26329-26334.

Chen, H.C., Appenddu, P.A., Parsons, J.T., Hildebrand, J.D., Schaller, M.D., and Guan, J.L. 1995. Interaction of focal adhesion kinase with cytoskeletal protein talin. *J. Biol. Chem.* 270: 16995-16999.

Chen, H.C. and Guan, J.L. 1994. Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA.* 91: 10148-10152.

Chen, K.D., Li, Y.S., Kim, M., Li, S., Yuan, S., Chien, S., and Shyy, J.Y. 1999. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J. Biol. Chem.* 274: 18393-18400.

Chen, Q., Kinch, M.S., Lin, T.H., Burridge, K., and Juliano, R.L. 1994. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J. Biol. Chem.* 269: 26602-26605.

Chiba, M., Sakai, S., Nakata, M., and Toshima, H. 1990. The role of basic fibroblast growth factor in myocardial hypertrophy. *Circulation.* 82: 760.

Chrzanowska-Wodnicka, M. and Burridge, K. 1994. Tyrosine phosphorylation is involved in reorganization of the actin cytoskeleton in response to serum or LPA stimulation. *J. Cell Sci.* 107: 3643-3654.

Chua, C.C., Chua, B.H., Zhao, Z.Y., Krebs, C., Diglio, C., and Perrin, E. 1991. Effect of growth factors on collagen metabolism in cultured human heart fibroblasts. *Connect. Tissue Res.* 26: 271-281.

Clark, E.A., and Hynes, R.O. 1996. Ras activation is necessary for integrin-mediated activation of extracellular signal-regulated kinase 2 and cytosolic phospholipase A2 but not for cytoskeletal organization. *J. Biol. Chem.* 271: 14814-14818.

Cleutjens, J.P., Kandala, J.C., Guarda, E., Guntaka, R.V., Weber, K.T. 1995. Regulation of collagen degradation in the rat myocardium after infarction. *J. Mol. Cell. Cardiol.* 27: 1281-1292.

Cochet, C., Filhol, O., Payrastre, B., Hunter, T., and Gill, G.N. 1991. Interaction between the epidermal growth factor receptor and phosphoinositide kinases. *J. Biol. Chem.* 266: 637-644.

Cohen, M.V., Vernon, J., Yaghdjian, V., and Hatcher, V.B. 1994. Longitudinal changes in myocardial basic fibroblast growth factor (FGF-2) activity following coronary artery ligation in the dog. *J. Mol. Cell. Cardiol.* 265: 683-690.

Coughlin, S.R., Escobedo, J.A., and Williams, L.T. 1989. Role of phosphatidylinositol in PDGF receptor signal transduction. *Science.* 243: 1191-1194.

Craelius, W. 1993. Stretch-activation of rat cardiac myocytes. *Exp. Physiol.* 78: 411-423.

Crawford, D.C., Chobanian, A.V., and Brecher, P. 1994. Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. *Circ. Res.* 74: 727-739.

Czerwinski, S.M., Novakofski, J., and Bechtel, P.J. 1993. Is insulin-like growth factor gene expression modulated during cardiac hypertrophy ? *Med. Sci. Sports Exerc.* 25: 495-500.

Dalton, S.L., Scharf, E., Briesewitz, R., Marcantonio, E.E., and Assoian, R.K. 1995. Cell adhesion to extracellular matrix regulates the life cycle of integrins. *Mol. Biol. Cell.* 6: 781-791.

Danker, K., Gabriel, B., Heidrich, C., and Reutter, W. 1998. Focal adhesion kinase $pp125^{FAK}$ and the β1 integrin subunit are constitutively complexed in HaCaT cells. *Exp. Cell Res.* 239: 326-331.

Davies, P.F., Robotewskyj, A.,and Griem, M.L. 1994. Quantitative studies of endothelial cell adhesion. Directional remodeling of focal adhesion sites in response to flow forces. *J. Clin. Invest.* 93: 2031-2038.

Davis, M.J., Donovitz, J.A., and Hoo, J.D. 1992. Stretch-activated single-channel and whole cell currents on vascular smooth muscle cells. *Am J. Physiol.* 262: C1083-C1088.

Debelle, L., Wei, S.M., Jacob, M.P., Hornbeck, W., and Alix, A.J. 1992. Predictions of the secondary structure and antigenicity of human and bovine tropoelastins. *Eur. Biophys. J.* 21: 321-329.

Dedhar, S. and Saulnier, R. 1990. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. *J. Cell Biol.* 110: 481-489.

Dickeson, S.K., and Santoro, S.A. 1998. Ligand recognition by the I domain-containing integrins. *Cell. Mol. Life Sci.* 54: 556-566.

Dickeson, S.K., Walsh, J.J., and Santoro, S.A. 1998. Binding of the alpha 2 integrin I domain to extracellular matrix ligands: structural and mechanistic differences between collagen and laminin binding. *Cell Adhes. Commun.* 5: 273-281.

Doering, C.W., Jalil, J.E., Janicki, J.S., Pick, R., Aghili, S., Abrahams, C., and Weber, K.T. 1988. Collagen network remodelling and diastolic stiffness of the rat left ventricle with pressure overload hypertrophy. *Cardiovasc. Res.* 22: 686-695.

Douglas, A.J., Clarke, E.W., and Goldspink, D.F. 1988. Influence of mechanical stretch on growth and protein turnover of rat uterus. *Am. J. Physiol.* 254: E543-E548.

Dulbecco, R., and Elkington, J. 1975. Induction of growth in resting fibroblastic cell cultures by Ca++. *Proc. Natl. Acad. Sci. USA.* 72: 1584-1588.

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, C., Sizeland, A.M., and Weinberg, R.A. 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature.* 363: 45-51.

Egbahli, M., Blumenfeld, O.O., Seifter, S., Butterick, P.M., Leinwand, L.A., Robinson, T.F., Zern, M.A., and Giambrone, M.A. 1989. Localisation of types I, III and IV

collagen mRNAs in rat heart cells by in situ hybridization. *J. Mol. Cell. Cardiol.* 21: 103-113.

Ehrlich, B.E., and Watras, J. 1988. Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature.* 336: 583-586.

Engel, J., and Prockop, D.J. 1991. The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. *Annu. Rev. Biophys. Chem.* 20: 137-152.

Exton, J.H. 1990. Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* 265: 1-4.

Eyden, B.P. 1993. Brief review of the fibronexus and its significance for myofibroblastic differentiation and tumor diagnosis. *Ultrastruct. Pathol.* 17: 611-622.

Farrugia, G., Holm, A.N., Rich, A., Sarr, M.G., Szurszewski, J.H., and Rae, J.L. 1999. A mechanosensitive calcium channel in human intestinal smooth muscle cells. *Gastroenterology.* 117: 900-905.

Ferrini, S., Sforzini, S., Cambiaggi, A., Poggi, A., Meazza, R., Canevari, S., Colnaghi, M.I., and Moretta, L. 1994. The LFA-1/ICAM cell-adhesion pathway is involved in tumor-cell lysis mediated by bispecific monoclonal-antibody-targeted T-lymphocytes. *Int. J. Cancer.* 56: 846-852.

Fogerty, F.J., Akiyama, S.K., Yamada, K.M., and Mosher, D.F. 1990. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin ($\alpha 5\beta 1$) antibodies. *J. Cell Biology.* 111: 699-708.

Fry, M.J., and Waterfield, M.D. 1993. Structure and function of phosphatidylinositol 3-kinase: A potential second messenger system involved in growth control. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 340: 337-344.

Fuller, S.J., Mynett, J.R., and Sugden, P.H. 1992. Stimulation of cardiac protein synthesis by insulin-like growth factors. *Biochem. J.* 282: 85-90.

Furuta, Y., Ilic, D., Kanazawa, S., Takeda, N., Yamamoto, T., and Aizawa, S. 1995. Mesodermal defect in late phase of gastrulation by a targeted activation of focal adhesion kinase, FAK. *Oncogene.* 11: 1989-1995.

Garcia, L.J., Rosado, J.A., Gonzalez, A., and Jensen, R.T. 1997. CCK-stimulated tyrosine phosphorylation of $p125^{FAK}$ and paxillin is mediated by phospholipase C-dependent and -independent mechanisms and requires the integrity of actin cytoskeleton and participation of $p21^{rho}$. *Biochem. J.* 327: 461-471.

Garcia, L.J., Rosado, J.A., Tsuda, T., and Jensen, R.T. 1997. CCK causes rapid tyrosine phosphorylation of $p125^{FAK}$ focal adhesion kinase and paxillin in rat pancreatic acini. *Biochimi. Biopysi. Acta.* 1358: 89-199.

Gehlsen, K.R., Dickerson, K., Argraves, W.S., Engvall, E., Ruoslahti, E. 1989. Subunit structure of a laminin-binding integrin and localization of its binding site on laminin. *J. Biol. Chem.* 264: 19034-19038.

Gilmore, A.P., and Romer, L.H. 1996. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decrease cell motility and proliferation. *Mol. Cell. Biol.* 7: 1209-1224.

Glagov, S. 1994. Intimal hyperplasia, vascular remodeling, and the restenosis problem. *Circulation.* 89: 2888-2891.

Go, Y-M., Park, H., Maland, M.C., Darley-Usmar, V.M., Stoyanov, B., Wetzker, R., and Jo, H. 1998. Phosphatidylinositol 3-kinase γ mediates shear stress-dependent activation of JNK in endothelial cells. *Am. J. Physiol.* 275: H1898-H1904.

Goldberg, A.L. 1967. Work-induced growth of skeletal muscle in normal and hypophysectomized rats. *Am. J. Physiol.* 312: 1193-1198.

Goldspink, G., Scutt, A., Loughna, P.T., Wells, D.J., Jaenicke, T., and Gerlach, G.F. 1992. Gene expression in skeletal muscle in response to stretch and force generation. *Am. J. Physiol.* 262: R356-R363.

Goldspink, 1978. The influence of passive stretch on the growth and protein turnover of the denervated extensor digitorum longus muscle. *Biochem. J.* 174: 295-302.

Goodship, A.E., Lanyon, R.E., and McFie, H. 1979. Functional adaptation of bone to increased stress: an experimental study. *J.B.J.S.* 61: 539-546.

Gozin, A., Franzini, E., Andrieu, V., Da Costa, L., Rollet-Labelle, and Pasquier, C. 1998. Reactive oxygen species activate focal adhesion kinase, paxillin and p130cas tyrosine phosphorylation in endothelial cells. *Free Radic. Biol. Med.* 25: 1021-1032.

Grove, D., Zak, R., Nair, K.G., and Aschenbrenner, V. 1969. Biochemical correlates of cardiac hypertrophy. IV. Observations on the cellular organization of growth during myocardial hypertrophy in the rat. *Circ. Res.* 25: 473-485.

Guan, J.L. and Shalloway, D. 1992. Regulation of pp125 FAK both by cellular adhesion and oncogenic transformation. *Nature.* 358: 690-692.

Guan, J.L., Trevithick, J.E., Hynes, R.O. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul.* 2: 951-964.

Hagiwara, N., Masuda, H., Shoda, M., and Irisawa, H. 1992. Stretch-activated anion currents of rabbit cardiac myocytes. *J. Cell. Physiol.* 456: 285-302.

Hall, A. 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Ann. Review Cell Biol.* 10: 31-54.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279: 509-514.

Hall, J.E., Mizelle, H.L., and Woods, L.L. 1986. The renin angiotensin system and long-term regulation of arterial pressure. *J. Hypertens.* 4: 387-397.

Hamasaki, K., Mimura, T., Furuya, H., Morino, N., Yamazaki, T., Komuro, I., Yazaki, Y., Nojima, Y. 1995. Stretching mesangial cells stimulates tyrosine phosphorylation of focal adhesion kinase pp125FAK. *Biochem. Biophys. Res. Commun.* 212: 544-549.

Hammond, G.L. and Market, C.L. 1982. The molecules that initiate cardiac hypertrophy are not species-specific. *Science.* 216: 529-531.

Hanks, S.K, Calalb, M.B, Harper, M.C, and Patel, S.K. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. U S A.* 89: 8487-9.

Hao, J., Ju, H., Zhao, S., Junaid, A., Scammell-La Fleur, T., and Dixon, I.M. 1999. Elevation of expression of Smads 2, 3, and 4, decorin and TGF-beta in the chronic phase of myocardial infarct scar healing. *J. Mol. Cell. Cardiol.* 31: 667-678.

Harada, M., Saito, Y., Nakagawa, O., Miyamoto, Y., Ishikawa, M., Kuwahara, K., Ogawa, E., Nakayama, M., Kamitani, S., Hamanaka, I., Kajiyama, N., Masuda, I.,

Itoh, H., Tanaka, I., and Nakao, K. 1997. Role of cardiac nonmyocytes in cyclic mechanical stretch-induced myocyte hypertrophy. *Heart Vessels.* Suppl 12: 189-200.

Harris, R.C., Haralson, M.A., and Badr, K.F. 1992. Continuous stretch-relaxation in culture alters rat mesangial cell morphology, growth characteristics, and metabolic activity. *Lab. Invest.* 66: 548-554.

Harte, M.T., Hildebrand, J.D., Burnham, M.R., Bouton, and Parsons, J.T. 1996. $p130^{Cas}$, a substrate associated with v-src and v-crk, localises to focal adhesions and binds to focal adhesion kinase. *J. Biol. Chem.* 271: 13649-13655.

Hawgood, S. 1989. Pulmonary surfactant apoproteins: a review of protein and genomic structure. *Am. J. Physiol.* 257: L13-22.

Hildebran, J.N., Absher, M., and Low, R.B. 1983. Altered rates of collagen synthesis in in vitro aged human lung fibroblasts. *In Vitro.* 19: 307-314.

Hildebrand, J.D., Schaller, M.D., and Parsons, J.T. 1995. Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. *Mol. Cell. Biol.* 6: 637-647.

Hildebrand, J.D., Schaller, M.D., Parsons, J.T. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125 FAK, to cellular focal adhesions. *J. Cell Biol.* 123: 993-1005.

Hilenski, L.L., Ma, X.H., Vinson, N., Terracio, L., and Borg, T.K. 1992. The role of beta 1 integrin in spreading and myofibrillogenesis in neonatal rat cardiomyocytes *in vitro*. *Cell Motil. Cytoskeleton.* 21: 87-100.

Hocking, D.C., Sottile, J., and McKeown-Longo, P.J. 1994. Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. *J. Biol. Chem.* 269: 19183-7.

Horowitz, A., Lanir, Y., Yin, F.C., Perl, M., Sheinman, I., and Strumpf, R.K. 1988. Structural three-dimensional constitutive law for the passive myocardium. *J. Biomech. Eng.* 110: 200-207.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C., and Burridge, K. 1986. Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. *Nature.* 320: 531-533.

Hoshiga, M., Alpers, C.E., Smith, L.L., Giachelli, C.M., and Schwartz, S.M. 1995. Alpha-v beta-3 integrin expression in normal and atherosclerotic artery. *Circ. Res.* 77: 1129-1135.

Howe, A.K., and Juliano, R.L. 1998. Distinct mechanisms mediate the initial and sustained phases of integrin-mediated activation of the Raf/MEK/mitogen-activated protein kinase cascade. *J. Biol. Chem.* 273: 27268-27274.

Huhtala, P. 1995. Cooperative signaling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J. Cell Biol.* 129: 867-879.

Hungerford, J.E., Compton, M.T., Matter, M.L., Hoffstrom, B.G., and Otey, C.A. 1996. Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *J. Cell Biol.* 135: 1383-90.

Hynes, R.O., and Bader, B.L. 1997. Targeted mutations in integrins and their ligands: their implications for vascular biology. *Thromb. Haemost.* 78: 83-87.

Hynes, R.O., and Wagner, D.D. 1997. Genetic manipulation of vascular adhesion molecules in mice. *J. Clin. Invest.* 100: S11-13.

Hynes, R.O. 1992. Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell.* 69: 11-25.

Ikeda, M., Takei, T., Mills, I., Kito, H., and Sumpio, B.E. 1999. Extracellular signal-related kinases 1 and 2 activation in endothelial cells exposed to cyclic strain. *Am. J. Physiol.* 276: H614-H622.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature.* 377: 539-44.

Ingber, D.E. 1991. Integrins as mechanochemical transducers. *Curr. Opin. Cell Biol.* 3: 841-848.

Ingber, D.E. 1997. Tensegrity: the architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.* 59: 575-599.

Ingber, D.E., Prusty, D., Sun, Z., Belensky, M., Wang, N. 1995. Cell shape, cytoskeletal mechanics, and cell cycle control in angiogenesis. *J. Biomech.* 28: 1471-1484.

Ingram, A.J., Ly, H, Thai, K., Kang, M, and Scholey, J.W. 1999. Activation of mesangial cell signaling cascades in response to mechanical strain. *Kidney Int.* 55: 476-485.

Iruela-Arispe, M.L., Hasselaar, P., and Sage, H. 1991. Differential expression of extracellular proteins is correlated with angiogenesis in vitro. *Lab. Invest.* 64: 174-186.

Isgaard, J., Wahlander, H., Adams, M.A., and Friberg, P. 1994. Increased expression of growth hormone receptor mRNA and insulin-like growth factor-1 mRNA in volume overloaded hearts. *Hypertension.* 23: 884-888.

Ishida, T., Peterson, T.E., Kovach, N.L., and Berk, B.C. 1996. MAP kinase activation by flow in endothelial cells-role of β1 integrins and tyrosine kinases. *Circ. Res.* 79: 310-316.

Jalil, J.E., Doering, C.W., Janicki, J.S., Pick, R., Clark, W.A. and Weber, K.T. 1988. Structural vs contractile protein remodelling and myocardial stiffness in the hypertrophied rat left ventricle. *Circ. Res.* 64: 1041-1050.

Jalil, J.E., Doering, C.W., Janicki, J.S., Pick, R., Shroff, S.G. Weber, K.T. 1989. The fibrillar collagen network and myocardial stiffness in the hypertrophied intact rat left ventricle. ***Circ. Res.*** 64: 1041-1050.

Jewell, K., Kapron-Bras, C., Jeevaratnam, P., and Dedhar, S. 1995. Stimulation of tyrosine phosphorylation of distinct proteins in response to antibody-mediated ligation and clustering of alpha 3 and alpha 6 integrins. *J. Cell Sci.* 108: 1165-1174.

Jo, H., Sipos, K., Go, Y.M., Law, R., Rong, J., and McDonald, J.M. 1997. Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in

endothelial cells. Gi2- and Gbeta/gamma-dependent signaling pathways. *J. Biol. Chem.* 272: 1395-401.

Jockusch, B.M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. 1995. The molecular architecture of focal adhesions. *Annu. Rev. Cell Dev. Biol.* 11: 379-416.

Jones, D.B. and Bingmann, D. 1991. How do osteoblasts respond to mechanical stimulation? *Scanning Microscopy Int.* 1: 329-340.

Kawaguchi, H., Sano, H., Iizuka, K., Okada, H., Kudo, T., Kageyama, K., Muramoto, S., Murakami, T., Okamoto, H., Mochizuki, N. 1993. Phosphatidylinositol metabolism in hypertrophied rat heart. *Circ. Res.* 72: 966-972.

Kawaguchi, H., Shoki, M., Sano, H., Kudo, T., Sawa, H., Mochizuki, N., Okamoto, H., Endo, Y., Kitabatake, A. 1992. Polyphosphoinositide metabolism in hypertrophic rat heart. *J. Mol. Cell. Cardiol.* 24: 1003-1010.

Kaye, D., Pimental, D., Prasad, S., Maki, T., Berger, H.J., McNeil, P.L., Smith, T.W., and Kelly, R.A. 1996. Role of transiently altered sarcolemmal membrane permeability and basic fibroblast growth factor release in the hypertrophic response of adult rat ventricular myocytes to increased mechanical activity in vitro. *J. Clin. Invest.* 97: 281-291.

Keeley, F.W., Elmoselhi, A., and Leenen, F.H.H. 1991. Effects of antihypertensive drug classes on regression of connective tissue components of hypertension. *J. Cardiovasc. Pharm.* 17: S64-S69.

Kim, H., Yoon, C.S., Kim, H., and Rah, B. 1999. Expression of extracellular matrix components fibronectin and laminin in the human fetal heart. *Cell. Struct. Funct.* 24: 19-26.

Kimura, T., Cheah, K.S., Chan, S.D., Lui, V.C., Mattei, M.G., van der Rest, M., Ono, K., Solomon, E., Ninomiya, Y., and Olsen, B.R. 1989. *J. Biol. Chem.* 264: 13910-13916.

King, W.G., Mattaliano, M.D., Chan, T.O., Tsichlis, P.N., and Brugge, J.S. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. *Mol. Cell. Biol.* 17: 4406-4418.

Kivirikko, K.I., Myllyla, R., and Pihlajaniemi, T. 1989. Protein hydroxylation: prolyl-4 hydroxylase, an enzyme with 4 cosubstrates and a multifunctional subunit. *FASEB J.* 3: 1609-1617.

Kohler, R., Distler, A., and Hoyer, J. 1999. Increased mechanosensitive currents in aortic endothelial cells from genetically hypertensive rats. *J. Hypertens.* 17: 365-371.

Kollros, P.R., Bates, S.R., Mathews, M.B., Horwitz, A.L., and Glagov, S. 1987. Cyclic AMP inhibits increased collagen production by cyclically stretched smooth muscle cells. *Lab. Invest.* 56: 410-417.

Kolpakov, V., Rekhter, M.D., Gordon, D., Wang, W.H., and Kulik, T.J. 1995. Effect of mechanical forces on growth and matrix protein synthesis in the in vitro pulmonary artery. Analysis of the role of individual cell types. *Circ. Res.* 7: 823-831.

Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. 1991. Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. Possible role of protein kinase C activation.. *J. Biol. Chem.* 266: 1265-1268.

Komuro, I., Kudo, S., Yamazaki, T., Zou, Y., Shiojima, I., and Yazaki, Y. 1996. Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. *FASEB. J.* 10: 631-636.

Kornberg, L., Shelton Earp, H., Parsons, J.T., Schaller, M., and Juliano, R.L. 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267: 23439-23442.

Kornberg, L.J., Shelton Earp, H., Turner, C.E., Prockop, C., and Juliano, R.L. 1991. Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β1 integrins. *Proc. Natl. Acad. Sci. USA.* 88: 8392-8396.

Kramer, R.H. and Marks, N. 1989. Identification of integrin collagen receptors on human melanoma cells. *J. Biol. Chem.* 264: 4684-2688.

Kramer, C.M., Rogers, W.J., Park, C.S., Seibel, P.S., Shaffer, A., Theobald, T.M., Reichek, N., Onodera, T., and Gerdes, A.M. 1998. Regional myocyte hypertrophy parallels regional myocardial dysfunction during post-infarct remodeling. *J. Mol. Cell. Cardiol.* 30: 1773-1778.

Krane, S.M. 1985. The turnover and degradation of collagen. *Ciba Found. Symp.* 114: 97-110.

Kulik, T.J., and Alvarado, S.P. 1993. Effect of stretch on growth and collagen synthesis is cultured rat and lamb pulmonary arterial smooth muscle cells. *J. Cell. Physiol.* 157: 615-624.

Kuppuswamy, D., Kerr, C., Narishige, T., Kasi, V., Menick, D., and Cooper, G. 1997. Association of tyrosine-phosphorylated c-Src with the cytoskeleton of hypertrophying myocardium. *J. Biol. Chem.* 272: 4500-4508.

Kurkinen, M., Vaheri, A., Roberts, P.J., and Stenman, S. 1980. Sequential appearence of fibronectin and collagen in experimental granulation tissue. *Lab. Invest.* 43: 47-51.

Languino, L.R., Gehlsen, K.R., Wayner, E., Carter, W.G., Engvall, E., Ruoslahti, E. 1989. Endothelial cells use α2β1 integrin on a laminin receptor. *J. Cell Biol.* 109: 2455-2462.

Lansman, J.B., Hallam, T.J., and Rink, T.J. 1987. Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature.* 325: 811-813.

Lanyon, L.E. 1984. Functional strain as a determinant for bone remodeling. *Calcif. Tissue. Int.* 36 (Suppl. 1): 556-561.

Laurent, G.J. and McAnulty, R. J. 1983. Protein metabolism during bleomycin-induced pulmonary fibrosis in rabbits. *Am. Rev. Respir. Dis.* 128: 82-88.

Lee Jie-O, Bankston, L.A, Arnaout, M.A., and Liddington, R.C. 1995. Two conformations of the integrin A-domain (I-domain): A pathway for activation? *Structure.* 3: 1333-1340.

Lee, A., Dillmann, W.H., McCulloch, A.D., and Villareal, F.J. 1995. Angiotensin II stimulates the autocrine production of transforming growth factor-beta in adult rat cardiac fibroblasts. *J. Mol. Cell. Cardiol.* 27: 2347-2357.

Lee, R.M.K.W., Forrest, J.B., Garfield, R.E. and Daniel, E.E. 1983. Ultrastructural changes in mesenteric arteries form spontaneously hypertensive rate. *Blood Vessels.* 20: 72-91.

Leslie, K.O., Taatjes, D.J., Schwarz, J., vonTurkivich, M., and Low, R.B. 1991. Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am. J. Pathol.* 139: 207-216.

Lethias, C., Labourdette, L., Wiltems, R., Comte, J., and Herbage, D. 1996. Composition and organisation of the extracellular matrix of vein walls: collagen networks. *Int. Angiol.* 15: 104-113

Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B., and Castelli, W.P. 1990. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham heart study. *N. Engl. J. Med.* 322: 1561-1566.

Lewis, J.M., and Schwartz, M.A. 1998. Integrins regulate the association and phosphorylation of paxillin by cAbl. *J. Biol. Chem.* 273: 14225-14230.

Li, S., Chen, B.P.C., Azuma, N., Hu, Y-L., Wu, S.Z., Sumpio, B.E., Shyy, J.Y-J., and Chien, S.I. 1999. Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *J. Clin. Invest.* 103: 1141-1150.

Li, S., Kim, M., Hu, Y-L., Jalali, S., Schaepfer, D.D., Hunter, T., Chien, S., and Shyy, J. Y-J. 1997. Fluid shear stress activation of focal adhesion kinase: Linking to mitogen-activated protein kinases. *J. Biol. Chem.* 272: 30455-30462.

Liang, F., Wu, J., Garami, M., and Gardner, D.G. 1997. Mechanical strain increases expression of the brain natiuretic peptide gene in rat cardiac myocytes. *J. Biol. Chem.* 272: 28050-28056.

Lim, L., Ahmed, S., Hall, A., Kozma, R., Leung, T., Manser, E., and Sarner, S. 1996. Morphological roles and interactions of the Rho family GTPases and their protein partners. *Mol. Biol. Cell.* 7: 13-23.

Lin, T.H., Aplin, A.E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., and Juliano, R.L. 1997. Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. *J. Cell Biol.* 136: 1385-1395.

Linz, W., Scholkens, B., and Ganten, D. 1989. Converting enzyme inhibition specifically prevents the development and induces the regression of cardiac hypertrophy in rats. *Clin. Exper. Hyper.-Theory and Practice.* A11: 1325-1350.

Lipfert, L., Haimovich, B., Schaller, M.D., Cobb, B.S., Parsons, J.T., and Brugge, J.S. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. *J. Cell Biol.* 119: 905-912.

Lo, S.H., Jammey, P.A., Hartwig, J.H. and Chen, L.B. 1994. Interactions of tensin with actin and identification of its three distinct actin-binding domains. *J. Cell Biol.* 125: 5495-5500.

Loeber, C.P., and Runyan, R.B. 1990. A comparison of fibronectin, laminin, and galactosyltransferase adhesion mechanisms during embryonic cardiac mesenchymal cell migration in vitro. *Dev. Biol.* 140: 401-412.

Lui, F., Hill, D.E., and Chernoff, J. 1996. Direct binding of the proline-rich region of protein tyrosine phosphatase 1B to the src homology domain of p130^{Cas}. *J. Biol. Chem.* 271: 31290-31259.

Lundgren, E., Terracio, L., Mardh, S., and Borg, T.K. 1985. Extracellular matrix components influence the survival of adult cardiac myocytes. *Exp. Cell Res.* 158: 371-381.

Lyman, S., Gilmore, A., Burridge, K., Gidwitz, S., and White, G.C. 1997. Integrin-mediated activation of focal adhesion kinase is independent of focal adhesion formation or integrin activation: Studies with activated and inhibitory β3 cytoplasmic domain mutants. *J. Biol. Chem.* 272: 22538-22547.

Machesky, L.M., and Hall, A. 1997. Role of actin polymerization and adhesion to extracellular matrix on rac and rho induced cytoskeletal re-organization. *J. Cell Biol.* 138: 913-926.

MacKenna, D.A., Dolfi, F., Vuori, K., and Ruoslahti, E. 1998. Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J Clin Invest.* 101: 301-10

Maler, J.A.M., Rusnati, M., Ragnotti, G., and Presta, M. 1990. Characterization of a MW 20, 000 basic fibroblast growth factor-like protein secreted by normal and transformed fetal bovine aortic endothelial cells. *Exp. Cell Res.* 186: 354-361.

Maniotis, A.J., Chen, C.S., and Ingber, D.E. 1997. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc. Natl. Acad. Sci. USA.* 94: 849-854.

Margolis, B., and Skolnik, E.Y. 1994. Activation of Ras by receptor tyrosine kinases. J. *Am. Soc. Nephrol.* 5: 1288-1299.

Marlin, S.D., and Springer, T.A. 1987. Purified intercellular adhesion molecule (ICAM-1) is a ligand for lymphocyte function associated antigen 1 (LFA-1). *Cell.* 51: 813-819.

Matsumoto, T., Kawakami, M., Kuribayashi, K., Takenaka, T., and Tamaki, T. 1999. Cyclic mechanical stress increases the growth rate and collagen synthesis of nucleus pulposus cells in vitro. *Spine.* 24(4): 315-319.

Mays, P.K., Bishop, J.E., and Laurent, G.J. 1988. Age related changes in the proportion of type I and III collagen. *Mech. Ageing. Dev.* 45: 203-212.

Mays, C and Rosenberry, T.L. 1981. Characterization of pepsin-resistant collagen-like tail subunit fragments of 18S and 14S acetylcholinesterase from Electrophorus electricus. *Biochemistry.* 20: 2810-2817.

McAnulty, R.J., Campa, J.S., Cambrey, A.D., and Laurent, G.J. 1991. The effect of transforming growth factor beta on rates of procollagen synthesis and degradation in vitro. *Biochim. Biophys. Acta.* 1091: 231-235.

McDonald, J.A, Quade, B.J, Broekelmann, T.J, LaChance, R., Forsman, K., Hasegawa, E., and Akiyama S. 1987. Fibronectin's cell-adhesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. *J. Biol. Chem.* 262: 2957-67.

McKeown-Longo, P.J, and Mosher, D.F. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. *J. Cell Biol.* 97: 466-72.

McNamee, H.P., Ingber, D.E., and Schwartz MA. 1993. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell Biol.* 121: 673-8.

Meazzine, M.C., Toma, C.D., Schaffer, J.H., Gray, M.L., and Gerstenfeld, L.C. 1998. Osteoblast cytoskeletal modulation in response to mechanical strain in vitro. *J. Orthop. Res.* 16: 170-180.

Medugorac, I. 1976. Different fractions in the normal and hypertrophied rat ventricular myocardium: an analysis of two models of hypertrophy. *Basic Res. Cardiol.* 71: 608-623.

Medugorac, I. and Jacob, R. 1983. Characterisation of left ventricular collagen in the rat. *Cardiovasc. Res.* 17: 15-21.

Meyer, U., Meyer, T., and Jones, D.B. 1997. No mechanical role for vinculin in strain transduction in primary bovine osteoblasts. *Biochem. Cell Biol.* 75: 81-87.

Meyers, R., and Cantley, L.C. 1997. Cloning and characterization of a wortmannin-sensitive human phosphatidylinositol 4-kinase. *J Biol Chem.* 272: 4384-90.

Meyrick, B., Reid, L. 1979. Hypoxia and incorporation of 3H-thymidine by cells of the rat pulmonary arteries and alveolar wall. *Am. J. Pathol.* 96: 51-70.

Michishita, M., Videm, V., and Arnout, M.A. 1993. A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. *Cell.* 72: 857-867.

Miller, E.J. 1985. The structure of fibril forming collagens. *Ann. NY. Acad. Sci.* 460: 1-13.

Mirsky, I. 1979. Factors which affect the diastolic pressure-volume curve. *Circ. Res.* 44: 592-595.

Miyamoto, S., Teramoto, H., Coso, O.A., Gutkind, J.S., Burbelo, P.D., Akiyama, S.K., and Yamada, K.M. 1995. Integrin function: Molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* 131: 791-805.

Miyamoto, S., Teramoto, H., Gutkind, J.S., and Yamada, K.M. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J. Cell. Biol.* 135: 1633-1642.

Monboisse, J.C., Randoux, A., Borel, J.P., and Braquet, P. 1986 . Effects of isaxonine phosphate and analogs on fibroblast metabolism in culture. *Experientia.* 42: 309-312.

Morino, N., Mimura, T., Hamasaki, K., Tobe, K., Ueki, K., Kikuchi, K., Takehara, K., Kadowaki, T., Yazaki, Y., and Nojima, Y. 1995. Matrix/integrin interaction activates the mitogen-activated protein kinase, p44 $^{erk-1}$ and p42 $^{erk-2}$. *J. Biol. Chem.* 270: 269-273.

Morioka, S., Honda, M., Ishikawa, S., Ishinaga, Y., Yano, S., Tanaka, K., and Moriyama, K. 1992. Changes in contractile and non-contractile proteins, intracellular Ca^{2+} and ultrastructures during the development of right ventricular hypertrophy and failure in rats. *Jpn. Circ. J.* 56: 469-474.

Morris, C.E. 1990. Mechanosensitive ion channels. *J. Membr. Biol.* 113: 93-107.

Mosher, D.F, Sottile, J., Wu, C., McDonald, J. 1992. Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* 4: 810-8.

Mosher, D.F. 1989. Role of fibronectins in embryogenesis. In: Fibronectin. San Diego, California, Academic. 181-212.

Mould, A.P., Akiyama, S.K., and Humphries, M.J. 1995. Regulation of integrin $\alpha5\beta1$ fibronectin interactions by divalent cations. Evidence for distinct classes of binding for Mn^{2+}, Mg^{2+}, and Ca^{2+}. *J. Biol. Chem.* 270: 26270-262777.

Mukherjee, D. and Sen, S. 1993. Alteration of cardiac collagen phenotypes in hypertensive hypertrophy: Role of blood pressure. *J. Mol. Cell. Cardiol.* 25: 185-186.

Nakamoto, T., Sakai, R., Honda, H., Ogawa, S., Ueno, H., Suzuki, T., Aizawa, S., Yazaki, Y., Hirai, H. 1997. Requirements for localization of p130cas to focal adhesions. *Mol. Cell. Biol.* 17: 3884-3897.

Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y., and Hirai, H. 1996. Direct binding of C-terminal region of $p130^{Cas}$ to SH2 and SH3 domains of Src kinase. *J. Biol. Chem.* 271: 8959-8965.

Nakamura, I., Jimi, E., Duong, L.T., Sasaki, T., Takahashi, N., Rodan, G.A., and Suda, T. 1998. Tyrosine phosphorylation of p130Cas is involved in actin organisation in osteoclasts. *J. Biol. Chem.* 273: 11144-11149.

Namba, T., Tsutsui, H., Tagawa, H., Takahashi, M., Saito, K., Kozai, T., Usui, M., Imanaka-Yoshida, K., Imaizumi, T., and Takeshita, A. 1997. Regulation of fibrillar collagen gene expression and protein accumulation in volume-overloaded cardiac hypertrophy. *Circulation.* 95: 2448-2454.

Nebe, B., Bohn, W., Sanftleben, H., and Rychly, J. 1996. Induction of a physical linkage between integrins and the cytoskeleton depends on intracellular calcium in an epithelial cell line. *Exp. Cell Res.* 229: 100-10.

Nimni, M.E., and Harkness, R.D. 1988. Molecular structure and functions of collagen. In: *Collagen.* CRC Press. 1-77.

Nojima, Y., Morino, N., Mimura, T., hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C, and Yazaki, Y. 1995. Integrin-mediated cell adhesion promotes tyrosine kinase phosphorylation of p130cas, a SH3-containing molecule having multiple SH2-binding motifs. *J. Biol. Chem.* 270: 15398-15402.

Nobes, C.D., Hawkins, P., Stephens, L., and Hall, A. 1995. Activation of the small GTP-binding proteins rho and rac by growth factors. *J. Cell Sci.* 108: 225-233.

Nojima, Y., Morino, N., Mimura, T., Hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C., Yazaki, Y., and Hirai, H. 1995. Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130cas, a src homology 3-containing molecule having multiple src homology 2-binding motifs. *J. Biol. Chem.* 270: 15398-15402.

Norman, J.C., Price, L.S., Ridley, A.J., Hall, A., and Koffer, A. 1994. Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. *J. Cell Biol.* 126: 1005-1015.

Nyui, N., Tamura, K., Mizuno, K., Ishigami, T., Hibi, K., Yabana, M., Kihara, M., Fukamizu, A., Ochiai, H., Umemura, S., Murakami, K., Ohno, S., and Ishii, M. 1997. Stretch-induced MAP kinase activation in cardiomyocytes of angiotensin-deficient mice. *Biochem. Biophys. Res. Commun.* 235: 36-41.

Ohayon, J., and Chadwick, R.S. 1988. Effects of collagen microstructure on the mechanics of the left ventricle. *Biophys. J.* 54: 1077-1088.

Ojanieui, M. and Vuori, K. 1997. Epidermal growth factor modulates tyrosine phosphorylation of $p130^{Cas}$. Involvement of phosphatidylinositol 3-kinase and actin cytoskeleton. *J. Biol. Chem.* 277: 25993-25998.

Okoshi, M.P., Matsubara, L.S., France, M., Cicogna, A.C., and Matsuara, B.B. 1997. Myocyte necrosis is the basis for fibrosis in renovascular hypertensive rats. *Braz. J. Med. Biol. Res.* 30: 1135-1144.

Okuda, M., Takahashi, M., Suero, J., Murry, C.E., Traub, O., Kawakatsu, H., and Berk, B.C. 1999. Shear stress stimulation of p130(cas) tyrosine phosphorylation requires calcium-dependent c-Src activation. *J. Biol. Chem.* 274: 26803-26809.

Olivetti, G., Capasso, J.N., Sonnenblick, E.H., and Anversa, P. 1990. Side-to-side slippage of myocytes participates in ventricular wall remodelling acutely after myocardial infarction in rats. *Circ. Res.* 67: 23-34.

Olivetti, G., Ricci, R., and Anversa, P. 1987. Hyperplasia of myocyte nuclei in long-term cardiac hypertrophy in rats. *J. Clin. Invest.* 80: 1818-1821.

Omura, T., Kim, S., Takeuchi, K., Ieao, H. and Takeda, T. 1994. Transforming growth factor-beta 1 and extracellular matrix gene expression in isoprenaline-induced cardiac hypertrophy: Effects of inhibition of the renin angiotensin system. *Cardiovasc. Res.* 28: 1835-1842.

Otey, C.A., Pavalko, F.M., Burridge, K. 1990. An interaction between alpha actinin and the beta 1 integrin subunit in vitro. *J. Cell Biol.* 111: 721-729.

Owens, G.K. and Schwartz, S.M. 1983. Vascular smooth muscle cell hypertrophy and hyperploidy in the Goldblatt hypertensive rat. *Circ. Res.* 53: 491-501.

Pacifici, M., and Iozzo, R.V. 1988. Remodeling of the rough endoplasmic reticulum during stimulation of procollagen secretion by ascorbic acid in cultured chondrocytes. A biochemical and morphological study. *J. Biol. Chem.* 263: 2483-2492.

Padua, R.R., and Kardami, E. 1993. Increased basic fibroblast growth factor (bFGF) accumulation and distinct patterns of localisation in isoproterenol-induced cardiomyocyte injury. *Growth Factors.* 8: 291-306.

Page K., and Lange, Y. 1997. Cell adhesion to fibronectin regulates membrane lipid biosynthesis through 5'-AMP-activated protein kinase. *J. Biol. Chem.* 272: 19339-19342.

Papakrivopoulou, E. 1999. The role of MAP kinases in the regulation of mechanical load induced procollagen gene expression in cardiac fibroblasts. PhD Thesis, London.

Parker, T.G., Packer, S.E., and Schneider, M.D. 1990. Peptide growth factors can provoke "fetal" contractile gene expression in cardiac myocytes. *J. Clin. Invest.* 85: 507-514.

Pavalko, F.M., Chen, N.X., Turner, C.H., Burr, D.B., Atkinson, Hseish, Y.F., Qiu, J., and Duncan, R.L. 1998. Fluid shear-induced mechanical signaling in MC3T3-EI osteoblasts requires cytoskeleton-integrin interactions. *Am. J. Physiol.* 275: C1591-1601.

Payrastre, B., Plantavid, M., Breton, M., Chambaz, E., Chap, H. 1990. Relationship between phosphoinositide kinase activities and protein tyrosine phosphorylation in plasma membranes from A431 cells. *Biochem J.* 272: 665-70

Petch, L.A., Bockholt, S.M., Bouton, A., Parsons, J.T., and Burridge, K. 1995. Adhesion-induced tyrosine phosphorylation of p130SRC substrate. *J. Cell Sci.* 108: 1371-1379.

Peterson, G and Barnes, S. 1993. Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor tyrosine autophosphorylation. *Prostate.* 22: 335-345.

Pender, N., and McCulloch, C.A. 1991. Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J. Cell Sci.* 100: 187-193.

Pick, R., Janicki, J.S., and Weber, K.T. 1989. Myocardial fibrosis in nonhuman primate with pressure overload hypertrophy. *Am. J. Pathol.* 135: 771-781.

Pike, L.J., and Eakes, A.T. 1987. Epidermal growth factor stimulates the production of phosphatidylinositol monophosphate and the breakdown of polyphosphoinositides in A431 cells. *J. Biol. Chem.* 262: 1644-1651.

Polte, T R and Hanks, S.K. 1997. Complexes of FAK and $p130^{Cas}$ are elevated in cytoskeleton-associated fractions following adhesion and src transformation. *J. Biol. Chem.* 272: 5501-5509.

Polte, T.R., and Hanks, S.K. 1995. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate $p130^{Cas}$. *Proc. Natl. Acad. Sci. USA*: 92: 10678-10682.

Pommerenke, H., Schreiber, E., Durr, F., Nebe, B., Hahnel, C., Moller, W., and Rychly, J. 1996. Stimulation of integrin receptors using a magnetic drag force device induces an intracellular free calcium response. *Eur. J. Cell Biol.* 70: 157-64.

Powis, G., Bonjouklian, R., Berggren, M.M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W.F., Dodge, J., Grindey, G., and Vlahos, C.J. 1994. Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res.* 54: 2419-2423.

Predel, H., Yanf, Z., von Segesser, L., Turina, M., Buhler, F.R., and Luscher, T.F. 1992. Implication of pulsatile stretch on growth of saphenous vein and mammary artery smooth muscle. *Lancet.* 340: 878-879.

Putney, J.W. Jr. 1987. Formation and actions of calcium mobilizing messenger, inositol 1,4,5-trisphosphate. *Am. J. Physiol.* 252: G149-G151.

Quade, B.J, McDonald, J.A. 1988. Fibronectin's amino-terminal matrix assembly site is located within the 29-kDa amino-terminal domain containing five type I repeats. *J. Biol. Chem.* 263: 19602-19609.

Ramachandran, G.N., and G. Kartha. 1954. Structure of collagen . *Nature.* 174: 269-270.

Rankin, S., Hooshmand-Rad, R., Claesson-Welsh, L., Rozengurt, E. 1996. Requirement for phosphatidylinositol 3-kinase activity in platelet-derived growth factor-stimulated tyrosine phosphorylation of p125 focal adhesion kinase and paxillin. *J. Biol. Chem.* 271: 7829-2834.

Rannels, D.E. 1989. Role of physical forces in compensatory lung growth. *Am. J. Physiol.* 257: L179-L189.

Reid, K.B. 1979. Complete amino acid sequences of the three collagen-like regions present in subcomponent C1q of the first component of human complement. *Biochem. J.* 179: 367-371.

Renshaw, M.W., Toksoz, D., and Schwartz, M.A. 1996. Involvement of the small GTPase-Rho in integrin-mediated activation of mitogen-activated protein kinase. *J. Biol. Chem.* 271: 21691-21694.

Reusch, H.P., Chan, G., Ives, H., and Nemenoff, R.A. 1997. Activation of JNK/SAPK and ERK by mechanical strain in vascular smooth muscle cells depends on extracellular matrix composition. *Biochem. Biophys. Res. Commun.* 237: 239-244.

Reusch, P., Wagdy, H., Reusch, R., Wilson, E., and Ives, H.E. 1996. Mechanical strain increases smooth muscle and decreases nonmuscle myosin expression in rat vascular smooth muscle cells. *Circ. Res.* 79: 1046-53.

Richardson, A., and Parsons, J.T. 1996. A mechanism for regulation of the adhesion of associated protein tyrosine kinase, pp125FAK. *Nature.* 380: 538-540.

Richardson, A., Shannon, J.D., Adams, R.B., Schaller, M.D., and Parsons, J.T. 1997. Identification of integrin-stimulated sites of serine phosphorylation in FRNK, the separately expressed C-terminal domain of focal adhesion kinase: A potential role for protein kinase A. *Biochem. J.* 324: 141-149.

Ridley, A.J., and Hall, A. 1992. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibres in response to growth factors. *Cell.* 70: 389-399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. 1992. The small GTP-binding protein rac regulates growth factor induced membrane ruffling. *Cell.* 70: 401-410.

Riley, D.J., Poiani, G.J., Low, R.B., Jensen, L., and Jacobs, T.P. 1990b. Effects of physical forces on lung structure, function and metabolism. *Am. Rev. Respir. Dis.* 142: 910-914.

Riser, B.L., Cortes, P., Zhao, X., Bernstein, J., Dumler, F., and Narins, R.G. 1992. Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. *J. Clin. Invest.* 90: 1932-1943.

Robins, S.P. 1979. Metabolism of rabbit skin collagen. *Biochem. J.* 181: 75-82.

Robinson, T.F., Cohen-Gould, L., and Factor, S.M. 1983. Skeletal framework of mammalian heart muscle. Arrangement of inter- and pericellular connective tissue structures. *Lab. Invest.* 49: 482-498.

Robinson, T.F., Cohen-Gould, L., Factor, S.M, Eghbali, M., and Blumenfeld, O.O. 1988. Structure and function of connective tissue in cardiac muscle: collagen types I and III in endomysial struts and pericellular fibers. *Scanning Miscrosc.* 2: 1005-1015.

Robinson, T.F., Cohen-Gould, L., Remily, R.M., Capasso, J.M., and Factor, S.M. 1985. Extracellular structures in heart muscle. *Adv. Myocardiol.* 5: 243-255.

Rodriguez-Fenandez, J.L., and Rozengurt, E. 1998. Bombesin, vasopressin, lysophosphatidic acid and sphingosylphosphorylcholin induce focal adhesion kinase activation in intact Swiss 3T3 cells. *J. Biol. Chem.* 273: 19321-19328.

Roman, J., LaChance, R.M., Broekelmann, T.J., Kennedy, C.J.R., Wayner, E.A., Carter, W.G., and McDonald, J.A. 1989. The fibronectin receptor is organizd by extracellular matrix fibronectin: implications for oncogenic transformation and for cell recognition sites. *J. Cell Biol.* 108: 2529-2543.

Romer, L.H., McLean, N., Turner, C.E., and Burridge, K. 1994. Tyrosine kinase activity, cytoskeletal organization, and motility in human vascular endothelial cells. *Mol. Biol. Cell.* 5: 49-61.

Rooney, P., Wang, M., Kumar, P., and Kumar, S. 1993. Angiogenic oligosaccharides of hyaluronan enhance the production of collagens by endothelial cells. *J. Cell Sci.* 105: 213-218.

Rosales, O.R., and Sumpio, B.E. 1992. Changes in cyclic strain increase inositol trisphosphate and diacylglycerol in endothelial cells. *Am. J. Physiol.* 262: C956-962.

Ross, L., Davis, C.M., and Molony, L. 1994. A truncated recombinant intercellular-adhesion molecule-1 inhibits adhesion of leukemic-cell lines to up-regulated endothelial cells. *In Vitro Cell. Dev. Biol. Animal.* 30A: 875-880.

Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57: 375-413.

Sachs, F. 1989. Ion channels as mechanical transducers, in Anonymous Cell Shape: determinants, regulation and regulatory role. *New York, Academic Press*: 63-92.

Sadoshima, J-I., and Izumo, S. 1993. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: Potential involvement of an autocrine/paracrine mechanism. *EMBO J.* 12: 1681-1692.

Sadoshima, J-I., and Izumo, S. 1997. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu. Rev. Physiol.* 59: 551-571.

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T.J., and Izumo, S. 1992. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J. Biol. Chem.* 267: 10051-10060.

Sadoshima, J., Takahashi, T., Jahn, L. and Izumo, S. 1992. Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate early gene expression and hypertrophy of cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* 89: 9905-9909.

Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., Hirai, H. 1994. A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO. J.* 13: 3748-3756.

Sano, H., Okamoto, H., Kitabatake, A., Iizuka, K., Murakami, T., and Kawaguchi, H. 1998. Increased mRNA expression of cardiac renin-angiotensin system and collagen synthesis in spontaneously hypertensive rats. *Mol. Cell. Biochem.* 178: 51-58.

Sawada, H. and Konomi, H. 1991. The α1 chain of type VIII collagen is associated with many but not all microfibrils of elastic fibre system. *Cell Struct. Funct.* 16: 455-466.

Schaller, M.D., and Parsons, J.T. 1995. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol. Cell. Biol.* 15: 2635-2645.

Schaller, M.D., Borgman, C.A., and Parsons, J.T. 1993. Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase $pp125^{FAK}$. *Mol. Cell. Biol.* 13: 785-791.

Schaller, M.D., Borgman, C.A., Cobb, B.C., Reynolds, A.B., and Parsons, J.T. 1992. pp125 FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions.*Proc. Natl. Acad. Sci. USA*. 89: 5192--5196.

Schaller, M.D., Hildebrand, J.D., Shannon, J.D., Fox, J.W., Vines, R.R., and Parsons, J.T. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol. Cell. Biol.* 14: 1680-1688.

Schaller, M.D., Otey, C.A., Hildebrand, J.D., and Parsons, J.T. 1995. Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. *J. Cell Biol.* 130: 1181-1187.

Schlaepfer, D.D., and Hunter, T. 1996. Evidence in *in vivo* phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by src-family protein-tyrosine kinase. *Mol. Cell Biol.* 16: 5623-5633.

Schlaepfer, D.D., Broome, M.A., and Hunter, T. 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: Involvement of the Grb2, $p130^{cas}$, and Nck adaptor protein. *Mol. Cell. Biol.* 17: 1702-1713.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P. 1994. Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesions. *Nature*. 372: 786-791.

Schlaepfer, D.D., Jones, K.C., and Hunter, T. 1998. Multiple Grb-2 mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: Summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol. Cell. Biol.* 18: 2571-2585.

Schmidt, C.E., Horwitz, A.F., Lauffenbruger, D.A., and Sheetz, M.P. 1993. Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell. Biol.* 123: 977-991.

Schmidt, C., Pommerenke, H., Durr, F., Nebe, B., Rychly, J. 1998. Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. *J. Biol. Chem.* 273: 5081-5085.

Schneider, M.D., and Parker, T.G. 1990. Cardiac myocytes as targets for the action of peptide growth factors. *Circulation.* 81: 1443-1456.

Schneider, M.D., and Parker, T.G. 1991. Cardiac growth factors. *Prog. Growth Factors.* 3: 1-26.

Seko, Y., Takahashi, N., Tobe, K., Kadowaki, T., and Yazaki, Y. 1999. Pulsatile stretch activates mitogen-activated protein kinase (MAPK) family members and focal adhesion kinase (p125(FAK)) in cultured rat cardiac myocytes. *Biochem. Biophys. Res. Commun.* 250: 8-14.

Seufferlein, T., and Rozengurt, E. 1994. Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130: Signalling pathways and cross-talk with platelet-derived growth factor. *J. Biol. Chem.* 269: 9345-9351.

Shaw, L.M., and Olsen, B.R. 1991. FACIT collagens: diverse molecular bridges in extracellular matrices. *Trends Biochem. Sci.* 16: 191-194.

Shyy, J.Y., and Chien, S. 1997. Role of integrins in cellular responses to mechanical stress and adhesion. *Curr. Opin. Cell Biol.* 9: 707-713. Review.

Sieg, D.J., Hauck, C.R., and Schlaepfer, D.D. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.* 112: 2677-2691.

Silvestre, J.S., Heymes, C., Oubenaissa, A., Robert, V., Aupetit-Faisant, B., Carayon, A., Swynghedauw, B., and Delcayre, C. 1999. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. *Circulation.* 99: 2694-2701.

Simionescu, D.T., and Kefalides, N.A. 1991. The biosynthesis of proteoglycans and interstitial collagens by bovine pericardial fibroblasts. *Exp. Cell Res.* 195: 171-176.

Singer, I.I., Kawaka, D.W., Kazazis, D.M., and Clark, R.A. 1984. In vivo co-distribution of fibronectin and actin fibers in granulation tissue: immunofluorescence and

electron microscope studies of the fibronexus at the myofibroblast surface. *J. Cell Biol.* 98: 2091-2106.

Sinnett-Smith, J., Zachery, I., Valverde, A.M., Rozengurt, E. 1993. Bombesin stimulation of pp125 focal adhesion kinase tyrosine phosphorylation. *J. Biol. Chem.* 268: 14261-14268.

Slack, B.E. 1998. Tyrosine phosphorylation of paxillin and focal adhesion kinase by activation of muscarinic m3 receptors is dependent on integrin engagement by the extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 95: 7281-7286.

Smith, P.G., Garcia, R., and Kogerman, L. 1998. Mechanical strain increases protein tyrosine kinase phosphorylation in airway smooth muscle cells. *Exp. Cell Res.* 239: 353-360.

Shimizu, N., Yoshiyamo, M., Omura, T., Hanatani, A., Kim, S., Takeuchi, K., Iwao, H., and Yoshikawa, J. 1998. Activation of MAP kinase and activator protein -1 in myocardial infarction in rats. *Cardiovasc. Res.* 38: 116-124.

Sottile, J., and Wiley, S. 1994. Assembly of amino-terminal fibronectin dimers into the extracellular matrix. *J. Biol. Chem.* 269: 17192-8.

Steinmann, B., Rao, V.H., and Gitzelmann, R. 1981. Intracellular degradation of newly synthesized collagen is conformation-dependent. *FEBS Lett.* 133: 142-144.

Stephens, L., Eguinoa, A., Corey, S., Jackson, T., and Hawkins, P.T. 1993. Receptor stimulated accumulation of phosphatidylinositol (3,4,5) trisphosphate by G-protein mediated pathways in human myeloid derived cells. *EMBO J.* 12: 2265-2272.

Stiemer, B., Springmeier, G., el-Jarad, L., and Schroter-Kermani, C. 1993. Matrix production of smooth muscle cells from rat aorta in vitro. *Histol. Histopathol.* 8: 63-72.

Steinberg, J. 1978. Collagen turnover and the growth state in 3T6 fibroblast cultures. *Lab. Invest.* 39: 491-496.

Story, M.T. 1989. Cultured human foreskin fibroblasts produce a factor that stimulates their growth with properties similar to basic fibroblast growth factor. *In vitro Cell. Dev. Biol.* 25: 402-408.

Sudhir, K., Wilson, E., Chatterjee, K., and Ives, H.E. 1993. Mechanical strain potentiates angiotensin II induced growth factor of vascular smooth muscle cells via autocrine secretion of platelet derived growth factor. *Circulation.* 88: 184.

Sullivan, M.J., Sharma, R.V., Wachtel, R.E., Chapleau, M.W., Waite, L.J., Bhalla, R.C., and Abbond, F.M. 1997. Non-voltage-gated Ca2+ influx through mechanosensitive ion channels in aortic barareceptor neurons. *Circ. Res.* 80: 861-867.

Sumbilla, C.M., Zielke, C.L., Reed, W.D., Ozand, P.T., and Zielke, H.R. 1981. Comparison of the oxidation of glutamine, glucose, ketone bodies and fatty acids by human diploid fibroblasts. *Biochim. Biophys. Acta.* 675: 301-304.

Sumpio, B.E., Banes, A.J., Levin, L.G., and Johnson, G. 1987. Mechanical stress stimulates aortic endothelial cells to proliferate. *J. Vasc. Surg.* 6: 252-256.

Sumpio, B.E., Banes, A.J., Link, G.W., and Iba, T. 1990. Modulation of endothelial cell phenotype by cyclic stretch. Inhibition of collagen production. *J. Surg. Res.* 48: 415-420.

Sutcliffe, M.C., and Davidson, J.M. 1990. Effect of static stretching on elastin production by porcine aortic smooth muscle cells. *Matrix.* 10: 148-153.

Suzuki, M., Naruse, K., Asano, Y., Okamoto, T., Nishikimi, N., Sakurai, T., Nimura, Y., and Sokabe, M. 1997. Up-regulation of integrin beta 3 expression by cyclic stretch in human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* 239: 372-376.

Tachibana, K., Sato, T., D'Avirro, N., and Morimoto, C. 1995. Direct association of pp125^{FAK} with paxillin, the focal adhesion-targeting mechanism of pp125^{FAK}. *J. Exp. Med.* 182: 1089-1100.

Takada, Y., Huang, C., and Hemler, M.E. 1987. Fibronectin receptor structures in the VLA family of heterodimers. *Nature.* 326: 607-609.

Takahashi M., and Berk B.C. 1996. Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J. Clin. Invest.* 98: 2623-31.

Takahashi M., Ishida T., Traub O., Corson M.A., and Berk B.C. 1997. Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J. Vasc. Res.* 34: 212-9.

Takahashi, T., Schunkert, H., Isoyama, S., Wei, J.Y., Nadal-Ginard, B., Grossman, W., and Izumo, S. 1992. Age-related differences in expression of proto-oncogenes and contractile protein genes in response to pressure overload in the rat myocardium. *J. Clin. Invest.* 89: 939-946.

Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H., and Takai, Y. 1997. Regulation of cell-cell adhesion by Rac and Rho small G proteins in MDCK cells. *J. Cell Biol.* 139: 1047-1059.

Tanaka, T., Yamaguchi, R., Sabe, H., Sekiguchi, K., and Healy, J. 1996. Paxillin association *in vitro* with integrin cytoplasmic domain peptides. *FEBS.* 399: 53-58.

Tang, D., Mehta, D., and Gunst, S.J. 1999. Mechanosensitive tyrosine phosphorylation of paxillin and focal adhesion kinase in tracheal smooth muscle. *Am. J. Physiol.* 276: C250-C258.

Tapley, P., Horwitz, A., Buck, C., Duggan, K., and Rohrschneider, L. 1989. Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene.* 4: 325-337.

Terracio, L., Tungstrom, A., Peters, W.H., and Borg, T.K. 1990. A potential role for mechanical stimulation in cardiac development. *Ann. NY. Acad. Sci.* 588: 48-60.

Thai, H.M., Van, H.T., Gaballa, M.A., Goldman, S., and Raya, T.E. 1999. Effects of AT1 receptor blockade after myocardial infarct on myocardial fibrosis, stiffness, and contractility. *Am. J. Physiol.* 276: H873-880.

Thiedemann, K.U. 1983. Connective tissue content and myocardial stiffness in pressure overload hypertrophy. A combined study of morphologic, morphometric, biochemical and mechanical parameters. *Basic Res. Cardiol.* 78: 140-155.

Thomas, C.V., Coker, M.L., Zellner, J.L., Handy, J.R., Crumbley, A.J., and Spinale, F.G. 1998. Increased matrix metalloproteinase activity and selective upregulation in LV myocardium from patients with end-stage dilated cardiomyopathy. *Circulation.* 97: 1708-1715.

Thompson, N.L., Bazoberry, F., Speir, E.H., Casscells, W., Ferrans, V.J., Flanders, K.C., Kondaiah, P., Geiser, A.G., and Sporn, M.B. 1988. TGF-β1 in acute myocardial infarction in rats. *Growth Factors.* 1: 91-99.

Tolstoshev, P., Berg, R.A., Rennard, S.I., Bradley, K.H., Trapnell, B.C., and Crystal, R.G. 1981. Procollagen production and procollagen messenger RNA levels and activity in human lung fibroblasts during periods of rapid and stationery growth. *J. Biol. Chem.* 256: 3135-3140.

Toma, C.D., Ashkar, S., Gray, M.L., Schaffer, J.L. and Gerstenfeld, L.C. 1997. Signal transduction of mechanical stimuli is dependent on microfilament integrity: Identification of osteopontin as a mechanically induced gene in osteoblasts. *J. Bone Miner. Res.* 12: 162-1636.

Tomasek, J.J., and Haaksma, C.J. 1991. Fibronectin filaments and actin microfilaments are organized into a fibronexus in Dupuytren's diseased tissue. *Anat. Rec.* 230: 175-182.

Traub, W., and Piez, K.A. 1971. The chemistry and structure of collagen. *Adv. Protein Chem.* 25: 243-352.

Turner, C.E., and Miller, J.T. 1994. Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: indentification of a vinculin and pp125^{FAK}-binding region. *J. Cell Sci.* 107: 1583-1591.

Turner, C.E., Glenney, J.R., and Burridge, K. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* 111: 1059-1068.

Turner, C.E., Schaller, M.D., and Parsons, J.T. 1993. Tyrosine phosphorylation of the focal adhesion kinase pp125^{FAK} during development: Relation to paxillin. *J. Cell Sci.* 105: 637-645.

Turner, J.E and Laurent, G.J. 1986. Increased type I collagen compared to type III during right ventricular hypertrophy in rabbits. *Biochem. Soc. Trans.* 14: 1079-1080.

Turner, J.E., Oliver, M.H., Guerreiro, D., and Laurent, G.J. 1986. Collagen metabolism during right ventricular hypertrophy following induced lung injury. *Am. J. Physiol.* 251: H915-H919.

Tyagi, S.C., Campbell, S.E., Reddy, H.K., Tjahja, E., and Voelker, D.J. 1996. Matrix metalloproteinase activity in infarcted, noninfarcted and dilated cardiomyopathic human hearts. *Mol. Cell. Biochem.* 155: 13-21.

Ueki, K., Mimura, T., Nakamoto, T., Sasaki, T., Aizawa, S., Hirai, H., Yano, S., Naruse, T., and Nojima, Y. 1998. Integrin-mediated signal transduction in cells lacking focal adhesion kinase $p125^{FAK}$. *FEBS Letters.* 432: 197-201.

van der Rest, M., and Garrone, R. 1991. Collagen family of proteins. *FASEB J.* 5: 2814-2823.

Vandenburgh, H.H. 1992. Mechanical forces and their second messengers in stimulating cell growth *in vitro. Am. J. Physiol.* 262: R350-R355.

Vandenburgh, H.H., Hatfaludy, S., Sohar, I., and Shansky, J. 1990. Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. *Am. J. Physiol.* 259: C232-240.

Veinot, J.P., Srivatsa, S., and Carlson, P. 1999. Beta3 integrin - a promiscuous integrin involved in vascular pathology. *Can. J. Cardiol.* 15: 762-770.

Vesely, I. 1998. The role of elastin in aortic valve mechanics. *J. Biomech.* 31: 115-123.

Villareal, F.J. and Dillman, W.H. 1992. Cardiac hypertrophy-induced changes in mRNA levels of TGF-beta 1, fibronectin, and collagen. *Am. J. Physiol.* 262: H1861-H1866.

Villareal, F.J., Kim, N.N., Ungab, G.D., Printz, M.P., and Dillmann, W.H. 1993. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation.* 88: 2849-2861.

Vincenti, M.P., White, L.A., Schroen, D.J., Benbow, U., and Brinckerhoff, C.E. 1996. Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): mechanisms that control enzyme activity, transcription, and mRNA stability. *Crit. Rev. Eukaryotic Gene Expr.* 6: 391-411.

Vlodavsky, I., Fridman, R., Sullivan, R., Sasse, J., and Klagsbrun, M. 1987. Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted. *J. Cell. Physiol.* 131: 402-408.

Vlodavsky, I., Sullivan, R., Fridman, R., Sasse, J., Folkman, J., and Klagsbrun, M. 1986. Heparin binding endothelial cell growth factor produced by endothelial cells and sequestered by the subendothelial extracellular matrix. *J. Cell Biol.* 103: 98a.

von Harsdorf, R., Lang, R.E., Fullerton, M., and Woodcock, E.A. 1989. Myocardial stretch stimulates phosphatidylinositol turnover. *Circ. Res.* 65: 494-501.

Vo, H.P., Lee, M.K., and Crowe, D.L. 1998. alpha2beta1 integrin signaling via the mitogen activated protein kinase pathway modulates retinoic acid-dependent tumor cell invasion and transcriptional downregulation of matrix metalloproteinase 9 activity. *Int. J. Oncol.* 13: 1127-1134.

Vuori, K., and Ruoslahti, E. 1993. Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading on fibronectin. *J. Biol. Chem.* 268: 21459-62.

Vuori, K., and Ruoslahti, E. 1995. Tyrosine phosphorylation of $p130^{Cas}$ and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. *J. Biol. Chem.* 270: 22259-22262.

Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. 1996. Induction of $p130^{Cas}$ signaling complex formation upon integrin-mediated cell adhesion: a role for src family kinases. *Mol. Cell. Biol.* 16: 2606-2613.

Wang, N., Butler, J.P, and Ingber, D.E. 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science.* 260: 1124-1127.

Wang, Z., Estacion, M., and Mordan, L.J. 1993. Ca2+ influx via T-type channels modulates PDGF-induced replication of mouse fibroblasts. *Am. J. Physiol.* 265: C1239-1246.

Wang, N., and Ingber, D.E. 1995. Probing transmembrane mechanical coupling and cytomechanics using magnetic twisting cytometry. *Biochem. Cell Biol.* 73: 327-335.

Watson, P.A. 1991. Function follows form: generation of intracellular signals by cell deformation. *FASEB J.* 5: 2013-2019.

Webb, D.L, Marlor, C.W., Conrad, P.J., Barkowskiclark, S., and Blue, M.L. 1993. Role of 3rd n-terminal domain of VCAM-1. *Biochem. Biophys. Res. Commun.* 197: 674-681.

Weber, K.T., and Brilla, C.G. 1991. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation.* 83: 1849-1865.

Weber, K.T., Janicki, J.S., Shroff, S.G., Pick, R., Abrahams, C., Chen, R.M. and Bashey, R.I. 1988a. Collagen compartment remodelling in the pressure overloaded left ventricle. *J. Appl. Cardiol.* 3: 37-46.

Weber, K.T., Janicki, J.S., Shroff, S.G., Pick, R., Chen, R.M. and Bashey, R.I. 1988b. Collagen remodelling of the pressure-overloaded, hypertrophied nonhuman primate myocardium. *Circ. Res.* 62: 757-765.

Weiser, M.C.M., Majack, R.A., Tucker, A., and Orton, E.C. 1995. Static tension is associated with increased smooth muscle cell DNA synthesis in rat pulmonary arteries. *J. Physiol.* 268: H1133-1138.

Welch, M.P., Odland, G.F., and Clark, R.A. 1990. Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression in wounds. *J. Cell Biol.* 110: 133-145.

Weng, Z., Taylor, J.A., Turner, C.E., Brugge, J.S., and Seidel-Dugan, C. 1993. Detection of Src homology 3-binding proteins, including paxillin, in normal and v-Src transformed Balb/c 3T3 cells. *J. Biol. Chem.* 268: 14956-14963.

Willems, M.E., and Huijing, P.A. 1994. Heterogeneity of mean sarcomere length in different fibres: effects on length range of active force production in rat muscle. *Eur. J. Appl. Physiol.* 68: 489-496.

Wilson, L., Carrier, M.J., and Kellie, S. 1995. pp125FAK tyrosine kinase activity is not required for the assembly of F-actin stress fibres and focal adhesions in cultured mouse aortic smooth muscle cells. *J. Cell Sci.* 108: 2381-2391.

Wilson, E., Mai, Q., Sudhir, K., Weiss, R.H., and Ives, H.E. 1993. Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. *J. Cell Biol.* 123: 741-747.

Wilson, E., Sudhir, and K., Ives, H.E. 1995. Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. *J. Clin. Invest.* 96: 2364-2372.

Wilson, E., Vives, F., Collins, T., and Ives, H. 1998. Strain-responsive regions in the platelet-derived growth factor-A gene promoter. *Hypertension.* 31: 170-175.

Winlove, C.P., Bishop, J.E., Chambers, R.C., and Laurent, G.J. 1995. The structure and function of the extracellular matrix in the pulmonary vasculature. *Pulmonary Vascular Remodelling.* p36-40. Portland Press.

Wolfe, B.L., Rich, C.B., Gould, H.D., Terpstra, A.J., Bashir, M., Rosenbloom, J., Sonneshein, G.E., and Foster, J.A. 1993. Insulin-like growth factor-1 regulates transcription of the elastin gene. *J. Biol. Chem.* 268: 12418-12426.

Wolinsky, H. 1970. Response of the rat aortic media to hypertension. *Circ. Res.* 26: 507-522.

Wolinsky, H. 1972. Long-term effects of hypertension on the rat aortic wall and their relation to concurrent ageing changes. *Circ. Res.* 30: 301-309.

Wright, M.O., Nishida, K., Bavington, C., Godolphin, J.L., Dunne, E., Walmsley, S., Jobanputra, P., Nuki, G., and Salter, D.M. 1997. Hyperpolarisation of cultured human

chondrocytes following cyclical pressure-induced strain: evidence of a role for alpha 5 beta 1 integrin as a chondrocyte mechanoreceptor. *J. Orthop. Res.* 15: 742-747.

Wu, C., Bauer, J.S., Juliano, R.L., and McDonald, J.A. 1993. The α5β1 integrin fibronectin receptor, but not the α5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. *J. Biol. Chem.* 268: 21883-21888.

Xu, J. and Clark, A.F. 1996. Extracellular matrix alters PDGF regulation of fibroblast integrins. *J. Cell Biol.* 132: 239-249.

Xu, Y.J, Yau, L., Yu, L.P., Elimban, V., Zahradka. P., and Dhalla, N.S. 1996. Stimulation of protein synthesis by phosphatidic acid in rat cardiomyocytes. *Biochem Pharmacol.* 52: 1735-40.

Yamazaki, T., Komuro, I., and Yazaki, Y. 1995. Molecular mechanism of cardiac cellular hypertrophy by mechancial stress. *J. Mol. Cell. Cardiol.* 27: 133-140.

Yamazaki, T., Komuro, I., and Yazaki, Y. 1996. Molecular aspects of mechanical stress-induced cardiac hypertrophy. *Mol. Cell. Biochem.* 162-164: 197-201.

Yano, Y., Geibel, J., and Sumpio, B.E. 1996. Tyrosine phosphorylation of pp125FAK and paxillin in aortic endothelial cells induced by mechanical strain. *Am. J. Physiol.* 271: C635-C649.

Yano, Y., Geibel, J., and Sumpio, B.E. 1997. Cyclic strain induces reorganization of integrin alpha 5 beta 1 and alpha 2 beta 1 in human umbilical vein endothelial cells. *J. Cell Biochem.* 64: 505-513.

Yasuda, T., Kondo, S., Homma, T., and Harris, R.C. 1996. Regulation of extracellular matrix by mechanical stress in rat glomerular mesangial cells. *J. Clin. Invest.* 98: 1991-2000.

Yazaki, Y., Komuro, I., Yamazaki, T., Tobe, K., Maemura, K., Kadowaki, T., and Nagai, R. 1993. Role of protein kinase system in the signal transduction of stretch-mediated protooncogene expression and hypertrophy of cardiac myocytes. *Mol. Cell. Biochem.* 119: 11-16.

Yurochko, A.D., Liu, D.Y., Eierman, D., and Haskill, S. 1992. Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. *Proc. Natl. Acad. Sci. USA.* 89: 9034-9038.

Zachary, I. and Rozengurt, E. 1992. Focal adhesion kinase (p^{125}FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell.* 71: 891-894.

Zachery, I., Sinnett-Smith, J., Turner, C. E., Rozengurt, E. 1993. Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *J. Biol. Chem.* 268: 22060-22065.

Zak, R. 1973. Cell proliferation during cardiac growth. *Am. J. Cardiol.* 31: 211-219.

Zhang, J., Fry, M.J., Waterfield, M.D., Jaken, S., Liao, L., Fox, J.E., Rittenhouse, S.E. 1992. Activated phosphatidylinositol 3-kinase associates with membrane skeleton in thrombin-exposed platelets. *J. Biol. Chem.* 267: 4686-4692.

Zhao, Q., and Lee, F.S. 1999. Mitogen-activated protein kinase/ERK kinase kinase 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. *J. Biol. Chem.* 274: 8355-8358.

Zhu, T., Goh, E. L.K., LeRoith, D., and Lobie, P.E. 1998. Growth hormone stimulates the formation of a multiprotein signaling complex involving $p130^{Cas}$ and CrkII. *J. Biol. Chem.* 273: 33864-33875.

Zhu, X, and Assoian, R.K. 1995. Integrin-dependent activation of MAP kinase: A link to shape-dependent cell proliferation. *Mol. Cell. Biol.* 6: 273-282.

Zou, Y., Hu, Y., Metzler, B., and Xu, Q. 1998. Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells. *Int. J. Mol. Med.* 1: 827-834.

ABSTRACTS ARISING FROM THIS THESIS

Reynolds LE, Zachary I and Bishop JE. 1999. FAK is involved in the mechanical load induced procollagen synthesis signalling pathway. *FASEB*: 13 (5 pt 2): A857.

Reynolds LE and Bishop JE. 1998. FAK phosphorylation is enhanced by mechanical load in cardiac fibroblasts. *Mol. Biol. Cell*: 9: 424a.

Reynolds LE and Bishop JE. 1998. Integrins act as mechanotransducers in human cardiac fibroblasts. *Circulation*: 98 (17): 678.

Reynolds LE and Bishop JE. 1998. Stimulation of cardiac fibroblast procollagen synthesis in response to serum is enhanced by mechanical load and fibronectin. *J. Mol. Cell. Cardiol.* 26: 120.

PAPERS ARISING FROM THIS THESIS

Reynolds, L.E. and Bishop, J.E. 1999. Stimulation of procollagen synthesis by mechanical load is $\alpha 5\beta 1$ and $\alpha v\beta 3$ dependent. Submitted to Cardiovascular Research

Reynolds, L.E., Zachary, I., and Bishop, J.E. 1999. Role for the focal adhesion complex in mechanical load induced collagen synthesis in human cardiac fibroblasts - mechanical load stimulates tyrosine phosphorylation of $p130^{Cas}$, FAK and paxillin. Manuscript in progress.