PROTECTIVE AND HYPERTROPHIC EFFECTS OF CARDIOTROPHIN-1 IN THE HEART

JULIA ELIZABETH RAILSON

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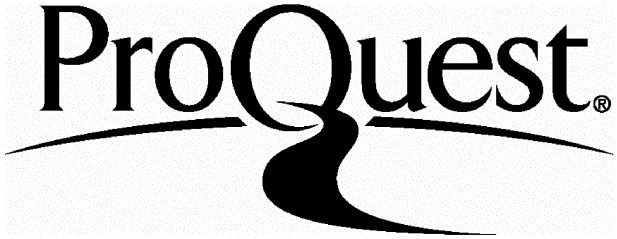
Medical Molecular Biology Unit
The Institute of Child Health
University College London



To Malcolm and Thomas,

For their constant support.

Abstract

Cardiotrophin-1 (CT-1) is a member of the interleukin-6 family of cytokines that was first isolated based on its ability to cause hypertrophy in cardiac myocytes. Hypertrophy is an initially compensatory response in the heart defined by enlargement of cells without division and reactivation of foetal genes. Chronic hypertrophy can be detrimental and may lead to heart failure. CT-1 is also a potent survival factor in cardiac myocytes and increases levels of cardioprotective heat shock protein 90 (hsp90). The subject of this thesis is an investigation of the mechanisms involved in cardiac protection, hypertrophy and heat shock protein induction by CT-1.

CT-1 increased hsp90 levels independently of RNA synthesis and without an increase in hsp90 mRNA level or activation of the promoter. This implies that CT-1 is acting via a post-transcriptional mechanism to increase hsp90 protein levels. Treatment with CT-1 prior to a stressful stimulus such as heat shock caused a reduction in the amount of hsp90 and hsp70 produced in response to the stress. The possible mechanisms for this novel antagonistic effect are discussed.

The protective effect of CT-1 is dependent on the p42/p44 Mitogen activated protein kinase (p42/p44 MAPK) pathway and the hypertrophic effect is independent from this pathway. It is demonstrated that the hypertrophic effect of CT-1 is dependent on the signal transducer and activator of transcription 3 (STAT3) pathway. The hypertrophic effect of CT-1 can therefore be blocked by inhibition of the STAT3 pathway while leaving the protective effect intact. This divergence in the signalling pathways stimulated by CT-1 may prove important if CT-1 were to be developed as a cardioprotective agent for use in the clinical setting.

Heat shock protein 56 (hsp56) (also known as FK506 binding protein 59) was shown to increase at both the mRNA and protein level in response to CT-1 treatment. Subsequent studies using herpes viral vectors or plasmid transfection to over express heat shock proteins in cardiac cells showed that elevated levels of hsp56 caused hypertrophy. Additionally, transfection of antisense hsp56 DNA inhibited the hypertrophic effect of CT-1. These data suggest that hsp56 is involved in the hypertrophic response to CT-1.

Urocortin is a peptide hormone of the Corticotrophin releasing factor family, which is cardioprotective via the same pathway as CT-1. It is shown here that urocortin is also hypertrophic, but via a distinct pathway to CT-1.

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Declaration

All the work presented in this thesis is the work of Julia Railson. Contributions by other researchers are acknowleged in the text.

Publications

1) Brar B.K., Jonassen A.K., Stephanou S., Santilli G., Railson J., Knight R.A., Yellon D.M., Latchman D.S. (2000) Urocortin protects against ischemic and ischemic reperfusion injury via a MAPK-dependent pathway. *Journal of Biological Chemistry*, Vol. 275, No. 12, pp. 8508-8514.

2) Railson J.E., Lawrence K., Stephanou A., Brar B.K., Pennica D., Latchman D.S. (2000) Cardiotrophin-1 reduces stress induced heat shock protein production in cardiac myocytes. *Cytokine (In Press).*

3) Railson J.E., Liao Z., Brar B.K., Pennica D., Stephanou A., Latchman D.S. (2000) Cardiotrophin-1 and Urocortin cause protection by the same pathway and hypertrophy via distinct pathways in cardiac myocytes. *Submitted to The Journal of Molecular and Cellular Cardiology.*

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CHAPTER 1
INTRODUCTION

1.1.0 Introduction

The cytokine Cardiotrophin-1 (CT-1) has two main effects on the heart: Protection and hypertrophy. The subject of this thesis is to investigate the effect of CT-1 on heat shock protein expression in heart cells. Additionally, the mechanisms involved in the cardioprotective effect and the hypertrophic effect of CT-1 will be studied. The cardioprotective hormone urocortin will also be characterised for any hypertrophic effect and the mechanism of such an effect compared with that for CT-1.

The introduction will commence with information about CT-1 and signalling pathways activated by CT-1, followed by sections on heat shock proteins, cardiac protection and cardiac hypertrophy. The final part of the introduction will introduce urocortin and discuss its effects on the heart.

1.1.1 Cardiotrophin-1 and the gp130 Receptor

Cardiotrophin-1 (CT-1) is a cytokine belonging to the Interleukin-6 (IL-6) family, first isolated for its ability to cause cardiac myocyte hypertrophy (Pennica *et al.*, 1995a). The IL-6 family includes: IL-6, IL-11, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), Oncostatin M (OSM), and CT-1, all of which share the common receptor component gp130 (see figure 1.1.1). Many functions of IL-6 family cytokines are redundant due to their common receptor component but most members have some specific effects. This specificity is conferred by other receptor subunits, which are specific to a particular cytokine and may only be expressed in target cells (see figure 1.1.1).

Gp130 is ubiquitously expressed and is involved in signal transduction, but does not bind directly to the cytokine itself (see figure 1.1.1). IL-6, IL-11 and CNTF each have their own receptors e.g. IL6-R, IL11-R, CNTF-R, which can be in soluble or membrane bound forms. The IL6/IL6R and IL11/IL11R complexes bind gp130 which homodimerizes initiating signal transduction. OSM binds to the membrane bound OSM receptor which is highly homologous to gp130, this complex then forms a heterodimer with gp130. OSM, uniquely can also bind directly to gp130 with low affinity. The CNTF/CNTFR complex binds to the LIF receptor β (LIFRβ), which is similar to gp130 and is membrane bound, this then forms a heterodimer with gp130. CT-1 and LIF both

bind directly to the LIFRβ, which then forms a heterodimer with gp130 (Taga & Kishimoto, 1997).

A third receptor component is also involved in CT-1 binding. Cross linking of iodinated CT-1 to the cell surface has lead to identification of an 80kDa glycoprotein which is tightly associated with the CT-1 / LIFRβ / gp130 complex (Robledo *et al.*, 1997b). This receptor component is known as CT-1Rα and is thought to be unique to the CT-1 receptor. Removal of glycoprotein-phosphatidylinositol (GPI) linked molecules from the cell surface with phospatidylinositol-specific phospholipase C (PIPLC) inhibited the survival effect of CT-1 but not LIF (Pennica *et al.* 1996a). This shows that a GPI linked receptor component is required for CT-1 activity and that this component is not shared with LIF. It is likely that this receptor component is the same 80kDa subunit described by (Robledo *et al.*, 1997b).

CT-1 binds to the LIFRβ receptor component, which dimerizes with gp130 and incorporates the CT-1Rα component (Pennica *et al.*, 1995b; Robledo *et al.*, 1997b). Several studies have indicated the importance of LIFRβ and gp130 in mediating the biological effects of CT-1. Monoclonal gp130 antibody completely inhibits induction of the immediate early gene c-fos by CT-1 (Wollert *et al.*, 1996). In the same study, a LIFRβ antagonist also blocked the CT-1 mediated induction of c-fos, showing the requirement for both gp130 and LIFRβ in CT-1 signalling. Antagonism of LIFRβ with an inactive LIF mutant inhibited the activity of CT-1, LIF, CNTF and OSM. In cells that also express the OSM receptor, OSM activity was not inhibited by antagonism of LIFRβ. This shows that CT-1, LIF and CNTF all require LIFRβ for their activity and OSM can signal through LIFRβ but also has an alternative receptor (Vernallis *et al.*, 1997). LIFRβ knockout mice were used to show that CT-1, LIF or CNTF are unable to promote the survival of motorneurones in the absence of LIFRβ (Arce *et al.*, 1999). Gp130 receptor complexes for IL-6 family cytokines are summarised in Figure 1.1.1.

Figure 1.1.1 Receptor complexes sharing gp130.

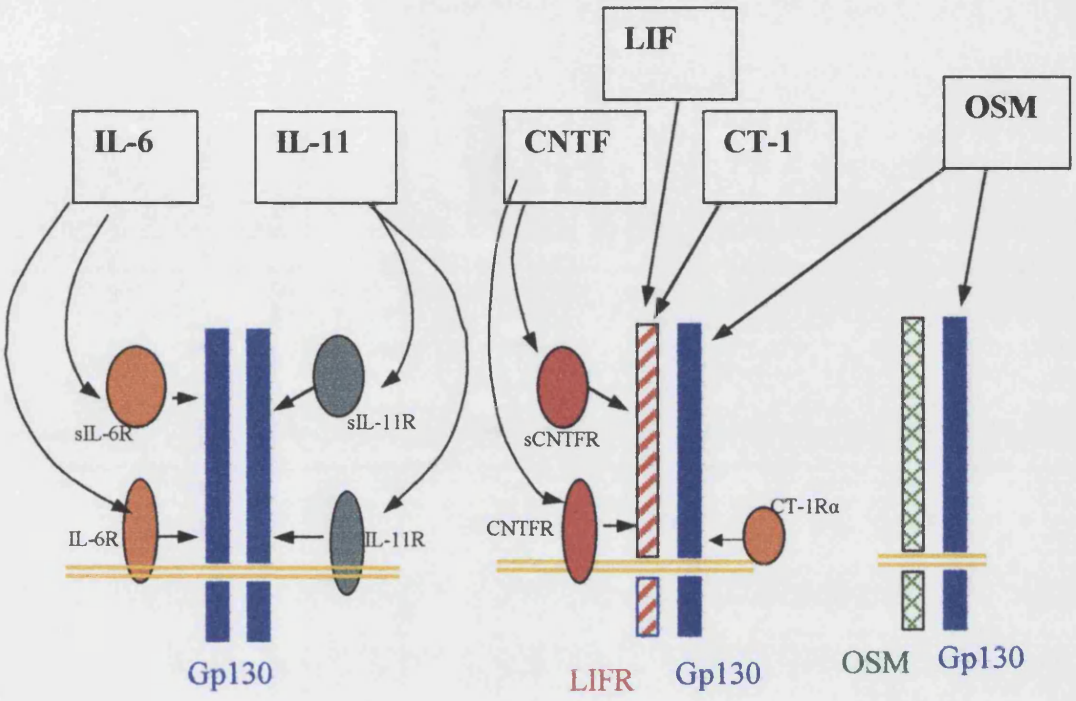


Figure 1.1.1 Receptor complexes sharing gp130. (adapted from (Taga & Kishimoto, 1997)). IL6 binds membrane bound IL6R or soluble (s) IL6R. The IL6/IL6R binds gp130 causing homodimerization. The same mechanism is thought to apply for IL-11. CNTF also has soluble and membrane bound receptors which, when bound cause hetrodimerization of gp130 with LIFR. LIF and CT-1 bind directly to LIFRβ causing dimerization with gp130. An 80kDa subunit CT-1Rα is also involved in the CT-1 receptor complex. OSM can bind directly to gp130 which then hetrodimerizes with LIFR or OSMR.

Dimerization of a gp130 molecule to another gp130 molecule or to a different receptor component such as LIFRβ causes activation of down stream signalling cascades. Activation of gp130 initiates signalling via two pathways; the janus activated kinase (Jak) – Signal transducer and activator of transcription (STAT) pathway and the p42/p44 mitogen activated protein kinase pathway (MAPK) (Kunisada *et al.*, 1996). A recent study also shows the activation of the phosphatidylinositol-3-OH kinase (PI3K) pathway by CT-1 in cardiac myocytes (Kuwahara *et al.*, 2000). These pathways will be described in turn, and are outlined in figure 1.1.2.

Figure 1.1.2 Signalling by the CT-1 receptor.

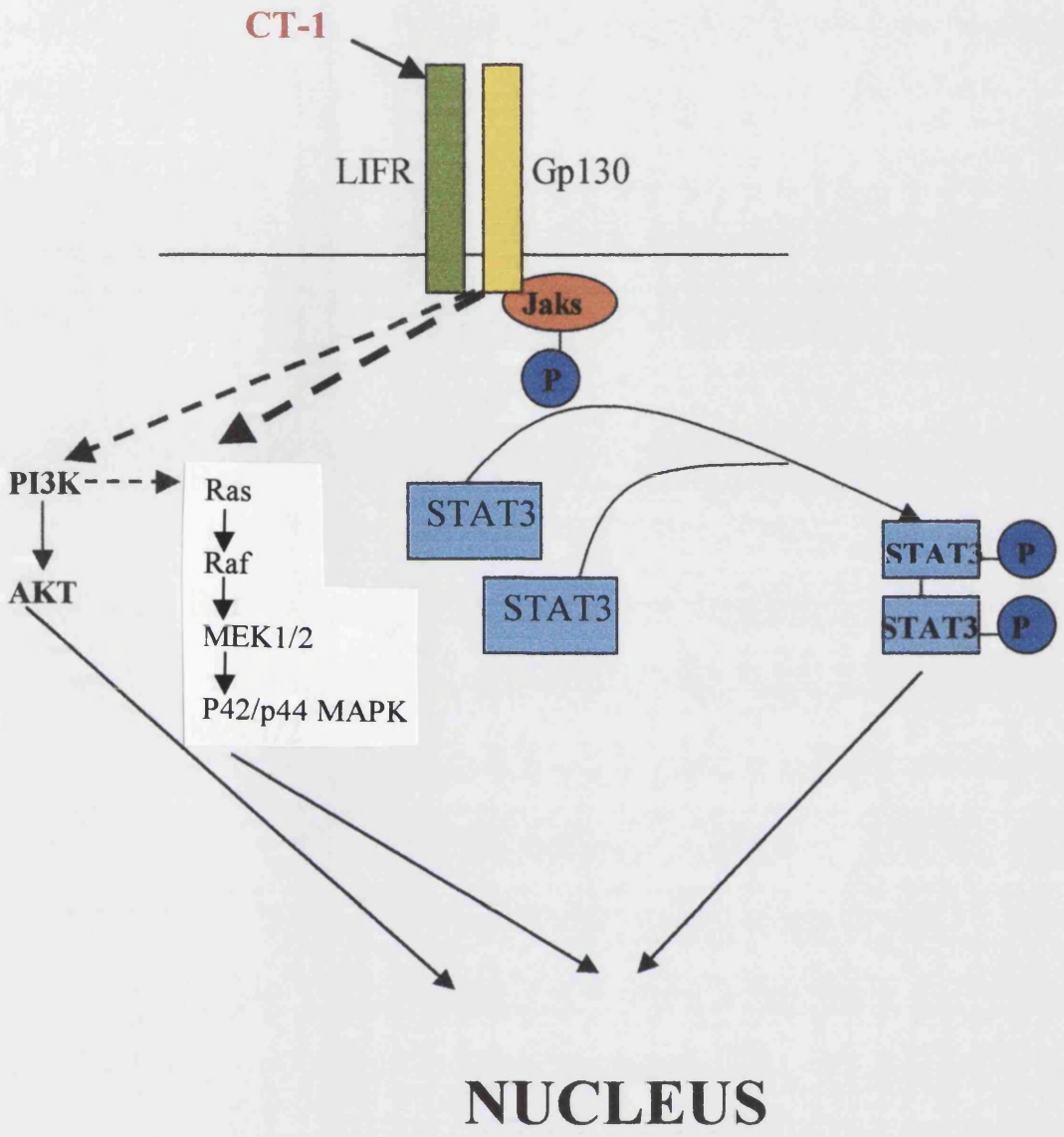


Figure 1.1.2 Cardiotrophin-1 mediated intracellular signalling. Dimerization of gp130 and LIFRβ activates the Jak/STAT pathway, the p42/p44 MAPK and PI3K signalling pathways.

1.1.2 The Jak/STAT Pathway

Dimerization of gp130 activates Janus activated kinases (Jaks), which are associated with the cytoplasmic region of gp130. Jak1, Jak2 and Tyk2 of the Jak family are associated with gp130 and are activated in response to IL6 family cytokines (Stahl *et al.*, 1995a). CT-1 has been shown to activate Jak1, Jak2 and Tyk2 through gp130 / LIFRβ receptor dimerization (Robledo *et al.*, 1997b). Jaks are intracellular tyrosine kinases that associate with gp130 and LIFRβ via box1 and box2 motifs (reviewed by (Heinrich *et al.*, 1998). Box1 is a proline-rich motif of eight amino acid residues and is essential for Jak association with gp130. Box 2 is a cluster of hydrophobic amino acids followed by positively charged amino acids, which enhances the binding of Jaks to gp130.

The kinase activity of Jaks comes from a carboxyl terminal domain known as the Jak homology domain (JH-1). This kinase domain contains an activation loop which is essential for tyrosine kinase activity (Duhe & Farrar, 1998). Dimerization of gp130 brings Jaks into close proximity with one another leading to their activation via inter or intra molecular phosphorylation. Activated Jaks phosphorylate cytoplasmic tyrosine residues on gp130 (Murakami *et al.*, 1991) creating docking sites for the SH-2 (Src homology) domains of STATs. Recruited STATs become tyrosine phosphorylated by activated Jaks and form homo or hetro dimers which translocate to the nucleus where they bind STAT response elements and regulate the transcription of target genes (Darnell-JE, 1997; Schindler & Darnell-JE, 1995; Wollert & Chien, 1997; Heinrich *et al.*, 1998; Zhong *et al.*, 1994).

Seven mammalian STAT family members have been identified and are known as STATs 1, 2, 3, 4, 5A, 5B and 6 (Darnell-JE, 1997) (see table 1.1.1)). Differential splicing of STAT mRNAs also occurs resulting in more protein variants. STAT3 (STAT3α), for example exists as a 89kDa protein, but a splice variant lacking an internal domain of 50 base pairs near the c-terminus also encodes a 80 kDa protein STAT3β. STAT3β is phosphorylated and binds to DNA when activated but fails to activate transcription, and acts as a repressor to the transactivation activity of STAT3. This repressor activity was demonstrated in response to IL-5 stimulation in COS cells (Caldenhoven *et al.*, 1996). In a separate study however, STAT3β activated a promoter containing an IL-6 responsive element in the absence of cytokine stimulation and co-

operated with c-jun in promoter activation (Schaefer *et al.*, 1995). A subsequent comparitive study showed that activated STAT3β was more stable and had greater DNA binding activity that STAT3α, but that STAT3α was more transcriptionally active than STAT3β (Schaefer *et al.*, 1997). This supports roles for STAT3β as a repressor and an activator under different conditions.

STATs 1, 3, 5A and 5B are activated by many different ligands but STATs 2, 4 and 6 have a defined activation profile (see table 1.1.1). STAT2 is activated only by IFN-α, STAT4 is activated by IL-12 and IFN-α in lymphocytes, and STAT6 is activated only by IL-4 and IL-13, which share a receptor chain (Darnell-JE, 1997).

All STAT family members have a single tyrosine residue in the region of amino acid 700 that is phosphorylated upon activation (Schindler & Darnell-JE, 1995). STAT molecules also contain an SH2 domain at their c-terminal end. The SH2 domain enables the STAT molecule to bind to tyrosine phosphorylated motifs on gp130 (Stahl *et al.*, 1995b). STAT1 binds to a pYXPQ motif and STAT3 binds to a pYXXQ motif (Gerhartz *et al.*, 1996). The importance of SH2 domains in STAT receptor binding and activation is demonstrated by the use of chimeric STATs where the SH2 domain of STAT1 was inserted into STAT3, replacing its own SH2 domain. This chimeric STAT molecule showed the same pattern of activation as STAT1 (Hemmann *et al.*, 1996). This shows that the SH2 domain is sufficient to confer specificity on STAT molecules. Once the STAT molecule is tyrosine phosphorylated, the SH2 domain allows homo or hetro dimerization between STATs (Heinrich *et al.*, 1998; Shuai *et al.*, 1994). Dimerization is a prerequisite for STAT DNA binding.

Table 1.1.1 The STAT family of transcription factors

STAT family member	**Isoforms**	**Examples of Activators**
STAT1	STAT1α STAT1β	Interferon-γ Interferon-α IL-6 Epidermal growth factor
STAT2		Interferon-α
STAT3	STAT3α STAT3β	CT-1, IL-6, LIF, OSM, IL-11, CNTF Leptin Interferon-α Interferon-γ Growth factor IL-2, IL-5
STAT4		Interferon-α IL-12
STAT5	STAT5a and STAT5b from a gene duplication, also multiple splice forms	IL-2 Erythropoietin Thrombopoietin Prolactin Growh hormone
STAT6		IL-4 IL-13

Table 1.1.1. Information taken from the following sources: (Darnell-JE, 1997; Shuai *et al.*, 1994; Zhong *et al.*, 1994)

The DNA binding domain is approximately in the middle of the STAT molecule and binds to the DNA element TTN_5AA. The exact sequence of the DNA element depends on the STATs present in the dimer. The number of base pairs between the palindromic motif determines which STAT will bind. TTN_5AA displays general STAT binding activity, while TTN_4AA selectively binds dimers containing STAT3 and TTN_6AA selectively binds STAT6 dimers activated by IL-4 (Seidel *et al.*, 1995). In addition to tyrosine phosphorylation, the activity of STATs is also regulated by serine phosphorylation (serine 727 in STAT1 and STAT3) (Chung *et al.*, 1997; Zhu *et al.*, 1997). STAT3 can be serine phosphorylated in response to growth factors by p42/p44 MAPK, this serine phosphorylation inhibits tyrosine phosphorylation of STAT3. IL-6 also stimulates STAT3 serine phosphorylation but by a p42/p44 MAPK independent pathway (Chung *et al.*, 1997). STAT1 is serine phosphorylated in a Jak2 dependent manner in response to Interferon-γ treatment. In this case serine phosphorylation is required for the maximal transcriptional activity of STAT1 (Zhu *et al.*, 1997).

Phosphorylated STATs dimerize and translocate to the nucleus where they bind specific enhancer elements and modulate the transcription of their target genes. Many STAT responsive genes have been identified, some IL-6 family cytokine responsive genes that are activated by STATs are listed below, and summarized in table 1.1.2 (Heinrich *et al.*, 1998).

The acute phase response gene for C-reactive protein (CRP) contains a STAT binding site and is transcriptionally activated by STAT3 (Zhang *et al.*, 1996). The AP-1 transcription factor family member Jun B has a STAT binding site in its promoter and is activated by STAT3 but not STAT1 in response to IL-6 treatment (Coffer *et al.*, 1995). The immediate early gene c-fos is regulated by two growth factor responsive promoter elements. Binding of STAT to the cis-inducible element is required, but not sufficient for c-fos gene trascription (Hill & Treisman, 1995). The binding of STAT1 to the interferon regulatory factor (IRF-1) promoter was one of the first examples of gene regulation by STATs (Harroch *et al.*, 1994). The CCAAT enhancer binding protein (C/EBP)δ (also known as NFIL6β) has a STAT binding site in its promoter and STAT3 is involved in its expression after IL-6 treatment. The promoter for Hsp90β contains binding sites for STAT3, NFIL6β and Heat shock factor-1 (HSF-1) (Stephanou *et al.*, 1998b). The anti-apoptotic gene bcl-x is induced by LIF treatment through STAT1

binding and is cardioprotective (Fujio *et al.*, 1997). The IL-6 family receptor component gp130 is also itself regulated by STATs. The gp130 promoter contains binding sites for STAT1 and STAT3 and the STAT binding domain was sufficient to confer inducibility by cytokines on the promoter (O'Brien & Manolagas, 1997).

Table 1.1.2 Genes activated by STAT1 or STAT3 in response to IL-6 family cytokines.

Promoters activated by STAT1	**Promoters activated by STAT3**
Interferon regulatory factor	C-reactive protein
bcl-x	jun B
gp130	NFIL-6β
	hsp90β
	gp130

It is not clear exactly how the binding of STATs to target DNA mediates their effect on transcription. One possible mechanism involves the observed interaction of STAT1 with CREB-binding protein (CBP) and p300 (Zhang *et al.*, 1996). STAT binding sites are often located too far upstream from the transcriptional start site to directly activate transcription. CBP and p300 are believed to act as bridges between transcription factors and RNA polymerase II (Kadonaga, 1998).

In addition to their direct transcriptional effects, STATs interact with other DNA binding proteins and affect their activity. The IFN-α-induced IFN–stimulated gene factor-3 (ISGF3) is composed of a STAT1 / STAT2 heterodimer and p48, an IFN regulatory factor 1 (IRF-1) family member. The DNA binding sites for STAT1 and p48 are only separated by 5-6 base pairs and interaction between STAT1 and p48 is essential for the IFN-α response (Qureshi *et al.*, 1995; Horvath *et al.*, 1996). Activated STAT3β interacts with the transcription factor c-jun and enhances its activity (Schaefer *et al.*, 1995). As previously mentioned, CBP and p300 transcription factors interact with STAT1. Neutralization of endogenous CBP and p300 by microinjection of antibodies

blocks transcriptional responses to IFNγ (Horvai *et al.*, 1997). INFγ signals through STAT1 so this demonstrates essential co-activation of STAT1 by CBP and p300.

STAT1 has been shown to interact with heat shock factor-1 (HSF-1) to enhance transcription of hsp70 and hsp90 genes (Stephanou *et al.*, 1999). STAT3 is unable to interact with HSF-1 but antagonizes the transcriptional activation of heat shock genes by HSF-1 (Stephanou *et al.*, 1998b). This antagonistic effect may be due to the fact that STAT3 binding sites overlap with heat shock elements in heat shock gene promoters so HSF-1 and STAT3 may compete for DNA binding. The regulation of heat shock genes by STATs is reviewed by (Stephanou & Latchman, 1999) and will be discussed further in the section on the regulation of heat shock protein expression.

Both STAT3 and STAT1 can be activated by gp130 dimerization, in response to various stimuli in various cell types (Gerhartz *et al.*, 1996; Zhong *et al.*, 1994). CT-1 treatment of neuronal cells was shown to result in STAT3 phosphoryation without detectable STAT1 phosphorylation (Robledo *et al.*, 1997b). Further evidence for STAT3 activation by CT-1 comes from experiments with LIF which binds to an almost identical receptor and has parallel effects to CT-1. LIF mediated dimerization of LIFRβ and gp130 in cardiac myocytes caused tyrosine phosphorylation of STAT3 within 5 minutes and returned to baseline within 60 minutes (Kunisada *et al.*, 1996). The same result was observed in murine hearts after in vivo administration of LIF (Kunisada *et al.*, 1996). STAT1 phosphorylation was not measured in this study. It is therefore clear that STAT3 is tyrosine phosphorylated in response to CT-1 treatment but it is unclear whether STAT1 is activated by CT-1 treatment in the heart.

1.1.3 Mitogen Activated Protein Kinase Pathway

In addition to the activation of STAT3, the p42/p44 Mitogen activated protein kinase (MAPK) pathway (also known as extracellular regulated kinase (ERK)) is activated in response to CT-1 stimulation (Sheng *et al.*, 1997). The activation of the p42/p44 MAPK pathway by cytokines and growth factors occurs via the well established Ras, Raf, MEK signalling cascade (Bokemeyer *et al.*, 1996; Cohen, 1996). This cascade will be discussed later in this section.

Unlike growth factor receptors such as epidermal growth factor receptor (EGF), gp130 receptors do not have integral tyrosine kinase activity. It was therefore difficult to elucidate the mechanism by which dimerized gp130 activates Ras. Ras is a small GTP-binding protein located at the cytoplamic membrane, it is a key regulator in cell growth, and activating mutations of Ras are found in an estimated 30% of all human tumours (Vojtek & Der, 1998). Growth factors activate Ras via the adapter protein Grb2 (growth factor receptor bound protein 2) which has both SH2 and SH3 domains. The SH2 domain of Grb2 binds to phosphotyrosines in an SH2 binding site on the activated growth factor receptor. The SH3 domain of bound Grb2 recruits the guanine-nucleotide exchange factor mSos (mammalian son of sevenless) (Egan *et al.*, 1993). Recruitment of mSos to the plasma membrane brings it into contact with Ras and Sos induces the dissociation of GDP from Ras, allowing the formation of an activated GTP-Ras complex (Egan *et al.*, 1993). The activation of Ras by gp130 may occur by the same mechanism as for growth factor receptors. SH2 binding sites are formed on dimerized gp130 by activated Jaks (see section on Jak/STAT pathway) Grb2 could bind to these sites and mediate the activation of the Ras cascade as described above. There is, however, no evidence for a Jak dependent association of Grb2 with gp130.

Another mechanism for the activation of Ras by gp130 involves the adapter proteins Gab1 (Grb2 associated binder-1) and SHP-2. Gab1 has structural similarities with the Drosophila protein DOS (daughter of sevenless), both have tyrosine residues, which when phosphorylated create SH2 binding sites (Takahashi-Tezuka *et al.*, 1998). Another protein, Gab2 has a high degree of homology to Gab1 at the protein level and has the same functions (Nishida *et al.*, 1999). SHP-2 is a protein tyrosine phosphatase that has two SH-2 domains in its N-terminal region and a C-terminal phosphatase domain. SHP-2 is recruited to tyrosine 759 on gp130 and is tyrosine phosphorylated in a Jak dependent manner in response to IL-6 stimulation (Stahl *et al.*, 1995b; Fukada *et al.*, 1996). Gab1 has been shown to form a complex with PI3-kinase (phosphatidylinositol-3-kinase) and SHP-2 in response to IL-6 stimulation (Takahashi-Tezuka *et al.*, 1998). Additionally, over expression of Gab1 enhanced gp130 dependent p42/p44 MAPK activation (Takahashi-Tezuka *et al.*, 1998). Both SHP-2 and Gab-1 have been shown to associate with Grb-2 (Schaper *et al.*, 1998; Yamada *et al.*, 1999; Schaeper *et al.*, 2000). As described above, Grb2 recruits mSos, which in turn activates Ras, so initiating the p42/p44 MAPK pathway. This is another possible mechanism for

the activation of Ras by gp130 and is likely to be important, but other mechanisms cannot be ruled out.

The involvement of PI3 kinase in activation of Ras is indicated by its presence in the IL-6 stimulated Gab1 / SHP-2 complex. CT-1 has been shown to activate the PI3 kinase pathway and to phosphorylate and activate its down stream target Akt (Kuwahara *et al.*, 2000). Additionally, inhibition of the PI3 kinase pathway with wortmannin or dominant negative p85 PI3 kinase inhbited p42/p44 MAPK activation by IL-6 (Takahashi-Tezuka *et al.*, 1998). There are therefore a number of potential mechanisms for the activation of the p42/p44 MAPK pathway by CT-1 stimulation, these are summarized in figure 1.1.3.

Figure 1.1.3 Activation of the p42/p44 MAPK pathway by CT-1

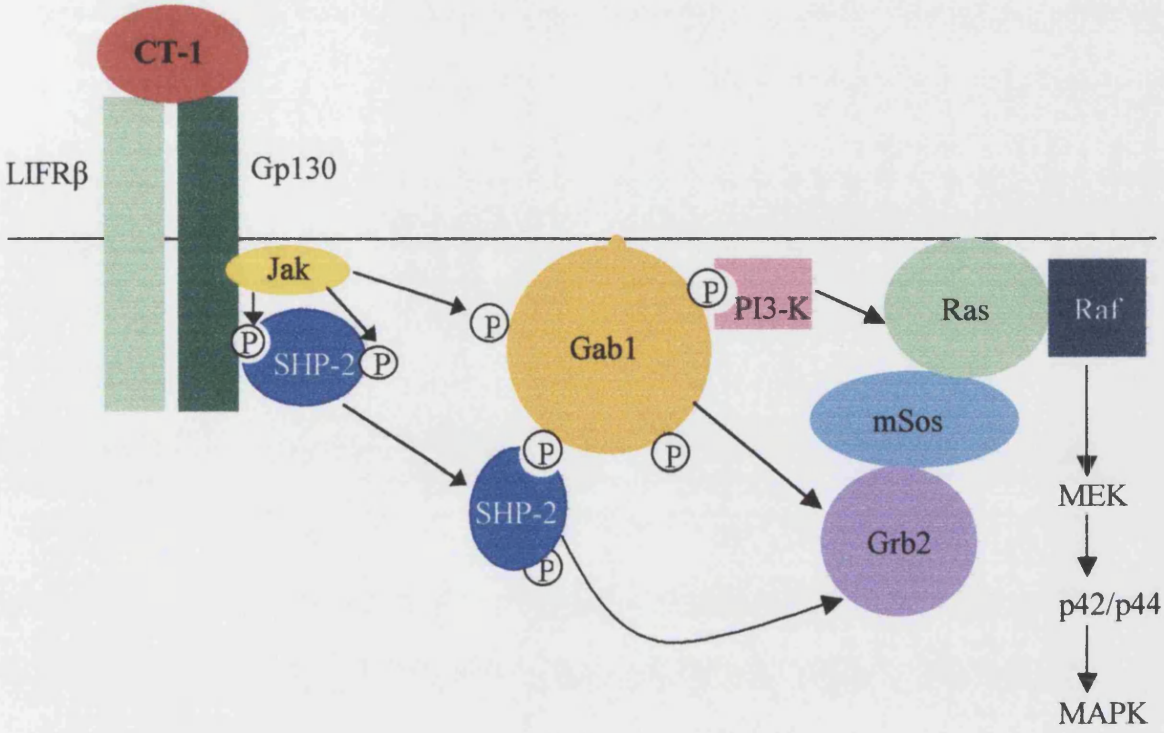


Figure 1.1.3 Schematic showing the activation of the p42/p44 MAPK pathway by gp130 receptor dimerization, adapted from (Takahashi-Tezuka *et al.*, 1998).

Activation of Ras initiates a signalling cascade resulting in the phosphorylation and activation of p42/p44 MAPK. Activated Ras binds to the N-terminus of the serine-threonine protein kinase Raf1, so recruiting Raf1 to the plasma membrane. Recruitment of Raf1 to the plasma membrane is sufficient for activation (Stokoe *et al.*, 1994). An unknown Ras independent mechanism activates Raf1 after it has been brought into contact with the plasma membrane by Ras. Activation of Raf1 specifically activates the p42/p44 MAPK pathway. Activation of the P38 and JNK pathways is thought to occur via other Ras binding molecules homologous to Raf1 (Vojtek & Der, 1998) as shown in figure 1.1.4. Raf1 phosphrorylates two regulatory serines on the MAP kinase kinases MEK1 and MEK2, so causing their activation (Yan & Templeton, 1994). There is also some evidence that MEK activity is modulated by phosphorylation of threonine residues (Matsuda *et al.*, 1993). MEK1 and MEK2 are highly specific activators of p42 and p44 MAPK (also known as Erk1 and Erk2) by phosphorylation of threonine and tyrosine regulatory sites (Seger *et al.*, 1992; Payne *et al.*, 1991). Upon activation p42 and p44 MAPKs translocate to the nucleus but MEK1 and MEK2 remain cytoplasmic (Lenormand *et al.*, 1993). p42 and p44 MAP kinases are thought to be functionally redundant and bind to and regulate many cytoplasmic and nuclear proteins.

All MAP kinases contain the regulatory tripeptide motif -Thr-X-Tyr- where X can be Glu,Gly or Pro. The p42/p44 MAP kinases have the specific –Thr-Glu-Tyr- motif (Cano & Mahadevan, 1995) and are proline directed protein kinases that phosphorylate Ser/Thr/Pro motifs (Gonzalez *et al.*, 1991). The best characterised target for phosphorylation by the p42/p44MAPKs is the transcription factor Elk1. Elk1, together with serum response factor (SRF) binds to the serum response element in the promoter regions of many genes. One such gene encodes the transcription factor and immediate early gene c-fos. C-fos gene expression has been shown to increase in response to p42/p44 MAPK stimulation (Gille *et al.*, 1995).

Many transcription factors are phosphorylated by p42/p44 MAPK some are described below and are summarised in table 1.1.3. NFIL-6, a basic leucine zipper transcription factor is phosphorylated at thr-235 by p42/p44 MAPK so causing its activation (Nakajima *et al.*, 1993). NFIL-6 is involved in the transcription of many IL-6 responsive genes including heat shock proteins (Stephanou *et al.*, 1998b). Expression of the cell cycle regulatory protein cyclin D1 is upregulated by the p42/p44 MAPK

pathway (Lavoie *et al.*, 1996). This upregulation occurs via activation of the transcription factor c-Ets-2 (Albanese *et al.*, 1995). The transciption factor c-Myc is phosphorylated on Ser-62 by P41 MAPK (human isoform of p42/p44 MAPK). This phosphorylation stimulates the activity of the N-terminal transactivation domain of c-Myc (Seth *et al.*, 1992).

Table 1.1.3 Transcription factors phosphorylated by the p42/p44 MAPK pathway.

Transcription Factor	**Role after phosphorylation by p42/p44 MAPK**
Elk1	Binds to serum response element and activates transcription of c-fos.
NFIL-6	Involved in transcription of IL-6 responsive genes such as heat shock proteins.
c-Ets-2	Up-regulation of cyclin D1.
c-Myc	Stimulates N-terminal transactivation domain.

Potential cytoplasmic targets for p42/p44 MAPKs include cytoskeletal elements (Minshull *et al.*, 1994), and various kinases including; ribosomal protein s6 kinases p70s6k and p90rsk (Chen *et al.*, 1993). The p42/p44 MAPK pathway has also been shown phosphorylate MAPKAP kinase-2, which has been shown to phosphorylate and regulate hsp27 (Stokoe *et al.*, 1992; Rouse *et al.*, 1994). Autoregulation of the p42/p44 MAPK pathway is indicated by regulation of upstream signalling elements such as SHP-2, mSos, Raf-1 and MEK. In particular a MEK dependent phosphorylation of mSos causes dissociation of the Grb2-mSos complex and inhibits the activation of Ras by mSos (Waters *et al.*, 1995). This is an example of negative feedback limiting the duration of Ras activation.

In addition to the p42/p44 MAPK cascade, other MAPK pathways are involved in signal transduction from a variety of stimuli (Figure 1.1.4). The JNK (c-jun N-terminal kinase) also known as SAPK (stress-activated protein kinase) cascade and P38 (also known as HOG1 for high osmolarity glycerol response-1) are the other main classes of MAPK in mammalian cells (Bokemeyer *et al.*, 1996). Both the JNK and P38 MAPK cascades are activated in response to stressful stimuli including heat shock (Force *et al.*, 1996). The JNK and P38 MAPKs phosphorylate and regulate numerous substrates including many that are also regulated by the p42/p44 MAPK pathway. JNK

phosphorylates and activates Jun, ATF2 and Elk1 transcription factors leading to induction of c-fos and c-jun gene expression (Gupta *et al.*, 1995; Karin, 1995). P38 MAPK phosphorylates ATF, Elk1 and MAPKAP kinase-2 (Raingeaud *et al.*, 1996; Rouse *et al.*, 1994). The P38 and JNK pathways are important in the stress response but no increase in these pathways has been documented after CT-1 stimulation. These pathways are outlined in figure 1.1.4.

Figure 1.1.4 Multiple MAPK signalling pathways

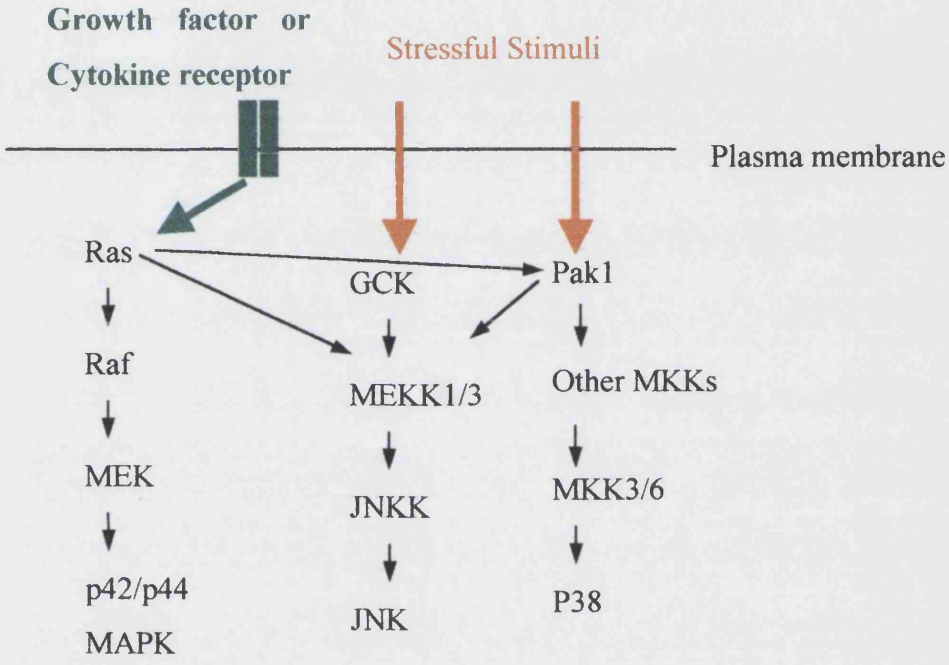


Figure 1.1.4 Multiple MAPK signalling pathways, adapted from (Bokemeyer *et al.*, 1996; Force & Bonventre, 1998).

1.1.4 Biological Activity of Cardotrophin-1

In the short time since CT-1 was cloned (Pennica *et al.*, 1995a) a considerable amount has been learned about its biological activity and mechanism of action. The two most well documented effects of CT-1 are cardiac myocyte hypertrophy and inhibition of cardiac myocyte apoptosis (Latchman, 1999).

1.1.5 Hypertrophic effect of Cardiotrophin-1

CT-1 was originally cloned based on its ability to induce cardiac myocyte hypertrophy (Pennica *et al.* 1995a). Hypertrophy is an increase in cell size without cell division, and will be discussed in a latter section in more detail. CT-1 was initially shown to cause an increase in size of cultured neonatal rat cardiac myocytes (Pennica *et al.* 1995a) and subsequent studies have further investigated this hypertrophic response to CT-1.

Hypertrophy can be determined by taking measurements from stained cells i.e. length, width and area, or by the reactivation of foetal genes. Stimulation with CT-1 resulted in the induction of a panel of immediate early genes including c-fos, egr-1, c-myc, c-jun, jun-B, and tis11 in a gp130 and LIFR dependent manner (Wollert *et al.*, 1996). CT-1 also increases gene expression and release of atrial natriuretic factor (ANF), which is a well characterised marker of cardiac hypertrophy (Wollert *et al.*, 1996).

CT-1 has been shown to induce a distinct form of hypertrophy to that caused by α-adrenergic stimulation. CT-1 causes an increase in cell length but not cell width, which is consistent to that seen in volume overload hypertrophy (Wollert *et al.*, 1996). Both CT-1 and LIF produce a highly significant increase in cell length and no change in cell width. CNTF does not cause any morphological changes, which suggests that direct binding of cytokine to the LIF receptor is required to trigger hypertrophy. CT-1 induced cardiac hypertrophy is achieved by accumulation of sarcomeres in series which is a characteristic feature of volume overload hypertrophy. In contrast, hypertrophy in response to α-adrenergic agonists results in an uniform increase in cell size (Wollert *et al.*, 1996).

Treatment with CT-1 increases Angiotensinogen mRNA levels in rat cardiac myocytes (Fukuzawa *et al.*, 2000). Angiotensinogen is a precursor protein, which is converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II causes

hypertrophy of the heart via an AT_1 receptor (Booz & Baker, 1996). The increase in angiotensinogen mRNA levels caused by CT-1 is mediated by STAT3 binding to a St-domain in the angiotensinogen promoter. Inhibition of angiotensin II receptor binding by an AT_1 receptor antagonist significantly attenuated the hypertrophic effect of CT-1 (Fukuzawa *et al.*, 2000). These results demonstrate an important role for angiotensin II activated by STAT3 in mediating the hypertrophic response to CT-1.

CT-1 is released from cultured cardiac nonmyocytes at a level 3.5 times greater than that released from cardiac myocytes. Cardiac nonmyocyte conditioned media can elicit a hypertrophic response in cardiac myocytes, and this response can be blocked by CT-1 antibodies. These findings suggest that CT-1 produced by cardiac nonmyocytes may act as a local trophic factor to cause hypertrophy in cardiac myocytes (Kuwahara *et al.*, 1999).

1.1.6 Protective Effect of Cardiotrophin-1

Followings its isolation as a hypertrophic factor, CT-1 has been shown to act as a survival factor in the heart and other tissues. CT-1 is expressed in the murine heart tube early in development and promotes survival of cardiac myocytes (Sheng *et al.*, 1996). Administration of CT-1 prevents serum withdrawal induced apoptosis in cultured neonatal rat cardiac myocytes (Sheng *et al.*, 1997). CT-1 can also protect cardiac myocytes against elevated temperatures (heat shock) or simulated ischemia (Stephanou *et al.*, 1998a). In these studies CT-1 is added prior to the stressful stimulus and reduces the amount of cell death. CT-1 has also been shown to be protective when added to cardiac myocytes after simulated ischemia, at the moment of reperfusion (Latchman, 1999)(Brar *et al.* unpublished observations). Ischemia-reperfusion injury will be discussed in greater detail in a later section. Studies with intact rat hearts on a Langendorff perfusion apparatus have confirmed in-vitro studies. Hearts on a Langendorff perfusion apparatus can be perfused and subjected to an ischemic insult by ligation of the coronary artery. The size of the risk zone – i.e. the area that has been deprived of perfusion and the size of the infarct – the area of cell death can then be determined. Perfusion of CT-1 prior to, during or following an ischemic episode reduces the infarct size to risk zone ratio, so is therefore protecting the heart (Brar *et al.* unpublished observation).

A number of studies have investigated the signalling pathways involved in the protective effect of CT-1 in the heart. Use of MAP kinase inhibitors and dominant negative mutants has shown that the anti apoptotic effects of CT-1 are mediated via the MAP kinase pathway in heart cells (Sheng *et al.*, 1997)(Brar *et al.* unpublished observations). Cardiac myocytes were transfected with a dominant negative MEK1 cDNA or treated with the MEK1 inhibitor PD98059. MEK1 is a MAPK kinase upstream in the p42/p44 MAPK pathway. Inhibition of this pathway blocked the anti-apoptotic effects of CT-1 but had no effect on its hypertrophic effect. This finding demonstrates a divergence in the signalling pathways leading to the protective and hypertrophic effects of CT-1. A recent study has demonstrated that CT-1 phosphorylates Akt via PI3 kinase and that activation of this pathway resulted in phosphorylation of the pro-apoptotic gene BAD (Kuwahara *et al.*, 2000). Phosphorylation of BAD inhibits its pro-apoptotic effects and promotes cell survival. Inhibition of the PI3 kinase pathway with the chemical inhibitor LY294002 blocked the protective and anti-apoptotic effect of CT-1 on serum starved ventricular myocytes (Kuwahara *et al.*, 2000). This shows that the PI3K / Akt pathway is also involved in the protective effect of CT-1. The finding that the p42/p44 MAPK and PI3K pathways are both required for the protective effect of CT-1 suggests overlap between the two pathways. PI3K is involved in the activation of the p42/p44 MAPK pathway through activation of Ras (see section on p42/p44 MAPK pathway). This could explain why inhibition of either pathway has the same effect of inhibiting the protective effect of CT-1.

Recently, CT-1 has been shown to induce heat shock protein (hsp) expression in cultured neonatal cardiac myocytes and to protect them from stressful stimuli (Stephanou *et al.*, 1998a). A dose of 1 ng/ml CT-1 resulted in a 'clear enhancement' of both hsp70 and hsp90 levels, as detected by western blotting. The same dose of CT-1 also caused a highly significant increase in cell survival when exposed to lethal ischaemic stress. A protective effect was also seen against a lethal heat stress but this was less significant than that seen with ischaemic stress. This study raises the possibility that the protective effect of CT-1 is mediated by hsps. This hypothesis is supported by the fact that hsps are well known to protect cardiac cells against stressful stimuli (Heads *et al.*, 1994; Cumming *et al.*, 1996b). Additionally, both pathways activated by CT-1 (STAT3 and MAPK) can result in HSP induction, as shown by

studies with IL-6 (Stephanou *et al.*, 1998b). Heat shock proteins will be discussed in greater detail in a later section.

Several studies document the protective effects of CT-1 on motoneurons. CT-1 is present in embryonic muscle and promotes long term survival of spinal motorneurones (Pennica *et al.* 1996a). Adenovirus mediated delivery of CT-1 prevents progressive motor neuronopathy (pmn) in pmn mice (Bordet *et al.*, 1999). Additionally, administration of CT-1 protects mouse motorneuronesin an LIFRβ dependent manner (Arce *et al.*, 1999).

CT-1 has also been shown to attenuate endotoxin induced lung injury by reducing the inflammatory response (Pulido *et al.*, 1999).

1.1.7 Other Roles for Cardiotrophin-1

An important developmental role for CT-1 in the heart is highlighted by measurement of CT-1 gene expression throughout development. CT-1 is predominantly expressed in the heart in murine embryogenesis, localized to myocardial cells. It is also expressed in skeletal muscle, liver and dorsal root ganglia after embryonic day 12.5. CT-1 and LIF promote neonatal cardiac myocyte survival; this effect is not seen with IL-6 and CNTF. CT-1 also triggers a two-fold increase in embryonic cardiac myocyte proliferation, (Sheng *et al.*, 1996). This developmental role for CT-1 is supported by the finding that gp130 knockout mice die in-utero with severe heart defects (Yoshida *et al.*, 1996). However, signalling of other IL-6 family cytokines would also be affected in a gp130 knockout, so a specific CT-1 knockout would be required to confirm the developmental role.

CT-1, in common with other IL6 family members can act as a hepatocyte-stimulating factor, inducing the hepatic acute phase response (Peters *et al.*, 1995).

It has been shown that CT-1 can stimulate IL-6 production in the KB epidermal cancer cell line. This may suggest an immunomodulatory role for CT-1 in some pathological states (Robledo *et al.*, 1997a).

1.1.8 In-vivo Effects of Cardiotrophin-1

In order to study the effects of CT-1 in vivo, a dose of 0.5 or 2μg was administered by intraperitoneal injection, twice a day for 14 days (Jin *et al.*, 1996). Treated mice showed a dose dependent increase in heart weight to body weight ratio compared with untreated controls. This indicates that CT-1 also causes cardiac hypertrophy in vivo. CT-1 treatment also caused an increase in size of the liver, kidney and spleen. Atrophy of the thymus was observed and no changes in overall body weight occurred. Both platelet and red blood cell counts were also increased in CT-1 treated animals. This study shows the diverse effects of CT-1 which are reflected by its expression in tissues other than the heart (Sheng *et al.*, 1996).

The haemodynamic effects of CT-1 were measured using in-dwelling catheters and flow probes in conscious, unrestrained rats (Jin *et al.*, 1998). CT-1 administered intravenously caused a dose dependent increase in heart rate and cardiac output. A dose dependent decrease was observed in mean arterial pressure and systemic vascular resistance. No change in stroke volume was observed in response to CT-1 treatment. Pretreatment with a specific nitric oxide synthase (NOS) inhibitor significantly attenuated the CT-1 mediated increase in heart rate and decrease in mean arterial pressure. This indicates that nitric oxide is a mediator of the haemodynamic effects of CT-1. In a subsequent study, CT-1 administered intravenously in conscious rats again produced an increase in heart rate and a decrease in blood pressure, with no change in the cardiac output. CT-1 also increased ventricular expression of the fetal genes for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Increased expression of inducible NOS (iNOS) was demonstrated in the lung and aorta but not heart or liver, and inhibition of iNOS blocked the depressor effect of CT-1 (Hamanaka *et al.*, 2000). Both these studies show that CT-1 reduces blood pressure by nitric oxide mediated vasodilation.

1.1.9 CT-1 Gene Expression

The mouse CT-1 gene produces an mRNA of 1.4kb and a protein of 21.5kDa from a 203 amino acid open reading frame. Comparison with other IL-6 family members shows that mouse CT-1 has 24% amino acid homology with LIF and 19% with CNTF (Pennica *et al.* 1995b). Shortly after the isolation of mouse CT-1, the human gene was cloned (Pennica *et al.* 1996b).

Human CT-1 is a 201 amino acid protein with 80% homology to mouse CT-1. The gene for human CT-1 is located on chromosome 16p11.1-16p11.2 and produces a 1.7kb mRNA in adult human heart, skeletal muscle, ovary, colon, prostate, testis and foetal kidney and lung. Human CT-1 is biologically active in mice, and binds to the LIF receptor in mouse and rat cells. Likewise, mouse CT-1 was able to bind to human LIF receptor indicating a lack of species specificity between mouse and human CT-1 (Pennica *et al.* 1995b). Conversely, mouse LIF failed to bind human LIF receptor indicating that a species barrier exists between human and mouse LIF (Layton *et al.*, 1994). CT-1 did not bind to the OSM receptor, this shows specificity for the LIF receptor over other gp130 containing IL-6 family cytokine receptors (Pennica *et al.* 1996b).

Cloning and characterisation of a 1.1kb region upstream of the human CT-1 gene has led to identification of a number of transcription factor binding sites (Erdmann *et al.*, 1998). These include two CAAT boxes, which may be involved in basal expression. The transcriptional activator C/EBPβ (also known as NFIL6) is able to bind the CAAT box at position –1076 (Erdmann *et al.*, 1998). Interestingly, NFIL6 has been shown to stimulate IL-6 expression (Akira *et al.*, 1990) which may indicate a role for this transcription factor in the regulation of CT-1 gene expression. The CT-1 promoter also contains a STAT binding site which may indicate autocrine regulation of CT-1, as STAT3 is phosphorylated by CT-1. Other transcription factor binding sites in the CT-1 promoter include; one Sp-1 site, nine AP2 like binding sites, one cAMP response element (CRE) and one GATA binding site. The roles of these transcription factors in regulation of CT-1 gene expression are as yet unknown. Upregulation of CT-1 gene expression has been demonstrated in response to norepinephrine stimulation. This transcriptional activation occurred through binding to the CRE in the CT-1 promoter

and is the first example of specific transcriptional regulation of the CT-1 gene (Funamoto *et al.*, 2000).

In the light of the developmental, protective and hypertrophic functions of CT-1, it is unsurprising that up regulation of CT-1 gene expression has been observed in a number of pathological states. Increased levels of CT-1 protein were observed in left ventricle of cardiomyopathic Syrian hamsters compared with normal controls (Tatsuguchi *et al.*, 1998). Additionally, an elevated level of CT-1 mRNA was observed in the ventricle of genetically hypertensive rats (Ishikawa *et al.*, 1996). These findings suggest that CT-1 may be elevated in the overloaded / overworked heart and may contribute to the hypertrophic response in such conditions. CT-1 levels are also increased in a canine model of congestive heart failure with a positive correlation between ventricular CT-1 mRNA levels and left ventricular mass index (Jougasaki *et al.*, 2000). Cultured cardiac myocytes subjected to hypoxic stress have been shown to produce increased levels of CT-1 mRNA (Hishinuma *et al.*, 1999). This shows that CT-1 expression is activated by hypoxic stress in vitro, as well as in response to the complex array stimuli that would be present in models of heart failure in vivo.

Development of a sensitive immunoluminometric assay has facilitated the detection of CT-1 in human plasma (Talwar *et al.*, 1999). In this study, levels of CT-1 were shown to be elevated in plasma of patients with heart failure compared with normal controls. In a subsequent study, plasma CT-1 levels in human subjects were correlated to left ventricular systolic dysfunction (LVSD) (Talwar *et al.*, 2000). Patients with heart failure and LVSD had elevated plasma CT-1 and reduced wall motion index, fractional shortening, stroke volume, end diastolic volume and systolic volume compared to control subjects. This study shows a direct correlation between plasma CT-1 levels and the severity of heart failure, demonstrating a real clinical relevance for CT-1 in the human heart.

1.2.0 The Stress Response

1.2.1 Overview

The stress response was first discovered in 1962 by Ritossa (Ritossa, 1962), who observed puffing of *Drosophila* salivary chromosomes in response to heat stress. Other stresses such as dinitrophenol and sodium salicylate were also shown to induce puffing, showing that the stress response can be elicited by many stimuli. Puffing of *drosophila* salivary chromosomes is indicative of transcription and was associated with production of a small number of novel proteins (Tissieres *et al.*, 1974). Subsequent studies have shown that the stress response exists in all organisms studied from yeast to man (Lindquist, 1986). Heat shock proteins have been well conserved through evolution in their protein coding and regulatory sequences. The ubiquity and conservation of the response indicates an important role for heat shock proteins in basic cell biology.

Heat shock proteins are also known as molecular chaperones based on their ability to prevent inappropriate protein – protein interactions under physiological and stress conditions. Many of the heat shock proteins are present in the absence of stressful stimuli and are critical to cell survival. Thus, the term heat shock protein becomes confusing, as not all heat shock proteins are heat inducible. The heat shock proteins have been grouped into families according to their molecular weight in kilodaltons and are named hspN where N is the molecular weight. In this thesis, common nomenclature is used and the heat shock proteins described are summarised in table 1.2.1.

Table 1.2.1 A summary of the major Mammalian heat shock proteins.

Heat Shock Protein	**Homologues**	**Proposed Function**
Ubiquitin		Tags proteins for degradation by the proteosome
Hsp27	Hsp25 (murine) Hsp26 (yeast) Hsp22, 23, 26, 28 (Drosophila)	Binds actin filaments Chaperones folding of citrate synthase Role in cell survival and thermotolerance Possible role in breast cancer
Hsp32 = Heme oxygnease-1 (HO-1)		Produced in response to Heme, catalyses degradation of Heme.
Hsp40 = Hdj-1		Hsp70 co-chaperone
Hsp47		Collagen chaperone found in brain increased in cerebral ischemia
Hsp56 (=P59, FKBP59)		Binds immunosuppressive drug FK506 Associated with Hsp70 and Hsp90 in steroid receptor complex
Hsp60	GroEL (prokaryotic)	Mitochondrial protein folding
Hsp70 (=Hsp72 or Hsp70i) inducible	DnaK	Important in cell survival and thermotolerance. Binding and refolding of denatured proteins.
Hsc70 (=Hsp73) constitutive		Binds nascent peptides and prevents formation of abnormal conformations. Uncoats clathrin baskets

Heat Shock Protein	**Homologues**	**Proposed Function**
Hsp90	Hsp83 (yeast, Drosophila) C62.5 (E.coli) GRP94 (Eukaryotic endoplasmic reticulum) HtpG (prokaryotic) Hsp75/TRAP1 (eukaryotic)	Abundant in unstressed cells, increased by stress. Maintains steroid receptor in inactive conformation. Associates with retroviral transforming protein $pp60^{src}$, maintaining it in an inactive state. α and β forms from distinct genes
Hsp100		Present in Golgi apparatus of normal cells. Glucose regulated
Hsp110		Present in nucleoli of normal cells. Glucose regulated.

1.2.2 Heat Shock Proteins As Molecular Chaperones

Folding to the correct conformation is a prerequisite for the function of many biological proteins. All the information required for tertiary protein structure is contained in the primary amino acid sequence. Although proteins can fold to their active conformation in vitro un-aided (Anfinsen, 1973), the situation in vivo is far more complex. Molecular chaperones are required at all stages of protein synthesis, from release from the ribosome to translocation to target organelle, to ensure the correct conformation is achieved. Incorrectly folded proteins also need to be unfolded and re-folded. Proteins must be unfolded in order to be transported across intracellular membranes. Additionally, irrepairably damaged proteins must be recognised and degraded to prevent formation of harmful aggregates. These are just some of the ways in which molecular chaperones function in the everyday running of the cell. In conditions of stress, such as elevated temperature or ischemia, proteins are damaged or do not fold correctly, so the role for molecular chaperones becomes even more critical. Functions of individual heat shock proteins will be described in detail in the next section.

1.2.3 Biology of the Heat Shock Proteins.

In this section the major mammalian heat shock proteins will be described in turn with emphasis being placed on those that are relevant to this thesis. Hsp32 and hsp47 will not be further described, ubiquitin, hsp27 and hsp60 will be introduced briefly but are not important to the subject of this thesis. Hsp56, hsp70 and hsp90 will be described in more detail.

1.2.4 Ubiquitin

Ubiquitin is a small heat shock protein involved in protein degradation. Ubiquitin binds to misfolded proteins and targets them to the proteosome for degradation (for review see (Pickart, 1999). The ubiquitin proteosome pathway is the principle mechanism for degrading short-lived proteins such as cyclins and transcription factors in eukaryotic cells. Ubiquitin molecules become covalently linked to lysine residues exposed on the misfolded protein and are activated by an enzyme cascade. The ubiquitinated substrate is recognised and degraded by the 26S proteosome. Free ubiquitin is released back into the cells upon protein degradation. The ubiquitin pathway is tightly regulated and the turnover of individual proteins can be controlled by ubiquitination signals. The

ubiquitin response is increased under conditions of stress to cope with the increased number of damaged proteins.

1.2.5 Hsp27 and the small heat shock proteins.

The small heat shock proteins are a large and relatively diverse group of proteins which have homology to αA,B-crystallin proteins from vertebrate eye (for review see (Arrigo & Preville, 1999)). Hsp27 enhances cellular resistance to stress (Fortin *et al.*, 2000) and acts as a negative regulator of apoptosis (Mehlen *et al.*, 1996). Hsp27 expression is correlated with the oncogenic status of the cell (Tetu *et al.*, 1995; Tetu *et al.*, 1992) and protects against cancer chemotherapy drugs and inflammatory mediators such as tumour necrosis factor α (TNFα) (Mehlen *et al.*, 1995; Wang *et al.*, 1996).

1.2.6 Mitochondrial Hsps

The major mitochondrial hsps are hsp60 and mhsp70. Their main function is to bind and stabilize substrate proteins within the mitochondria and to fold proteins entering the mitochondria and unfold proteins for export.

1.2.7 Hsp56

Also known as FKBP56, FKBP59 or hsp59. The confusing nomenclature of this protein is due to its dual roles as an immunophillin binding to the immunosuppressive drug FK506 and its function as a molecular chaperone in the steroid receptor complex. Hsp56 was first isolated from rabbit as a 59 kDa component of the steroid receptor (Tai *et al.*, 1986), the human 56kDa isoform was subsequently shown to be a heat shock protein, so was named hsp56.

The immunophillin, cyclosporin A binding protein 40 (CyP-40) has been identified in the oestrogen receptor complex, CyP-40 and hsp56 share a high degree of homology in their carboxyl terminal domains. Hsp56 and CyP40 can both bind directly to hsp90, but they cannot both bind to the same hsp90 molecule and compete for binding, so they probably bind to the same site on hsp90 (Owens-Grillo *et al.*, 1995). Hsp70 is also found in the steroid receptor complex but does not interact directly with hsp56 (Czar *et al.*, 1994). The hsp56/hsp70/hsp90 complex exists in the cytoplasm independently from the presence of steroid receptor.

Like all immunophilins, hsp56 has peptidyl prolyl cis-trans isomerase (PPIase) activity (Chambraud *et al.*, 1993). The cis-trans isomerism of proline residues is a rate limiting step in protein folding, so the PPIase activity of hsp56 may be important in the correct folding of newly synthesised and denatured proteins. FK506 binds to the PPIase site of hsp56, inhibiting its catalytic function. FK506 has no effect on steroid receptor complex formation which suggests that PPIase activity is not required for complex formation (for review see (Pratt, 1993)). The steroid receptor complex has been implicated in receptor trafficking in and out of the nucleus. Particles move through the cytoplasm to the nucleus along microtubules driven by tubulin assisted dynein motors (Gibbons, 1988). Hsp56 has been shown to bind several filamentous proteins and binds to intermediate and heavy chains of dynein (for review see (Pratt, 1993)). This suggests a direct role for hsp56 in protein trafficking.

FK506 (also known as Tacrolimus) is an immunosuppressive drug which inhibits the activation of T-cells by antigens (for review see(Schreiber & Crabtree, 1992)). FKBP-12 is the best characterised FK506 binding protein and the FKBP-12 / FK506 complex has been shown to bind to and inhibit the activity of the calmodulin dependent phosphatase calcineurin (reviewed by (Andreeva *et al.*, 1999)). It is thought that calcineurin inhibition in T-cells may be the target for the immunosuppressive effect of the FK506-immunophilin complex (Liu *et al.*, 1991). It is likely that the hsp56 / FK506 complex has a similar effect on calcineurin inhibition as FKBP12 / FK506, but this is yet to be investigated (Yem *et al.*, 1992). Calcineurin inhibition by FK506 or cyclosporin A has been shown to prevent cardiac hypertrophy (Molkentin *et al.*, 1998; Olson & Molkentin, 1999; Oie *et al.*, 2000). This will be discussed in detail in the section describing cardiac hypertrophy.

1.2.8 Hsp70

The 70kDa Heat shock proteins are probably the best-characterised and most highly conserved family of HSPs. A constitutive form of hsp70 (also known as hsp73 or hsc 70 for heat shock constitutive or heat shock cognate) is present in the normal cytoplasm and nucleus. The highly inducible form (hsp72) is synthesised in the cytoplasm at very high levels in response to stress. There are also forms of hsp70 in the endoplasmic reticulum (BiP) and mitochondria (mhsp70). The different isoforms of hsp70 are encoded by multiple gene copies in eukaryotes (Becker & Craig, 1994). In Prokaryotes

e.g. *E.coli*, a single copy gene encodes a 70 kDa hsp known as dnaK, which is related to eukaryotic hsp70.

The substrate binding activity of hsp70 is ATP dependent. All hsp70 family members bind ATP with high affinity and have weak ATPase acivity, which is triggered by binding to unfolded proteins or synthetic peptides . ATP binds the amino terminal domain of hsp70 and is hydrolysed causing the substrate binding pocket to open. Hsp70 family members bind unfolded and short polypeptides with their C-terminal end, and within their own sub-cellular compartment interact with proteins undergoing synthesis on the ribosome or translocation into organelles. In general, the hsp70 family of proteins is thought to be important in the early stages of protein maturation by binding to and stabilizing the unfolded state of a nascent polypeptide. hsp70 is released when protein synthesis is complete or the protein has translocated into the appropriate organelle. In order to be translocated in to an organelle a protein must be unfolded to pass through a membrane pore. An important role for hsp70 is binding to, and unfolding partially folded polypeptides (Haas & Wabl, 1983), so allowing them to pass through a membrane pore.

Hsp70 is also part of the steroid receptor complex and binds to hsp90 through Hop (hsc70 / hsp90 organising protein, also known as p60). Hsp70 also interacts with Hip (hsc70 interacting protein, also known as p48) in the steroid receptorcomplex. Hip and Hop are thought to act as co-chaperones for hsp70. As proteins are synthesised on polyribosomes, many interact with the early protein folding complex. Hsp70 is central to the early protein folding complex where it binds to an unfolded polypeptide chain. Binding of hsp70 to the polypeptide requires hydrolysis of hsp70 bound ATP, this is stimulated by the co-chaperone hsp40 and the binding of Hip. Hop can bind to both hsp70 and hsp90 so assists in the assembly of the intermediate complex, which includes hsp90, hsp70, hip and hop. In order to become competent for ligand binding the receptor must be bound by the mature folding complex which consists of hsp90, hsp56 and the co-chaperone p23. Upon hormone binding the hsp90 complex dissociates from the receptor (for review of the protein folding complex see (Neckers *et al.*, 1999).

The constitutive form of hsp70, hsp73 or heat shock cognate 70 (hsc70) has been identified as a clathrin uncoating ATPase. This is an enzyme that removes clathrin

cages from coated vesicles (Braell *et al.*, 1984). Clathrin aids vesicle formation from the cell membrane by causing them to curve and bud off. Once the vesicle is formed the clathrin is removed by an uncoating ATPase such as hsp73.

The hsp70 family member localised to the endoplasmic reticulum is known as immunoglobulin heavy chain binding protein (BiP). As its name suggests, BiP binds to immunoglobulin heavy chains formed in the absence of light chain (Haas & Wabl, 1983). This, however, is just one of its roles, BiP binds proteins as they enter the endoplamic reticulum, preventing aggregation and aiding protein folding.

Heat shock or other stressful stimuli can cause an insoluble aggregation of proteins to develop. In the presence of ATP, HSP-70 can dissociate these proteins so they may be refolded correctly or reach their target organelle (Schlesinger, 1990).

1.2.9 Hsp90

Hsp90 is a constitutively expressed, abundant protein constituting 1-2% of total cellular protein. Hsp90 exists as α and β isoforms which are the result of a gene duplication. Hsp90α and hsp90β share 76% amino acid homology, hsp90β being slightly larger and less inducible than hsp90α. A 94kd hsp90 family member known as glucose regulated protein 94 (grp94) is found in the endoplasmic reticulum and increases in response to glucose starvation. Grp94 is 50% identical to hsp90. Hsp75 or TRAP1 is a recently isolated eukaryotic hsp90 family member (Song *et al.*, 1995) which closely resembles the prokaryotic hsp90 homologue high temperature protein G (htpG). Deletion of eukaryotic hsp90 is incompatible with survival (Parsell & Lindquist, 1993; Voss *et al.*, 2000), conversely, deletion of bacterial htpG is not lethal but does make the bacteria more heat sensitive (Bardwell & Craig, 1988).

Hsp90 exists as phosphorylated dimers (predominantly homodimers) with 2-3 covalently bound phosphates per monomer (Iannotti *et al.*, 1988). Both phosphorylation and dimerisation are necessary for many of the chaperoning functions of hsp90 (Minami *et al.*, 1994). Hsp90 is also methylated on 1-3 lysine residues after translation; the importance of this post-translational modification is unknown. Hsp90 consists of an amino (N) terminal domain connected to a carboxyl (C) terminal domain by a highly charged flexible loop. The N-terminal domain has a weak ATP/ADP

binding domain which is also the site for geldanomycin binding. Geldanomycin is an anti tumour drug which can be used as an inhibitor for hsp90. The N-terminus of hsp90 is also involved in substrate binding. The C-terminal domain has a calmodulin binding site and the dimerisation site. The highly charged connecting loop resembles DNA and can be bound by many transcription factors e.g. HSF-1 (Nadeau *et al.*, 1993).

The main function of hsp90 is its ability to bind and stabilise partially formed proteins to prevent inappropriate interactions. Hsp90 preferentially binds positively charged or hydrophic proteins. The list of proteins, which interact with hsp90, is seemingly endless so will not be discussed in full here. Further information can be gained from an excellent review (Csermely *et al.*, 1998). Some of the proteins that interact with hsp90 are as follows: protein kinases e.g. casein kinase, phosphatases e.g. phoshatase S, nuclear hormone receptors, cytoskeltal proteins e.g. actin, transcription factors e.g. Heat shock factor 1 (HSF-1), and G-proteins. Hsp90 is necessary for the correct folding and therefore activity of many kinases. Of interest to this thesis is the ability of hsp90 to bind to Raf kinase and Mitogen activated protein kinase kinase 1 (MEK-1) which are both upstream signalling molecules in the p42/p44 Mitogen activated protein kinase pathway. This pathway will be discussed in greater detail later in this thesis. A stable complex of hsp90 with a transcription factor is said to reduce DNA binding activity (Pratt, 1997) but a low affinity complex enhances binding (Shaknovich *et al.*, 1992). This implies a possible modulatory role for hsp90 in transcription factor DNA binding.

The best characterised function of hsp90 is its role in the steroid receptor complex. Hsp90 is necessary for steroid action in-vivo (Picard *et al.*, 1990). The steroid receptor complex also contains, hsp70, hsp56 and the co-chaperones Hip, Hop and p23. Binding of the steroid receptor to the complex maintains it in a partially unfolded state to allow optimal steroid binding. Binding of steroid to the receptor causes dissociation of the complex. It is also thought that hsp90 may be involved in transport of steroid receptors to and from the nucleus through its interaction with the cytoskeleton. There is a considerable amount of literature about steroid receptor complexes with hsp90 but it will not be discussed further here (for review see (Baulieu, 1991)).

Purified hsp90 can suppress the aggregation of unstable proteins. Hsp90 alone cannot refold proteins but maintains them in a folding competent state allowing them to be

rescued by addition of an hsc70/hsp40 complex (Freeman & Morimoto, 1996). Unlike hsp70, hsp90 does not require ATP for its activity. It seems that hsp70 recognises unfolded proteins while hsp90 recognises early folding intermediates with defined secondary structure (Buchner, 1996).

In conditions of stress, levels of hsp90 increase and the predominantly cytoplasmic protein shifts to the nucleus. Stressful stimuli also cause the usually dimerised hsp90 to preferentially form oligomers. Hsp90 can bind to tubulin and protects fragile microtubules after heat shock (Williams & Nelsen, 1997). In stressful conditions ATP levels are depleted in the cell. Hsp90 binds actin with greater affinity under conditions of reduced ATP (Koyasu *et al.*, 1986), this may be another way of protecting the cytoskeleton under stressful conditions.

1.3.10 Protective Effect of Hsps

Given the important functions of hsps in protein folding, translocation and degradation, it is unsurprising that hsps are critical to cell survival. The protective effect of hsps was originally demonstrated through the preconditioning response (also known as thermotolerance) where a mild stress protects against a subsequent lethal stress (Gerner & Scheider, 1975). This response has been demonstrated in numerous systems and the amount of heat shock protein induction correlated with the protective effect. Heat shock proteins can also protect against apoptotic cell death when increased by heat shock or stable transfection of plasmids to overexpress hsp27 or hsp70 (Samali & Cotter, 1996).

There have been many studies showing the protective effect of heat shock protein induction in the heart, some examples are as follows: Hsp72 increased by exercise improves the recovery of the heart after an ischemic insult (Locke *et al.*, 1995). Mild and chronic hyperthermia selectively increased constitutive hsp70 in a rat heart cell line and protected the cells against oxidative stress (Su *et al.*, 1999). Whole body hyperthermia increased the amount of hsp72 produced in rat heart and the hsp72 level could be directly correlated with the degree of myocardial protection (Hutter *et al.*, 1994). Treatment of cardiac cells with the tyrosine kinase inhibitors Herbimycin A or geldanamycin was shown to increase hsp25, hsp70 and hsp90 and to decrease cell death in response to simulated ischemia (Morris *et al.*, 1996; Conde *et al.*, 1997).

More direct evidence for the role of heat shock proteins in myocardial protection comes from studies where one or more heat shock proteins are specifically over expressed. Rat hearts transfected in vivo with an hsp70 expression vector showed better functional recovery after an ischemic insult than control hearts (Suzuki *et al.*, 1998). Transfection of heart cells with a plasmid to over express hsp70 was protective against heat and ischemic stress. In the same study, overexpression of hsp90 protected against heat stress only and hsp60 was not protective (Cumming *et al.*, 1996b). Transgenic mice have also been created over expressing hsp70 and have improved myocardial recovery after ischemic injury (Marber *et al.*, 1995; Plumier *et al.*, 1995). Overexpression of hsp70 by adenovirus mediated gene transfer protects heart cells against simulated ischemia (Mestril *et al.*, 1996). In a similar way, Herpes virus mediated overexpression of hsp70 or hsp27 protected cardiac cells against thermal or hypoxic stress and from apoptotic stimuli, whereas hsp56 over expression had no protective effect (Brar *et al.*, 1999b).

It is traditionally thought that the hsps mediate their protective effect by their actions as molecular chaperones to increase integrity of cellular proteins. A direct anti-apoptotic effect has been demonstrated for hsp70 through inhibition of the stress kinase JNK (Gabai *et al.*, 1998). There can be little doubt therefore of the protective effect of hsp70 in the heart. There is less evidence for the other hsps, as hsp70 has been studied far more intensively. It does seem, however that a protective effect can be correlated with increased levels of hsp90 in the heart. One reason for the lack of evidence for a specific protective effect of hsp90 may be the difficulty of expressing this abundant protein to levels significantly higher than background levels. A substantial decrease in hsp90 levels has been reported in the ventricular tissues of sudden death pigs with hypertrophic cardiomyopathy (HCM) compared to surviving HCM or normal pigs (Lee *et al.*, 1996). This is further evidence that hsp90 may have a protective role in the heart.

Due to their established protective effect, it is therefore important to find novel ways to increase hsp levels in the heart, without the use of potentially damaging stressful stimuli. The gene therapy approach, although highly specific is a long way off in the clinical setting and a pharmacological means to induce hsps safely in the heart would be potentially important. It is therefore of interest to study the signalling pathways involved in hsp induction in the heart.

1.2.11 Regulation of hsp gene expression

The family of transcription factors involved in hsp induction are the heat shock factors (HSFs). Four HSFs have been described in vertebrates and are known as HSF-1-4. Despite an overall homology of less than 40%, the HSFs all have two highly conserved features: A 100 amino acid amino terminal DNA binding domain and a carboxyl terminal oligomerization motif of hydrophobic heptad repeats (Morimoto *et al.*, 1992). The DNA binding domain allows HSF to bind to heat shock elements (HSE) in target promoters. The heptad repeats allow formation of HSF trimers. The carboxyl terminus of HSF is less conserved and contains the transcriptional activation domains. Carboxyl terminal deletion studies have revealed that different regions are required for constitutive and inducible transcriptional activation of hsp genes (reviewed by (Sorger, 1991)).

HSF-1 activation can be closely related to the stress induced activation of heat shock genes and the protein acquires binding activity in response to stressful stimuli such as elevated temperatures, ischemia/reperfusion, oxidative stress, heavy metals and amino acid analogues (Morimoto *et al.*, 1992; Sarge *et al.*, 1993). HSF-1 will be discussed further below.

HSF-2 is not stress regulated and seems to be important in development. HSF-2 is activated in human K562 erythroleukemia cells during hemin stimulated differentiation (Sistonen *et al.*, 1992), but as HSF-2 cannot be directly activated by hemin or heat stress, its activation may be secondary to the effects of hemin on differentiation (Zhu & Mivechi, 1999). HSF-2 is constitutively active in mouse embryonic stem cells and in spermatogenic cells of mouse testes (Murphy *et al.*, 1994; Sarge *et al.*, 1994). HSF-2 displays DNA binding activity in unstressed mouse blastocysts, this is another example of a possible role for HSF-2 mediated hsp gene expression in cellular differentiation (Mezger *et al.*, 1994).

HSF-3 is stress activated but has a different activation threshold to HSF-1. HSF-3 is activated more slowly and at higher temperatures than HSF-1, so is thought to be involved in the response to severe stress (Tanabe *et al.*, 1997).

HSF-4 is the most recently isolated HSF and differs from other HSF family members as it lacks the carboxyl terminal heptad repeats that are present in all the other HSFs. HSF-4 lacks the properties of a transcriptional activator and represses the expression of heat shock genes (Nakai *et al.*, 1997).

HSF-1 exists in the cytoplasm as inactive monomers and is activated by stressful stimuli. Activation of HSF-1 involves trimerization, acquisition of DNA binding activity and inducible phosphorylation (Rabindran *et al.*, 1993; Sarge *et al.*, 1993; Westwood *et al.*, 1991). Activated HSF-1 trimers rapidly translocate to the nucleus to form nuclear stress granules (Jolly *et al.*, 1997). These stress granules disappear on recovery from the stress and reform in the same locations if cells are stressed again (Jolly *et al.*, 1999). The stress granules are distinct from the sites of transcription of the major hsp70 and hsp90 genes, and it is thought that they may be localising to particular chromosomal loci (Jolly *et al.*, 1997).

The exact mechanism to explain how HSF-1 detects stressful stimuli is unknown. The most likely hypothesis seems to be the binding and negative regulation of HSF-1 by an hsp90 chaperone complex (Ali *et al.*, 1998; Nadeau *et al.*, 1993; Zou *et al.*, 1998). In this model hsp90 forms a complex with inactive HSF-1 so reducing the amount of free HSF-1 available for trimer formation. HSF-1 trimer formation is a highly concentration dependent process (Larson *et al.*, 1988), so if HSF-1 is bound to an hsp90 complex, trimer formation will not occur. In conditions of stress, misfolded or unfolded proteins would compete with HSF-1 for binding to hsp90, resulting in more free HSF-1. This increased HSF-1 would preferentially form trimers so initiating its activation. Hsp70 has also been shown to bind to the transactivation domain of HSF-1, inhibiting its activity once bound to DNA (Shi *et al.*, 1998). Depletion of Hsp90 from the chaperone complex is sufficient to cause HSF-1 trimer formation, while depletion of hsp70 is not (Zou *et al.*, 1998). This implies that while hsp70 has inhibitory effects on transcriptional activation by HSF-1, hsp90 is the most important regulator of trimer formation.

The promoters of all heat shock genes contain heat shock elements (HSE), which are inverted repeats of the pentameric sequence nGAAn. The hsp70 promoter contains multiple HSEs, and a basal promoter region containing multiple cis-regulatory elements,

including binding sites for the transcription factors Sp1 and ATF. The human hsp90α promoter contains one 'perfect' and several 'imperfect' HSEs, an SP1 binding site and a serum response element. The human hsp90β promoter contains two HSEs in its first intron, which are responsible for 80% of constitutive transcriptional activity and almost all inducible activity (Shen *et al.*, 1997). This is unusual, as most heat shock genes do not contain introns and mRNA splicing is inhibited after heat shock.

Targeted disruption of HSF-1 has been shown to abolish thermotolerance but had no effect on the constitutive expression of hsps (McMillan *et al.*, 1998). Additionally, delivery of a constitutively active form of HSF-1 with a herpes viral vector protected neuronal cells from thermal or ischaemic stress, but not apoptosis (Wagstaff *et al.*, 1998). These findings demonstrate the importance of HSF-1 in stress induced hsp induction and implicate the other HSFs in constitutive regulation.

Other transcription factors can also interact with HSF to regulate hsp gene transcription. Examples of this are the interaction of c-Myb with HSF-3, causing its activation in unstressed cells. This is important in hsp gene regulation during cellular proliferation (Kanie-ishii *et al.*, 1997). HSF-1 and STAT1 can also interact to enhance transcription of heat shock genes (Stephanou *et al.*, 1999). Activated NFIL6 and STAT3 synergistically activate the hsp90β promoter in response to Interleukin-6 stimulation. This is in contrast to the interaction with HSF-1. Over expressed STAT3 is antagonistic with active HSF-1, (either over expressed artificially or activated by heat shock), causing less hsp90β promoter activity than with either stimuli alone. NFIL6, however, acts synergistically with HSF-1 to enhance activity of the hsp90β promoter (Stephanou *et al.*, 1998b).

Stimulation of the p42/p44MAPKpathway has been shown to phosphorylate serine residues at amino acids 303 and 307 of mammalian HSF-1 (He *et al.*, 1998). Phosphorylation of these residues inhibits the HSF-1 dependent control of hsp70 basal expression (Chu *et al.*, 1996). An additional study also shows that the MAPK pathway can negatively regulate the heat shock response in NIH3T3 cells (Mivechi & Giaccia, 1995). These studies show that the activity of HSF is modulated by intracellular transcription factors as well as stressful stimuli.

It seems therefore that regulation of heat shock genes is dependent on a balance of HSF activation and other transcription factors that interact with HSF or bind to cis-regulatory sites in heat shock gene promoters.

1.3.0 Cardiac Injury

1.3.1 Infarction and Ischemia Reperfusion Injury

In the United Kingdom, an estimated 180,000 people are hospitalised due to acute myocardial infarction each year. Of these, 10-15% die during hospitalisation and a further 10-15% die in the following year (Dana & Walker, 1999). Myocardial infarction (MI) is death of an area of heart muscle due to reduced blood flow (ischaemia) to that area. Lack of blood flow deprives the tissue of oxygen, glucose, and allows toxic waste products to build up. The most effective treatment for MI is rapid restoration of blood flow (reperfusion) to the ischaemic myocardium (Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI), 1986; ISIS-2 (Second International Study of Infarct Survival) Collaborative Group, 1988). Although essential to prevent further damage, reperfusion can itself cause additional damage to the myocardium known as reperfusion injury (Matsumura *et al.*, 1998).

1.3.2 Mechanisms of cell death in the heart

There are two types of cell death that can be identified in eukaryotic cells. Necrosis is an uncontrolled event where a cell dies rapidly, losing membrane integrity and allowing leakage of intracellular contents. This uncontrolled 'bursting' of cells can cause a potentially damaging immune response and infiltration of macrophages to clear up the cellular debris (Darzynkiewicz *et al.*, 1997). Necrosis can be detected by the loss of membrane integrity which allows release of cytoplasmic enzymes such as lactate dehydrogenase (LDH) or creatinine kinase (CK) (Lowe *et al.*, 1988). Loss of membrane integrity can also be detected using dyes such as Trypan Blue which can only enter cells where the membrane is not intact (Leite *et al.*, 1999; Mascotti *et al.*, 2000). Techniques for measuring necrotic cell death cannot exclude detection of apoptotic cells so are generally thought of as measuring total cell death.

Apoptosis, also known as programmed cell death is a controlled event triggered by a signalling cascade. Cell death from apoptosis is a slower process than necrosis and usually takes 12-24 hours, compared to just a few minutes for necrosis (Collins *et al.*, 1997).

Apoptosis results in rapid cell shrinkage and phagocytosis by neighbouring cells. In apoptosis there is no cytoplasmic leakage and therefore no immune response

(Darzynkiewicz *et al.*, 1997; MacLellan & Schneider, 1997). Apoptosis can be detected in cells in a number of ways. One of the first events in apoptosis is the 'flipping' of a phosphatidyl-serine residue from the inside of the plasma membrane to the outside. This event can be detected with Annexin V labelling which stains cells with the phosphatidy-serine residue on the outer surface of their plasma membranes (Willingham, 1999). Another characteristic of apoptotic cells is DNA cleavage. This can be detected by the appearance of a DNA ladder on an agarose gel or by specific labelling of 3'OH ends with techniques such as TUNEL labelling (using terminal transferease) (Willingham, 1999; Mesner-PW & Kaufmann, 1997). Despite the many quantitative methods for the detection of apoptosis, detection of cellular morphological changes remains the most well recognised indicator of apoptosis. Apoptotic morphological changes include; cell shrinkage, nuclear hypersegmentation and chromatin condensation, cell surface blebbing, and formation of apoptotic bodies (Collins *et al.*, 1997).

The first genes to be connected to apoptosis were the *ced* (cell death abnormal) genes in *Caenorhabditis elegans* (Yuan *et al.*, 1993). These genes are required for apoptosis in the worm and share a high degree of homology to the human gene for Interleukin-1 converting enzyme (ICE), which is a protease that cuts out IL1 from its precursor protein (Yuan *et al.*, 1993). More than ten human CED/ICE family members have since been identified and are known as caspases (Alnemri *et al.*, 1996). All caspases are cysteine proteases that have a cysteine residue in their active site and cleave their target proteins at specific aspartic acid residues. Caspases are synthesised as large precursors (procaspases) and are cleaved to their active form by other caspases in a proteolytic cascade. There are many targets for activated caspases including cleavage of structural and adhesion proteins, helping the cell to round up and be taken up by adjacent cells (Nicholson & Thornberry, 1997). The caspase cascade can be activated by many different mechanisms, a few of those known are briefly described below.

Apoptosis can be triggered from outside the cell, for example by a killer lymphocyte, which may recognsise a cell as being virally infected. Lymphocytes can secrete proteins such as perforin onto the target cell membrane causing channels to open up. These channels allow the entry of apoptotic agents such as granzyme B to activate the caspase cascade (Atkinson & Bleackley, 1995).

Receptor mediated apoptosis can be caused by binding of extracellular death signals to death receptors on the cell membrane (for review see (Ashkenazi & Dixit, 1998)). The best characterised death receptors are Fas (also called CD95), which binds to Fas ligand and TNFR1 (also called p55) which binds to TNF (tumour necrosis factor). Death ligands and receptors can be produced by lymphocytes or by cells subjected to stress. Activated Fas receptors aggregate and via an adapter molecule recruit procaspase 8 proteins, which when brought into close proximity with one another cleave and activate each other. Activation of TNFR1 activates the transcription factors AP-1 and NF-κB. Unlike Fas, it rarely causes apoptosis unless protein synthesis is blocked (Ashkenazi & Dixit, 1998). Increased levels of TNF have been detected in patients with heart failure (Levine *et al.*, 1990).

Damage to mitochondria causes the electron transport chain protein cytochrome c to be released into the cytoplasm. Once in the cytoplasm, cytochrome c binds to, and induces oligomerization of the adapter protein Apaf-1 which binds to procaspase 9. Apaf-1 bound procaspase 9 molecules then cleave each other resulting in activation. Active caspase 9 then cleaves and activates procaspase 3, active caspase 3 mediates the apoptotic response (Li *et al.*, 1997). Cytochrome c release occurs in most forms of apoptosis, in some cases it may be the trigger and in others a downstream effect (Green & Reed, 1998).

It is important for survival that cells can regulate their apoptotic machinery to ensure that cell death is not triggered accidentally. For this reason there are many endogenous regulators of the apoptotic cascade. The bcl2 family of proteins are important cellular regulators of apoptosis. Bcl2 and bclx inhibit apoptosis, while bax, bad and bid are pro-apoptotic (Adams & Cory, 1998; Vaux *et al.*, 1992). The tumour suppressor p53 is a DNA binding protein that has been implicated in cell cycle arrest in some forms of apoptosis (Dulic *et al.*, 1994). Additionally, p53 induces apoptosis in response to DNA damage, so preventing the propagation of faulty genes (Lane, 1993). The anti-apoptotic effects of bcl2 are partly due to inhibition of p53 (Miyashita *et al.*, 1994). This shows that control of apoptosis depends on a balance of pro and anti apoptotic genes.

Animal models of heart failure provide a useful tool in the study of the diseased heart. Ischemia reperfusion injury can be induced in vivo by coronary artery ligation for a defined ischemic period followed by reperfusion. Coronary artery ligation in the rat resulted in $2.8x10^6$ cells that were labelled as apoptotic and only 90,000 necrotic after two hours. Up regulation of the apoptotic marker genes bcl2 and Fas was also detected in the infarcted area (Kajstura *et al.*, 1996). Apoptosis has also been demonstrated in human heart failure. A 232 fold increase in apoptotic myocytes was shown in failing hearts compared with control hearts (Olivetti *et al.*, 1997). In this study, apoptosis was identified by DNA fragmentation and morphological characteristics such as chromatin condensation. In a separate study, Narula *et al* characterised apoptosis in human failing hearts by cytochrome c release, caspase 3 activation and ultrastructural examination (Narula *et al.*, 1999). This study also showed that many cells which had cytochrome c release and activated caspases did not have any morphological markers of apoptosis. This implies that either heart cells have some mechanism to block apoptosis following activation of caspase 3 or the cells are commited to apoptosis but have not begun the physical process (Reed & Paternostro, 1999). These studies show that apoptosis is likely to be an important factor in the myocyte loss seen in heart failure.

1.4.0 Cardiac Hypertrophy

1.4.1 Overview

Hypertrophy of the heart is seen in many pathological conditions. In its initial stages it is an important compensatory mechanism to maintain cardiac function in response to increased workload. Increased workload could be defined by increased wall tension in the heart, which occurs in conditions such as hypertension, abnormalities in the valves, or following a myocardial infarction (MI). Wall tension is inversely proportional to the thickness of the heart wall, therefore thickening of the heart as in hypertrophy reduces wall tension and thereby reduces the oxygen demand of the myocardium (Force *et al.*, 1999). Adult cardiac myocytes have traditionally been described as terminally differentiated and unable to divide. There is recent evidence that adult cardiac myocytes can divide and that an increase in cell number may contribute to the hypertrophic phenotype (Anversa & Kajstura, 1998). It is still accepted, however that the majority of hypertrophic growth occurs through an increase in cell size without division (Neyses & Pelzer, 1995). Chronic hypertrophy can occur in many forms of cardiovascular disease and is potentially damaging. In the Framingham study, left ventricular mass as measured by echocardiography was related to the incidence of cardiovascular disease, mortality from cardiovascular disease and mortality from all causes. An increase in left ventricular mass could be correlated with increased death from all causes, showing the detrimental effects of chronic hypertrophy in humans (Levy *et al.*, 1990).

1.4.2 The hypertrophic phenotype

In vivo, hypertrophy is commonly measured by an increased heart to body weight ratio. Assessment of hypertrophy in cultured cardiac myocytes in vitro can be achieved by measurement of cell size, protein synthesis, sarcomeric organisation, and altered gene expression (Force *et al.*, 1999). Contractile proteins that are present in the normal adult myocardium are upregulated in hypertrophy e.g. myosin light chain-2 (MLC-2) and are involved in the formation of additional sarcomeric units in the hypertrophied cell (Lee *et al.*, 1988). Reactivation of embryonic contractile protein isoforms such as skeletal α-actinin (Schwartz *et al.*, 1986) and β-myosin heavy chain (β-MHC) (Izumo *et al.*, 1987) occurs from two days after a hypertrophic stimuli. The enhanced expression of β-MHC improves the economy of contraction so preserves ATP, but leads to slower contraction and relaxation phases (Force *et al.*, 1999). Other, non contractile fetal genes such as

atrial natriuretic factor (ANF) (also known as atrial natriuretic peptide ANP) and Brain natriuretic peptide (BNP) are also reactivated during hypertrophy (Knowlton *et al.*, 1991; Harada *et al.*, 1998; Hanford *et al.*, 1994). These peptides are vasodilators and diuretics that lower blood pressure and enhance excretion of excess fluid, so reducing cardiac wall tension. Expression of ANF has become one of the most well characterised markers of hypertrophy in the heart (Lee *et al.*, 1988).

One of the earliest events in hypertrophy is the expression of immediate early genes such as c-fos, c-myc, c-jun and egr1, which occurs within one hour of the hypertrophic stimuli (Izumo *et al.*, 1988). Immediate early genes encode transcription factors, which are involved in many aspects of cell cycle regulation and are also referred to as proto-oncogenes. It is likely that immediate early gene products are involved in transduction of the hypertrophic response (Chien *et al.*, 1991). Changes in the expression of proteins involved in calcium handling are also observed in cardiac hypertrophy. Expression of the sarcoplasmic reticulum calcium pump (SERCA2a) is decreased during cardiac hypertrophy and heart failure (Nagai *et al.*, 1989; Mercadier *et al.*, 1990). SERCA2a clears calcium from the cytoplasm, allowing the myocyte to relax after contraction. Elevated intracellular calcium is associated with heart failure and cardiac hypertrophy and is likely to have a causative role in the pathology seen (Richard *et al.*, 1998).

1.4.3 Hypertrophic Stimuli

As already discussed, CT-1 and LIF are capable of causing cardiac hypertrophy (Pennica *et al.* 1995a). In addition to these IL-6 family cytokines, other stimuli can also induce a hypertrophic response.

Physical events such as cell stretch can directly activate the hypertrophic response (for review see (Yamazaki *et al.*, 1995)). Cell stretching occurs in the heart when wall tension is increased by volume or pressure overload. Stretching of cultured cardiac myocytes stimulates protein synthesis and induces a hypertrophic pattern of gene expression without participation of neural or humoral factors (Komuro *et al.*, 1990; Komuro *et al.*, 1991; Mann *et al.*, 1989). Mechanical overload increases cardiac protein synthesis in isolated hearts (Kira *et al.*, 1984) and in-vivo (Cooper *et al.*, 1985). Mechanical stretch also induces the release of neurohormonal agents, which can themselves stimulate a hypertrophic response (Force *et al.*, 1999). These agents include

Angiotensin II (AngII), Endothelin-1 (ET-1) and α-adrenergic agonists such as noradrenaline, adrenaline and phenylephrine (Dostal & Baker, 1998; Force *et al.*, 1999). The activity of the Na^+ / H^+ ion exchanger (NHE) is increased in the heart in response to mechanical stretch. The NHE regulates cellular pH, cell volume and various intracellular signalling pathways and is thought to have an important role in mediating growth in the pressure-overloaded myocardium (Dostal & Baker, 1998).

Peptide growth factors such as transforming growth factor β1 (TGFβ-1) and fibroblast growth factor (FGF) stimulate expression of fetal contractile genes such as α-actinin and βMHC in cardiac myocytes (Parker *et al.*, 1990). It is also likely that peptide growth factors contribute to the hypertrophic phenotype through their effects on other cells in the heart, such as FGF causing proliferation of fibroblasts and thus causing fibrosis, which is a pathological feature of hypertrophy.

Endothelin-1 (ET-1) is a 21 amino acid vasoconstrictor peptide secreted from endothelial cells. High affinity receptors for ET-1 are found in the mammalian atria and ventricles (Galron *et al.*, 1989; Hirata, 1989). ET-1 has a potent positive inotrophic effect in the myocardium, possibly caused by sensitisation of myofilaments to calcium (Watanabe *et al.*, 1989). ET-1 has been shown to cause hypertrophy in serum starved rat ventricular cardiocytes (Shubeita *et al.*, 1990). Hypertrophy was assessed by induction of immediate early gene expression, reactivation of foetal genes including ANP, and increase in cell size. Additionally, plasma ET-1 levels were shown to double in dogs with heart failure induced by rapid ventricular pacing (Margulies *et al.*, 1990). ET-1 has been shown to activate Phospholipase C, which causes activation of protein kinase C (PKC) and an increase in intracellular calcium in many cell types including cardiac myocytes (Takuwa *et al.*, 1989; Shubeita *et al.*, 1990). ET-1 induces PKC dependent expression of IL-6 and LIF but not CT-1 in cardiac myocytes (Saito *et al.*, 1999). The induction of ANP and skeletal α-actinin gene expression by ET-1 is also dependent on PKC activation (Ito *et al.*, 1991). Over expression of a dominant negative gp130 suppressed ET-1 induced activation of ANP and β-MHC promoters (Saito *et al.*, 1999). These results suggest that the hypertrophic effects of ET-1 are mediated by PKC and partly by the induction of IL6 and LIF.

The rennin-angiotensin system is an endocrine loop involved in cardiovascular and renal fluid homeostasis. Active angiotensin II (AngII) is cleaved from its inactive precursor angiotensinogen by angiotensin converting enzyme (ACE), and binds to an AT_1 G-protein receptor. A number of studies have implicated AngII in the hypertrophic response to pressure overload (Lorell, 1995; Iwai *et al.*, 1995). AngII can also cause hypertrophy in cardiac myocytes as measured by immediate early gene expression and reactivation of the fetal genes α-actinin and ANF. Ang II is secreted by neonatal cadiomyocytes in response to passive stretch and acts in an autocrine / paracrine manner to promote hypertrophy (Sadoshima *et al.*, 1993). Ang II mediated induction of the immediate early gene c-fos is dependent on PKC activation (Sadoshima & Izumo, 1993), implicating a role for PKC in the hypertrophic response to AngII. AngII also activates the Jak/STAT pathway, in particular STATs 3 and 6, which bind to and activate transcription of the angiotensinogen promoter in a positive feed back loop (Mascareno *et al.*, 1998). AngII is also involved in activation of the Jak/STAT pathway in response to pressure overload in the rat heart (Pan *et al.*, 1997). Cardiotrophin-1 has been shown to induce expression of angiotensinogen in cardiac myocytes through STAT3 (Fukuzawa *et al.*, 2000). These results show that AngII causes hypertrophy and may be involved in the hypertrophic response to CT-1. Evidence of an important role for AngII in hypertrophy comes from the use of ACE inhibitors and AT_1 receptor antagonists to induce regression of hypertrophy in both animals and humans (Monopoli & Ongini, 1994).

Catecholamines are a group of closely related hormones and neurotransmitters, which include adrenaline (also known as epinephrine) and noradrenaline (also known as norepinephrine) (for review see (Hadley, 1996)). Adrenaline acts as a hormone and is released from the adrenal medulla. Noradrenaline is neurotransmitter released from sympathetic nerves. There are two classes of adrenergic recptors (AR); α-AR and β-AR and each class have several subtypes. Adrenaline stimulates both α-AR and β-AR simultaneously but its actions on the α-AR dominate. The α-AR stimulates smooth muscle contraction and the β-AR stimulates relaxation. A number of drugs can specifically stimulate subsets of ARs. Phenylephrine (PE), for example is a specific α_1-AR agonist and isoproterenol is a specific β-AR agonist. Adrenaline increases both the force and rate of heartbeat through stimulation of β-ARs, this is the only known example of β-ARs causing contraction. Adrenaline stimulation causes blood to be

shunted from low priority area such as skin, mucosa, connective tissue and kidneys, in all these tissues α-ARs predominate to mediate contraction. Smooth muscle of coronary arteries, bronchi and skeletal muscle contain β-ARs to allow relaxation in the presence of adrenaline. This selective receptor distribution allows blood to be shunted from the periphery to vital organs for the 'fight or flight' response to adrenaline.

There is evidence for a hypertrophic effect of catecholamines in the heart. Noradrenaline administration was shown to produce cardiac hypertrophy in dogs (Gans & Cater, 1970) but this effect may be due to its hypertensive effect. A subsequent study used a subhypertensive dose of noradrenaline in conscious dogs, and a significant degree of left ventricular hypertrophy was detected despite the fact that blood pressure had remained within the normotensive range (Laks & Morady, 1976). Administration of noradrenaline or isoproterenol to cultured cardiac myoctes produced an increase in cell size and protein content (Simpson *et al.*, 1982a). The α_1-AR agonist phenylephrine has well documented hypertrophic effects both on cell size, and ANF and BNP gene expression (Hanford *et al.*, 1994). Transgenic mice with myocardial over expression of a constitutively active α_{1B}-AR display marked cardiac hypertrophy (Chen *et al.*, 1995). ARs, like AngII and ET-1 receptors are Gq protein coupled so activate PKC through phospholipase C. It is likely therefore that the hypertrophic effects of adrenergic agonists are mediated by PKC activation.

1.4.4 Signalling pathways in cardiac hypertrophy

Several signalling pathways are known to be important in the hypertrophic response to various stimuli. The p42/p44 MAPK and STAT3 pathways are activated by CT-1 and have both been implicated in cardiac hypertrophy.

Involvement of the p42/p44 MAPK pathway in cardiac hypertrophy has been suggested by a number of studies. Transgenic mice with constitutively active ras, an upstream activator of the p42/p44 MAPK pathway, develop ventricular hypertrophy (Hunter *et al.*, 1995; Gottshall *et al.*, 1997). Constitutively active Ras, src or raf stimulate an increase in size and a hypertrophic pattern of gene expression when transfected into cardiac myocytes (Fuller *et al.*, 1998). Src is a tyrosine kinase, which is involved in the activation of Ras by G-protein coupled receptors. Raf is a kinase activated by Ras that activates MEK-1 and thus the p42/p44 MAPK pathway. Transfection of constitutively

active MEK-1 or p44 MAPK increased ANF promoter activity in cultured cardiac myocytes (Gillespie-Brown *et al.*, 1995). These results show that activation of the p42/p44 MAPK pathway at many different levels is able to cause hypertrophy in cultured cardiac myocytes. Inhibition of this pathway by antisense oligonucleotides to p42 and p44 MAPKs down regulates the phenylephrine (PE) induced hypertrophic response (Glennon *et al.*, 1996). Transfection of dominant negative MEK-1 also reduced the induction of ANF promoter activity by PE but did not affect sarcomeric organisation (Gillespie-Brown *et al.*, 1995; Thorburn *et al.*, 1994). This implies that the p42/p44 MAPK pathway is required for the induction of ANF by PE but not for other hypertrophic effects of PE. Conversely, in a recent study, inhibition of MEK-1 with PD98059 or dominant negative MEK-1 had no effect on ANF promoter activation by PE, but inhibited activation by AngII (Aoki *et al.*, 2000). It is therefore evident that the p42/p44 MAPK pathway is involved in some aspects of the hypertrophic response but it is unclear which hypertrophic agents utilize this pathway to mediate their hypertrophic effects. Sheng et al (Sheng *et al.*, 1997) report that activation of the p42/p44 MAPK pathway is not required for CT-1 mediated cardiac hypertrophy, indicating that the alternative STAT3 pathway may be involved.

Transgenic mice with cardiac specific over expression of STAT3 develop cardiac hypertrophy at 12 weeks of age with increased expression of ANF, β-MHC and CT-1 genes (Kunisada *et al.*, 2000). STAT3 transgenics also had increased survival rates after administration of a cardiotoxic drug suggesting both hypertrophic and protective effects for STAT3 in the heart (Kunisada *et al.*, 2000). Phosphorylation (activation) of STAT3 occurs in the heart in response to hypertrophic stimuli such as pressure overload (Pan *et al.*, 1997) and mechanical stretch (Pan *et al.*, 1999). In both these cases, STAT3 phosphorylation was mediated by gp130 (Pan *et al.*, 1998; Pan *et al.*, 1999). Additionally, CT-1 has been shown to activate the angiotensinogen promoter through STAT3 in cardiac cells and this is thought to be a cellular signal for hypertrophy in cardiac muscle (Fukuzawa *et al.*, 2000; Mascareno *et al.*, 1998). More direct evidence for the role of STAT3 signalling in CT-1 mediated cardiac hypertrophy comes from a study by Kunisada et al (Kunisada *et al.*, 1998), where replication-defective adenovirus vectors carrying wild type or mutant STAT3 were used to infect cardiac myocytes. The hypertrophic effect of the CT-1 related factor, LIF (as assayed by induction of c-fos, ANP and protein synthesis) was augmented by wild type STAT3 and attenuated by

mutant STAT3. These effects occurred independently from any changes in MAPK activity. These findings strongly implicate the STAT3 pathway as a mediator of the hypertrophic response to CT-1, but as yet no direct link has been shown.

There is some evidence that the P38 and JNK MAPK pathways are also involved in the hypertrophic response. Much information is provided by a study comparing the effects of the JNK and P38 MAPK pathways on the hypertrophic response (Nemoto *et al.*, 1998): P38 activity was stimulated by and required for ANF expression by hypertrophic agonists such as ET-1 and PE in rat cardiac myocytes. Activation of P38 alone was sufficient to stimulate ANF gene expression and inhibition of P38 with the chemical inhibitor SB202190 blocked ANF gene expression by ET-1 and PE (Nemoto *et al.*, 1998). In the same study, JNK was shown to negatively regulate ANF expression by ET-1 and PE. Additionally, transfection of an inactive JNK mutant potentiated ANF expression. In contrast, activation of JNK by infection with adenoviral vectors containing constitutively active MKK7 (a specific activator of JNK) caused an increase in cell size, elevated ANF expression and sarcomeric organisation (Wang *et al.*, 1998). Co-activation of both the P38 and JNK pathways resulted in increased cell death and cytopathic responses (Wang *et al.*, 1998). Co-activation of P38 and JNK has been demonstrated in ischemia reperfusion so may be involved in cell death in response to such stressful stimuli (Bogoyevitch *et al.*, 1996; Yin *et al.*, 1997). The role of the P38 and JNK MAPKs in hypertrophy is therefore uncertain due to many contradictory studies. LIF was also shown to slightly activate both P38 and JNK MAPKs, which may indicate a role for these pathways in CT-1 signalling (Nemoto *et al.*, 1998).

PKC is implicated in the hypertrophic response as it is activated by many hypertrophic stimuli, including norepinephrine (Simpson, 1983), phenylephrine (Izumo *et al.*, 1987), ET-1 (Shubeita *et al.*, 1990), and AngII (Sadoshima & Izumo, 1993). PKC exists as a family of at least eight related enzymes that can be divided into two subfamilies (Bogoyevitch *et al.*, 1993). The calcium dependent PKCs were first isolated in brain and include PKC α, β_1, β_2, and γ. The calcium independent PKCs lack the calcium binding domain and include PKC δ, ε, ζ, and η. The only PKC isoform to be detected at significant levels in unstimulated cardiac myocytes is PKCε. Treatment of cardiac myocytes with ET-1 or adrenaline caused translocation of PKCε to the membrane and activation after one minute of stimulation (Bogoyevitch *et al.*, 1993). Over expression

of constitutively active PKCβ activates the β-MHC promoter and PKCβ was activated by α_1-adrenergic agonists in cardiac myocytes (Kariya *et al.*, 1991). These studies show that PKC isoforms are activated by hypertrophic stimuli and can mediate some hypertrophic effects.

Calcineurin is a serine/threonine protein phosphatase that is activated by elevated intracellular calcium through calmodulin binding (Klee *et al.*, 1998). A sustained calcium plateau is required for the activation of calcineurin and it is insensitive to transient calcium fluxes such as in cardiomyocyte contraction (Dolmetsch *et al.*, 1997). Calcineurin is bound and inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506, which has enabled the identification of its many roles in the transduction of calcium signals (Shenolikar, 1994).

Calcineurin exists as a heterodimer of a 58-64 kDa catalytic and calmodulin binding subunit, calcineurin A and a regulatory 19 kDa calcium binding subunit calcineurin B (Klee *et al.*, 1988). Calcineurin regulates target genes through dephosphorylation of a family of transcription factors known as NF-ATs (nuclear factors of activated T cells) (Loh *et al.*, 1996). Although originally demonstrated in immune cells, a calcineurin dependent pathway for the activation of NF-AT transcription factors has also been identified in the heart (Molkentin *et al.*, 1998).

Four NF-AT family members have been identified of which only NF-AT3 is expressed in the heart (Hoey *et al.*, 1995). Activated NF-ATs translocate to the nucleus and bind to the consensus DNA sequence GGAAAAT through a Rel homology domain. A study by Molkentin *et al* reported the involvement of calcineurin in cardiac hypertrophy (Molkentin *et al.*, 1998). NF-AT3 was shown to interact with the cardiac transcription factor GATA-4 and these factors synergistically activate the BNP promoter. The calcineurin inhibitors CsA and FK506 were shown to inhibit the hypertrophic effects of AngII and PE as measured by cell size and ANF mRNA levels. AngII and PE were shown to upregulate NF-AT through a calcineurin dependent pathway. Transgenic mice with heart specific constitutively active calcineurin had an increased heart to body weight ratio of 2-3 times that of control mice. Dilatation of the ventricular chambers was observed in transgenic mice with increasing age and such mice were also susceptible to sudden death. More transgenic mice were created with a construct

encoding constitutively active NF-AT expressed in the heart. These mice also had cardiac hypertrophy showing that NF-AT activation alone is sufficient to cause cardiac hypertrophy. This study introduced calcineurin as a novel mediator of the hypertrophic response (Molkentin *et al.*, 1998) and was particularly interesting as a calcium regulated hypertrophic pathway due to the fact that elevated intracellular calcium is associated with many forms of hypertrophy (Richard *et al.*, 1998). Since this finding, many studies have investigated the importance of calcineurin in hypertrophy induced by various stimuli.

CsA and FK506 prevented the development of hypertrophy in mice that were genetically pre-disposed to develop hypertrophy by cardiac specific expression of tropomodulin, myosin light chain-2 or fetal β-tropomyosin (Sussman *et al.*, 1998). Expression of these proteins in the adult heart causes disruption of sarcomeric proteins, loss of contractility and heart failure. In the same study, another transgenic hypertrophic model with a constitutively active retinoic acid receptor still developed hypertrophy in the presence of calcineurin inhibition. This shows that not all forms of hypertrophy are blocked by calcineurin inhibition and hypertrophy that is not triggered by disruption of sarcomeric proteins may not depend on calcineurin signalling.

CsA treatment was able to inhibit hypertrophy caused by pressure overload due to aortic banding (Sussman *et al.*, 1998). Inhibition of calcinurin with an adenovirus expressing a non competitive peptide inhibitor of calcineurin (known as cain) was also able to attenuate the hypertrophic effects of AngII, PE and fetal bovine serum (Taigen *et al.*, 2000). This shows that the observed effects of calcineurin inhibition on cardiac hypertrophy are not due to non specific effects of CsA or FK506. Interestingly, calcineurin mediated hypertrophy has been shown to protect cardiomyocytes from apoptosis (De Windt *et al.*, 2000b). Treatment with FK506 has also been shown to protect cardiac myocytes against severe thermal or ischemic stress (Cumming *et al.*, 1996a). Additionally, inhibition of hypertrophy with CsA enhances cardiac dysfunction after infarction in rats subjected to coronary artery ligation (Oie *et al.*, 2000). These studies imply that calcineurin mediated hypertrophy may act as a compensatory mechanism to improve heart viability in some cases.

There is therefore a great deal of evidence to implicate calcineurin as an important factor in the development of hypertrophy. This, however, does not preclude the involvement of the other signalling pathways mentioned. It is likely that calcineurin interacts with other signalling pathways and that regulation of hypertrophy depends on cooperation of many factors. A recent study has investigated the interdependence of calcineurin, p42/p44 MAPK, JNK MAPK, P38 MAPK and PKC isoforms on regulation of cardiac hypertrophy (De Windt *et al.*, 2000a). Several experimental approaches were used and collectively showed that the hypertrophic signalling of calcineurin is interconnected with PKCα, PKCθ and JNK, while PKCε, PKCβ, PKCλ, p42/p44MAPK and P38 could not be linked to calcineurin induced hypertrophy (De Windt *et al.*, 2000a). It is likely that further studies of this nature will demonstrate increased cross talk between hypertrophic signalling pathways in the heart.

1.5.0 Urocortin

1.5.1 The CRH family of hormones

Urocortin is a member of the corticotrophin releasing hormone (CRH) family of peptides. CRH is a 41 amino acid peptide that was originally isolated from ovine hypothalamus (Vale *et al.*, 1981). CRH is produced by the hypothalamus and acts on the pituitary to stimulate adrenal corticotrophic hormone release, which goes on to increase steroidogenesis in the adrenal gland. Urocortin and CRH share 45% amino acid homology. Both peptides are synthesised as inactive precursors and are proteolytically processed to a mature peptide. CRH family members share an essentially conserved amino acid motif (PPLSIDLTFHLLR) in their N-termini and a completely conserved GK sequence immediately before the stop codon (Vaughan *et al.*, 1995; Donaldson *et al.*, 1996).

1.5.2 The CRH receptor

Two distinct genes encode the receptors for the CRH family of peptides and are known as CRH-R1 and CRH-R2 (Chen *et al.*, 1993; Lovenberg *et al.*, 1995). The CRHR2 receptor exists in three splice variants; α, β and δ. Urocortin has a higher binding affinity for the CRH-R2β receptor than CRH and it is this receptor that is predominantly expressed in the rat heart (Stenzel *et al.*, 1995). The CRH-R2α receptor is the predominant isoform in human heart (Chen *et al.*, 1993). CRH-R1 receptors show little specificity between CRH family members (Gottowik *et al.*, 1997). CRH family receptors are coupled to Gs proteins and adenylate cyclase (Vaughan *et al.*, 1995). G-protein receptor binding causes cAMP release and phosphorylation of protein kinase A (PKA). PKA phosphorylates components of calcium channels, allowing calcium to enter the cell. The involvement of PKA in signalling by urocortin is inferred by data showing that urocortin increases intracellular cAMP in cardiac myocytes (Ikeda *et al.*, 1998). Urocortin has also been shown to activate the p42/p44 MAPK pathway in cardiac myocytes (Brar *et al.*, 2000). No phosphorylation of P38 or JNK MAPKs could be detected in cardiac myocytes in response to urocortin (Brar *et al.*, 2000).

1.5.3 Biological effects of urocortin

Urocortin and CRH both have anti-inflammatory effects through their induction of corticosteriods. It has also been shown that CRH and urocortin can mediate anti-inflammatory effects independently from corticosteroid production (Poliak *et al.*, 1997).

Subsequently a corticosteroid independent inhibition of tumour necrosis factor (TNF) production has been demonstrated in response to urocortin (Agnello *et al.*, 1998). This shows a direct inhibitory effect of urocortin on production of a pro-inflammatory cytokine.

IL-6 is released in response to non-inflammatory stresses such as immobilization, and this IL-6 release is inhibited by CRH antagonists (Ando *et al.*, 1998). This inferred a role for CRH in IL-6 release and was confirmed by data showing the release of IL-6 in response to CRH or urocortin administration (Ando *et al.*, 1998). It is unknown whether CRH or urocortin are involved in regulation of IL-6 levels under physiological conditions.

One of the main avenues of research into urocortin has been its role in appetite suppression (reviewed by (Heinrichs & Richard, 1999). In particular, urocortin reduces food intake and gastric emptying in *ob/ob* obese mice resulting in significant weight loss (Asakawa *et al.*, 1999).

Urocortin is present in the brain and causes relaxation of cerebral arteries (Schilling *et al.*, 1998). Urocortin is also found in the placenta where it has been shown to cause vasodilation (Leitch *et al.*, 1998). These studies seem to point to a general vasodilatory role for urocortin in the circulatory system.

1.5.4 Urocortin in the heart

A number of studies have indicated an important role for urocortin in the heart: Intravenous injection of urocortin in conscious sheep caused a dose dependent increase in cardiac contractility, heart rate, cardiac output and coronary blood flow (Parkes *et al.*, 1997). Urocortin levels are increased in cardiac myocytes in response to simulated ischemia, and the protective effects of ischemic conditioned media are blocked by inhibitors of CRH family peptides. In the same study, exogenous urocortin protected cardiac myocytes against lethal ischaemic injury (Brar *et al.*, 1999a; Okosi *et al.*, 1998). The cardio-protective effect of urocortin is abrogated by inhibition of the p42/p44 MAPK pathway, indicating dependence on this pathway (Brar *et al.*, 2000). Urocortin has also been shown to increase expression of hsp90 in cardiac myocytes, which could be a potential mechanism for its protective effect (B.K. Brar, unpublished observations).

In addition to its protective effects, urocortin stimulates atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) secretion from neonatal rat cardiomyocytes (Ikeda *et al.*, 1998). Urocortin induced BNP and ANP production was reduced by a specific inhibitor of PKA and a voltage dependent calcium channel blocker. This suggests that urocortin is increasing ANP and BNP levels via activation of PKA and its associated phosphorylation of calcium channels. In the same study, urocortin was also shown to increase protein synthesis in cardiac myocytes (Ikeda *et al.*, 1998). This induction of ANP and BNP levels and increase in protein synthesis suggests that in addition to its protective effects, urocortin, like CT-1 may also be acting as a cardiac hypertrophic factor.

Urocortin shares many of its effects with CT-1. They are both expressed in the heart along with their receptors, they are both cardioprotective by the same pathway, and both induce hsp expression in cardiac myocytes. It is therefore important to find out whether urocortin is also hypertrophic like CT-1, and if so whether they share a common signalling pathway for this effect.

1.6.0 Aims and Objectives

In view of the fact that CT-1 has protective and hypertrophic effects in the heart, the subject of this thesis is to investigate the signalling pathways involved in these effects, with the aim of preventing the damaging hypertrophic effect and promoting the beneficial protective effect.

CT-1 has been shown to increase levels of cardioprotective heat shock proteins in the heart. This induction of heat shock proteins will be further investigated and the mechanism of induction explored. If CT-1 were to be used as a therapeutic agent, it is likely that the heart being treated would be subject to stressful stimuli such as ischemia. The interaction of CT-1 stimulation with stressful stimuli such as heat shock will therefore be investigated, in particular for the effect of both stimuli together on heat shock protein levels.

A method will be developed to enable measurement of hypertrophy to be carried out in transfected cells. This will allow specific manipulation of signalling pathways with genetic inhibitors or activators and the selective measurement of successfully transfected cells.

The corticotrophin releasing factor (CRF) family hormone urocortin mediates protection in the heart by the same pathway as CT-1 but has not been investigated for a hypertrophic response. Urocortin will be tested for a hypertrophic effect, and any such effect compared to the known hypertrophic agents CT-1 and phenylephrine. Additional studies will be carried out to test the effects of CT-1, urocortin and phenylephrine in isolated adult rat cardiac myocytes. Most studies are carried out in myocytes from neonatal rats, as these are easier to culture. Adult cells provide a model system that is morphologically more similar to human adult cardiac myocytes, but have not previously been used to measure the hypertrophic response to CT-1.

An investigation will be carried out into the signalling pathways that mediate the hypertrophic effects of CT-1 and urocortin. Transfection of dominant negative mutants and genetic inhibitors will be carried out, in addition to studies with chemical inhibitors to attempt to identify the pathways involved in the hypertrophic response.

If the hypertrophic effect of CT-1 or urocortin can be blocked by manipulation of a signalling pathway, the effects of manipulation of the same pathway will be tested for its effect on the protective effect of CT-1 or urocortin. This will show whether there is divergence in the signalling pathways involved in the protective and hypertrophic responses.

Heat shock proteins have well-established protective functions, but have not been tested for a possible role in the hypertrophic response. Over expression of heat shock proteins will be achieved using plasmid and viral vectors, cells will be measured to see whether over expression of heat shock proteins has any effect on cell size.

Some initial studies will be carried out in cardiac derived cell lines but the majority of experiments will use primary neonatal rat cardiac myocytes which are a well established model system for the heart.

CHAPTER 2

MATERIALS AND METHODS

2.1.0 Laboratory Reagents

General laboratory chemicals were of analytical grade and were purchased from the following companies:

SIGMA Chemical Company Ltd., Poole, Dorset, U.K.

BDH Merck Ltd., Lutterworth, Leicestershire, U.K.

Boehringer Mannheim, Lewes, East Sussex, U.K.

Molecular biology enzymes including restriction endonucleases, hexanucleotides [pd$(N)_6$] for random prime labelling, and dNTPs were purchased from Promega, Southampton, U.K.

Radiochemicals, Hybond nitrocellulose membranes, protein molecular weight 'rainbow' marker, Enhanced chemiluminescence system (ECL) and Kodak X-OMAT imaging photographic film were purchased from Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.

Photographic developing and fixing chemicals were obtained from X-OGRAPH Ltd., Tetbury, U.K.

30% (w/v) acrylamide / bisacrylamide solution for polyacrylamide gels was obtained from Amresco Ltd., Ohio, USA.

Collagenase enzyme was obtained from Worthington Biochemical Corporation, New Jersey, USA.

Yeast extract and tryptone for bacterial growth medium was from Duchefa, Harlem, Netherlands.

Secondary antibodies for Western blotting were purchased from DAKO Ltd., Glostrup, Denmark. Primary antibodies were purchased from Stressgen Biotechnologies, York, U.K.

Tissue culture reagents were purchased from GIBCO BRL through Life Technologies, Paisley, U.K.

General laboratory plastic ware and tissue culture plastic was purchased from BDH Merck Ltd, and Eppendorf, Cambridge, U.K.

3MM chromatography Whatman paper and thin layer aluminium chromatography plates were supplied by BDH Merck Ltd.

Disposable sterile filters were purchased from Millipore, Watford, U.K.

Phosphate buffered saline (PBS) was made from PBS tablets (GIBCO BRL), 1 tablet per 500ml ddH_2O, and autoclaved.

All soulutions were made with reverse osmosis (RO) or Millipore water and autoclaved where necessary.

2.2.0 Cell Culture

All cell culture work was carried out under sterile conditions in a laminar flow cabinet. Media and reagents were purchased sterile, autoclaved or filtered with a 0.2μm filter.

2.2.1 Cell lines

Stably transformed rat foetal heart derived BW and Clem cells were a kind gift from G. Engelmann (Engelmann *et al.*, 1993). Cells were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS), at 37°C, 5% CO_2 in a standard tissue culture incubator. Cells were allowed to divide until an approximately 80% confluent monolayer was formed. To detach cells to enable cultures to be split, cells were washed once in, and then incubated for 1 minute in 10% trypsin in versine. FBS was added to the cell suspension in excess to neutralize trypsin and cells were centrifuged at 145g for 5 minutes. The cells were then re-suspended in fresh growth medium and plated on uncoated dishes at approximately $1x10^3$ cells/ml. 24 hours later cells had formed a 50-80% confluent monolayer, media was removed and replaced with DMEM supplemented with 1% FBS. Cells were then ready for experimentation.

ND7 cells were created by the fusion of immortalised HGPRT-mouse neuroblastoma cells (N18Tg2) with rat post-mitotic neonatal dorsal root ganglion neurons (Wood *et al.*, 1990). Cells were grown in Liebovitz L15 medium containing 10% FBS, 100 units/ml penicillin and streptomycin, 0.35% (w/v) glucose, 2mM L-glutamine and 0.375% (w/v) sodium bicarbonate. Cells were maintained at 37°C, 5% CO_2 until 80% confluent. Cells were then passaged by washing in sterile PBS, and dislodging from the dish by a sharp tap while in PBS. Cells were then centrifuged at 145g for 5 minutes and re-suspended in fresh growth medium. Cells were plated at approximately $1x10^3$ cells/ml, 24 hours after plating, media was removed and replaced with growth media supplemented with 1% FBS. Cells were then ready for transfection.

2.2.2 Primary neonatal rat cardiac cell culture:

Neonatal Rat Cardiocyte cultures were prepared as described by Simpson and Savion (Simpson and Savion, 1982b). Hearts from one or more litters of neonatal (1-2 day old) Sprague Dawley rats were removed and trisected into ADS buffer (6.8g NaCl, 4.76g HEPES, 0.12g NaH_2PO_4, 1.0g Glucose, 0.4g KCl, 0.1g $MgSO_4$). The tissue was then dissociated with repeated incubations in an oxygenated enzyme solution (30mgs collagenase, 500 µl pancreatin, in 100mls ADS buffer). Six, 15 minute digestions were carried out with the enzyme solution being removed from the hearts each time, the cells spun down (145g, 5 minutes) and fresh enzyme added to the hearts. Pelleted cells were re-suspended in FBS and pooled. The pooled cells were spun down and re-suspended in plating media (DMEM supplemented with 10% FBS, 1% penicillin and streptomycin). Cells were pre-plated for 45 minutes in uncoated flasks to allow the fibroblasts to stick down. The non-adhered cells were then plated in gelatine coated dishes (1% gelatine / PBS) at approximately $1x10^5$ cells / ml, unless stated otherwise. Cells were incubated for 24 hours in plating media and were then ready to treat or transfect. Cells could be seen beating spontaneously in a confluent monolayer 24 hours after plating. Prior to stimulation medium was changed to maintenance medium (DMEM, 1% FBS, 1% penicillin and streptomycin).

2.2.3 Primary adult rat cardiac cell culture

Primary adult rat cardiac myocytes were prepared from 6 month old female Sprague Dawley rats, as described previously (Lundgren *et al.*, 1984; Piper *et al.*, 1982). Rats were sacrificed by cervical dislocation, hearts removed immediately, and immersed in

buffer A (130mM NaCl, 5mM HEPES, 10mM Glucose, 5.4mM KCl, 3.5 mM $MgCl_2$, 0.4mM NaH_2PO_4) with 5 units/ml heparin. The aorta was identified and mounted on the canula of a Langendorf perfusion apparatus. The heart was retrogradely perfused at 9ml/min with buffer A containing 750μM $CaCl_2$ for 2 minutes, followed by buffer A containing 100μM EGTA for 4 minutes, and buffer A with 0.8mg/ml collagenase typeII and 200μM $CaCl_2$ for 15 minutes. The heart was then taken off the rig, cut up and placed in buffer A with 0.8mg/ml collagenase, 10% BSA and 200μM $CaCl_2$ for 5 minutes at 37°C with agitation. Cells were filtered through a 200μm nylon gauze and re-suspended in buffer A with 10% BSA and 200μM $CaCl_2$. Cells were allowed to settle for 5-10 minutes at 37°C, 5% CO_2, the supernatant was then removed and the wash repeated. Cells were then re-suspended in DMEM with 80μM EGTA, 1% Penicillin/Streptomycin, 1% FBS, and incubated at 37°C with 5% CO_2, for 1 hour on laminin coated plates (10-15μg/ml). The media was then changed and cells were returned to the incubator and treated as required. Cells had a rod like shape with visible myofibrils and could be seen to beat spontaneously.

2.2.4 Stimulation of Cells

Cardiotrophin-1 (CT-1) (mouse), a kind gift from D. Pennica (Genentech Inc, California, USA) was dissolved in sterile PBS and administered to cells at doses varying from 1-50ng/ml for varying times, as stated in the results section.

Phenylephrine (PE) (Sigma) was dissolved in ethanol and used at a dose of 50μM for varying times (see results section).

Urocortin (Rat synthetic, Sigma) was dissolved in ethanol and used at a dose of $1x10^{-7}$M.

The RNA synthesis inhibitor Actinomycin D (Sigma) was dissolved in ethanol and administered to cells at a concentration of 5μg/ml or 25μg/ml for 1 hour prior to stimulation with CT-1.

The Mek-1 inhibitor PD98059 (New England Biolabs, Beverly, Massachusetts, USA.) was dissolved in DMSO and used at a concentration of 50μM.

The P38 MAPK inhibitor SB202190 (Calbiochem-Novabiochem Corp., La Jolla, California, USA.) was dissolved in DMSO and used at a concentration of 20μM. SB202580 is light sensitive so care was taken to avoid exposure to light.

The tyrosine kinase inhibitor wortmannin (Sigma) was dissolved in ethanol and used at a concentration of 50nM, at which it specifically inhibits PI3 kinase.

In experiments where reagents dissolved in ethanol or DMSO were used, the final concentration of DMSO or ethanol added to cells did not exceed 1 in 1000. In each case, the appropriate amount of ethanol or DMSO vehicle was added to control cells.

Heat shock was administered by wrapping the plate of cells in parafilm and floating on a water bath at 43°C for 30 minutes. Pre-warming the medium was found to make no difference when small volumes of media were used. For this reason, the medium was not changed on cells prior to or after heat shock. This enabled other stimuli, such as CT-1 to be present throughout the heat shock. After the heat shock, the parafilm was removed, the plate dried and returned to the incubator.

Hypoxic stress was administered using a hypoxic chamber consisting of a sealed chamber with a constant flow through of 95% Argon 5% CO_2 (BOC special gases), and a constant temperature of 37°C. Cells were covered with a minimal volume of media (0.5ml per well of 6 well plate) and sealed in the chamber for 6 hours. After the hypoxic period, 1.5ml of fresh growth medium was added to each well of cells and the cells were returned to the incubator at 37°C, 5% CO_2.

2.3.0 Transient Transfection

Transient transfections were carried out using the calcium phosphate method. The DNA precipitate was prepared as follows: 100μl 2xHBS (200μl 70mM Na_2HPO_4 added to 10ml 280mM NaCl, 50mM HEPES pH7.1) was added dropwise to 50μl plasmid DNA in water mixed with 50μl $CaCl_2$ and mixed thoroughly. This mixture was left to precipitate for 10-20 minutes at room temperature. 200μl precipate was added to each well (based on 6 well plate), 100μl for 24 well plate and incubated overnight. Cells were washed once with serum free DMEM then 1ml DMEM (1% FCS) was added per well. Cells were incubated for 4-6 hours then treated as required.

Primary neonatal rat cardiac cells were transfected 24 hours after plating while cells were still in plating medium. The transfection mixture was left on the cells for 24 hours, then removed, cells washed with DMEM, and cells were incubated in DMEM supplemented with 1% FBS for 6 hours before treatment.

Cell lines were transfected 20 hours after plating. The media was changed to growth media supplemented with 1% FBS, the transfection mixture was then added and cells incubated for 6 hours. The cells were then washed with serum free medium and incubated in growth media supplemented with 1% FBS for 24 hours before treatment.

DNA plasmids used in transfections are summarised in table 2.3.1. The propagation and isolation of DNA plasmids is described in section 2.4.

Table 2.3.1 DNA Plasmids

Plasmid	**Description**	**Reference / Source**
Hsp90α	-1700 to +40 of the Hsp90α promoter driving expression of CAT reporter gene.	(Hickey *et al.*, 1989)
Hsp90β-A	-1044 to +36 of the hsp90β promoter upstream from CAT reporter gene	(Rebbe *et al.*, 1989)
Hsp90β-C	-299 to +36 of the hsp90β promoter upstream from CAT reporter gene	(Rebbe *et al.*, 1989)
HSE	Heat shock element up stream of a viral thymidine kinase promoter, driving expression of CAT reporter gene	(Stephanou *et al.*, 1999)
PIAS1	Protein inhibitor of activated STAT1 driven by the cmv promoter	(Liu *et al.*, 1998)
PIAS3	Protein inhibitor of activated STAT3 driven by the cmv promoter	(Chung *et al.*, 1997)
dn MEK-1	Dominant negagative MEK-1 driven by cmv promoter	(Lavoie *et al.*, 1996)
Rccmv	Empty vector with cmv promoter but no insert	Invitrogen, 9351 NV Leek, Netherlands.
cmv-β-gal	Lac-z cDNA driven by cmv promoter	(Liu *et al.*, 1998)
pR19-27	Chinese hamster hsp27 cDNA driven by cmv promoter	(Wagstaff, 1997)

Plasmid	Description	Reference / Source
pR19-56	Rabbit hsp56 cDNA driven by cmv promoter	(Wagstaff, 1997)
pR19-70	Human hsp70 cDNA driven by cmv promoter	(Wagstaff, 1997)
pR19-90	Human hsp90β cDNA driven by cmv promoter	(Wagstaff, 1997)
pR19-β-gal	Lac-z cDNA driven by cmv promoter	(Wagstaff, 1997)
pR19-gfp	Green fluorescent protein cDNA driven by cmv promoter	(Wagstaff, 1997)
ANF-luciferase	-3003 to +62 of the rat ANF gene driving expression of a Firefly luciferase reporter gene.	(Knowlton *et al.*, 1991)
TK-*Renilla* luciferase	Viral thymidine kinase (TK) promoter driving expression of *Renilla* luciferase reporter gene.	Promega

2.4.0 Propagation, purification and manipulation of plasmid DNA

All manipulations of bacterial cultures were carried out under sterile conditions. All media and glassware were autoclaved before use at 120°C, 10lb/square inch for 20 minutes and sterile plastic ware was used. Manipulations were carried out under a Bunsen burner flame to prevent contamination.

2.4.1 Transformation of *E.coli*

The *E.coli* strain XL1-Blue was used for the propagation of plasmid DNA. XL1-blues were streaked on a plate of Luria Bertani (LB) Agar (1% (w/v) Bacto®-tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto®-yeast extract, and 2% Bacto®-agar), and incubated at 37°C. 24 hours later a single colony was picked and used to inoculate 5mls of LB medium (1% (w/v) Bacto®-tryptone, 1% (w/v) NaCl, and 0.5% (w/v) Bacto®-yeast extract). This starter culture was grown over night in an orbital shaker at 37°C, 200 rpm.

In order to allow transformation, bacteria must be made competent to allow entry of plasmid DNA. 100µl of starter culture was used to inoculate 100mls of LB and cultured in the orbital shaker until a swirl of bacteria could be seen (4-6 hours). The cultures were then pelleted in sterile 50ml tubes by centrifugation at 2500g at 4°C for 10 minutes. The supernatant was then discarded and the pellets re-suspended in 10ml ice-cold 100mM $CaCl_2$. The bacteria were then centrifuged as above and re-suspended in 2ml ice cold 100mM $CaCl_2$, and incubated on ice for at least 30 minutes prior to use. Competent cells were stored on ice and used within 72 hours or discarded.

For transformation, 200µl of competent cells were aliquoted into 1.5ml sterile microcentrifuge tubes, mixed with approximately 1µl of plasmid DNA and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and cooled on ice for 2 minutes. 800µl of LB medium was added and cells incubated in orbital shaker for one hour. The cells were then centrifuged at 145g, for one minute at 20°C. 900µl of supernatant was discarded and cells re-suspended in the remaining 100µl. The cell suspension was then spread on LB agar plates containing 100µg/ml Ampicillin (All the plasmids used in this study contained an Ampicillin resistance marker gene). Plates were incubated at 37°C over night and then stored for up to one month at 4°C.

2.4.2 Large scale plasmid DNA extraction from *E.coli.*

'Qiagen-tip 100' (Qiagen Ltd, Crawley, U.K.) plasmid midi prep kits were carried out according to manufactures instructions to prepare plasmid DNA for transfections. 200ml of LB medium containing 100μg/ml Ampicillin was inoculated with an E.coli colony containing the plamid of interest, and grown up overnight in an orbital shaker at 37°C, 200 rpm. Isolation of plasmid DNA was achieved by alkaline lysis of cells followed by purification of plasmid on the Qiagen resin, elution and precipitation in isopropanol. Precipitated DNA was centrifuged at 12000g for 30 minutes at 15°C, washed in 70% ethanol, dried and re-suspended in 100μl sterile water. Details of this technique can be found in the Qiagen plasmid purification handbook.

DNA was quantified by spectrophotemetry, reading absorbance at A_{260} and A_{280} wavelengths. Average yields were 100μg DNA per 'tip 100' prep. DNA was stored at –20°C. Concentration and purity of DNA was calculated with the following equations:

1 OD (A_{260}) = 50μg/ml double-stranded DNA
OD260 / OD280 = approximately 1.7 in a pure sample of DNA.

2.4.3 Small scale plasmid DNA extraction from *E.coli.*

Small scale plasmid mini preps were used to purify DNA from several colonies to enable screening for the correct plasmid insert. 5ml of LB media containing 100μg/ml Ampicillin was inoculated with a single colony of transformed XL1-blues, and grown overnight in an orbital shaker at 37°C, 200rpm. 1.5mls of culture was transferred to a 1.5ml microcentrifuge tube and centrifuged at 12000g for 1 minute. The remaining culture was stored at 4°C. Pelleted cells were re-suspended by vortexing in 100μl solution 1 (50mM Tris-HCl pH 7.5, 10mM EDTA pH 8, 100μg/ml RNAse-A). 200μl solution 2 was then added (200mM NaOH, 1% Triton X-100), mixed and 150μl solution 3 added (3M NaOAc pH 5.5). Tubes were vortexed, centrifuged at 12000g for 3 minutes and the pellet removed and discarded by lancing with a hypodermic needle bent at the tip. 500μl isopropanol was added to the supernatant, vortexed and centrifuged for 5 minutes at 12000g. The supernatant was discarded and the pellet washed with 500μl ethanol, air dried and re-suspended in 30μl ddH_20.

2.4.4 Examination of DNA by restriction digests.

Plasmid DNA was digested with restriction enzymes to characterise plasmid structure. 1μg DNA was incubated with 10 units enzyme, 1μl enzyme buffer in a total volume of 10μl made up with ddH_20, at 37°C for 1-2 hours. The enzyme buffer and enzyme concentration varies for different enzymes and was carried out as specified by the Promega catalogue.

Agarose gel electrophoresis was carried out to examine the size of plasmid fragments generated by restriction digests. 1% agarose (w/v) was dissolved in 1xTAE (0.4M Tris, 0.2M sodium acetate, 20mM EDTA, adjusted to pH 8.3 with HCl) and melted in a microwave oven. After cooling for 5-10 minutes, ethidium bromide was added to a final concentration of 0.5μg/ml, swirled and poured into a gel casting tray containing a suitable comb. Once set, the comb was removed and the gel placed in an electrophoresis tank containing 1x TAE. DNA loading buffer 10x stock (1x TAE, 50% (v/v) glycerol, 0.025% (w/v) bromophenol blue) was added to DNA to a final 1x concentration. Samples were loaded along side a marker (1kb ladder, Gibco BRL) and run at 50-100 volts depending on size of gel until the loading dye had travelled a sufficient distance along the gel.

DNA bands were observed on a transilluminator and photographed on black and white Polaroid film.

2.4.5 Isolation of DNA fragments from agarose gels

DNA bands were purified from agarose gels using a 'geneclean II' kit (BIO 101, California, USA) according to manufactures handbook (BIO 101 guide to protocols and procedures). The appropriate band was cut out of the gel on a transilluminator and placed in a 1.5ml microcentrifuge tube. The DNA was bound to a silica resin in a high salt buffer, washed, and eluted from the resin in ddH_20. The DNA could then be used in subsequent procedures without further purification.

2.4.6 Ligation of DNA

In the work presented here, all ligations were 'double sticky ended' i.e. occurred between complementary codons as a result of digestion with restriction endonucleases. The 'sticky ends' were different at either end of the plasmid backbone so phosphatase

treatment was not required to prevent religation of the backbone without the insert. The plasmid backbone was ligated with an excess of insert in a ratio of 1:2, backbone: insert. Ligations were carried out in a total volume of 6µl containing 1x ligase buffer (from 5x stock), 1-3 units of T_4 DNA ligase (Promega), made up to 6µl with ddH_20. The reaction was incubated at 16°C overnight and then transformed as described previously.

2.5.0 Preparation of radiolabelled cDNA probes

cDNA probes were made by cutting the insert out of its respective plasmid by restriction digest, and purifying the DNA band (using gene clean II kit). Radiolabelling was achieved with a random prime labelling reaction, adapted from the method of (Feinberg & Vogelstein, 1983).

When probing RNA filters all solutions were treated with diethyl pyrocarbonate (DEPC) and autoclaved or made fresh with DEPC treated water to avoid contamination with RNAses.

Oligolabelling buffer (OLB) was prepared by mixing solutions A:B:C in the ratio 100:250:150.

Solution O: 1.25M Tris-HCl (pH 8.0), 0.125M $MgCl_2$

Solution A: 1ml Solution O, 18µl β-mercaptoethanol, 5µl 0.1M dATP, 5µl 0.1M dGTP, 5µl 0.1M dTTP.

Solution B: 2M HEPES pH 6.6

Solution C: 90 Units/ml randon hexamers [$pd(N)_6$] dissolved in TE pH 8.

2-3µl of DNA fragment was denatured in 30µl ddH_20 at 97°C for 10 minutes and snap cooled on ice. 10µl OLB, 2µl bovine serum albumin (BSA), 5 units of DNA polymerase large fragment 1 (Klenow), and 50µCi of α-[^{32}P]-dCTP were added to the single stranded DNA and incubated for 1 hour at 37°C. The appropriate safety precautions were taken when handling radioactivity. The probe was then filtered through a G50 Sephadex column by centrifugation for 5 minutes at 400g.

2.5.1 Hybridisation

100x Denhardt's reagent: 2% (w/v) BSA, 2% (w/v) ficoll (type 400), 2% polyvinylpyrrolidone.

20x SSC: 150mM sodium chloride, 15mM sodium citrate, pH 8.

Hybridisation solution: 5 x SSC, 5x Denhardt's 0.5% (w/v) SDS, 100µg/ml denatured herring sperm DNA, made up to volume with ddH_20.

Filters were pre-hybridised in hybridisation solution at 65°C for 1 hour. The hybridisation solution was then replaced with fresh hybridisation solution pre-warmed to 65°C. The labelled probe was denatured by incubation at 97°C for 5 minutes and snap cooled on ice. The denatured radiolabelled probe was then added to the hybridisation solution with the membrane and incubated over night at 65°C in a rotating hybridisation oven. The hybridisation solution was then discarded and the filter washed in 2x SSC, 0.1% SDS in ddH_2O for 15 minutes at 65°C. The membranes were then wrapped in cling film and exposed to film over night. Additional washes and exposures to film were performed where necessary.

2.5.2 Southern Blotting

DNA was digested and run on an agarose gel as described previously. The gel was photographed on a transilluminator and placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 minutes, rinsed in ddH_2O, then placed in neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA) for 30 minutes. The DNA from the gel was transferred to Hybond N+ membrane (Amersham) by capillary action. The DNA was then fixed the membrane by U.V. cross linking. The membrane was then probed with a radiolabelled cDNA probe as described above.

2.6.0 Analysis of RNA levels

When handling RNA precautions were taken to prevent contamination with RNAses. Gloves were worn at all times, solutions were DEPC treated and autoclaved where possible or made up with DEPC treated water. Sterile glass and plastic ware was used

and gel rigs and other equipment cleaned with RNAse Zap (Invitrogen) and rinsed in DEPC treated water.

2.6.1 Extraction of RNA

Cells were washed twice in sterile PBS then all liquid was removed from the culture dish with an aspirator. 500µl of RNAsol (Genyosys) was added per well (based on 6 well plate), cells scraped with a rubber policeman, and transferred to sterile 1.5ml microcentrifuge tubes. Total RNA was isolated according RNAsol manufacturers instructions. Cells were incubated in RNAsol at room temperature for 30 minutes, centrifuged at 12000g for 30 seconds to pellet cellular debris and the supernatant transferred to a sterile 1.5ml microcentrifuge tube. 500µl chloroform was added to the RNAsol solution, vortexed thoroughly, and incubated on ice for 2 minutes. The mixture was then centrifuged at 12000g for 2 minutes at 4°C and the upper layer transferred to a sterile 1.5ml microcentrifuge tube. 500µl of isopropanol was added to the transferred upper layer, mixed, and stored at –80°C over night to allow the RNA to precipitate. The RNA was then pelleted by centrifugation at 12000g for 25 minutes at 4°C. The supernatant was discarded, the pellet washed in 70% ethanol, air dried and re-suspended in 20µl DEPC treated water. RNA samples were stored at –80°C and used within 1 week.

RNA was quantified by measuring absorbance at A_{260} and A_{280} on a spectrophotometer.

2.6.2 Northern blotting

10x MOPS buffer: 120mM MOPS (morpholinopropane sulphonic acid), 30mM sodium acetate, 60mM EDTA, stored in dark.

10µg RNA made up to 6µl was incubated with 12.5µl deionised formamide, 2.5µl 10x MOPS buffer, and 4µl formaldehyde, for 5 minutes at 65°C. The denatured RNA was then chilled on ice and 2.5µl loading dye (50% (v/v glycerol, 0.1mg/ml bromophenol blue) added. Samples were then run on a 1% denaturing agarose gel (1g agarose, 10ml 10x MOPS, 73ml H_2O, 17ml formaldehyde from 37% v/v solution) with 1xMOPS as running buffer. The gel was run at 50 volts until the loading dye was near the end of the gel. The RNA was then transferred to Hybond N+ membrane by capillary action. The

RNA was fixed to the membrane by UV cross linking and then probed with radiolabelled cDNA probes as previously described.

The membrane was stripped after probing by placing it in a solution of 0.5% SDS at 100°C and allowing to cool to room temperature. The membrane could then be re-probed with a cyclophillin cDNA probe to equalize for RNA loading.

2.6.3 Slot blot

10µg RNA was incubated in 3 volumes of denaturing solution (500µl formamide, 163µl formaldehyde (37% solution), 100µl 10x MOPS buffer) at 65°C for 5 minutes. The denatured RNA was then chilled on ice and 1 volume of ice cold 20x SSC added. RNA was then spotted on to Hybond N+ membrane using a slot blotting apparatus, and fixed to the membrane by UV cross linking. The membrane was then probed with a radiolabelled cDNA probe as described.

2.7.0 Analysis of protein levels

2.7.1 Western Blotting

Cells were washed in PBS, harvested by scraping in sample treatment buffer (50mM Tris, 100mM Dithiotheitol, 2% SDS, 0.1% bromohenol blue, 10% glycerol) and heated to 100°C for 5 minutes. Polyacrylamide gel electrophoresis was carried out using a 10% acrylamide gel (10% acrylamide (from 30% acrylamide (w/v), 0.8% bisacrylamide), 375mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% ammonium persulphate, 0.05% NNNN-tetraethylethalinediamine (TEMED)), with a 5% stacking gel (5% acrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED). A molecular weight marker (rainbow marker, Amersham) was run to identify the size of protein bands. The gel was run in Tris-glycine running buffer (25mM Tris, 250mM glycine (pH 8.3), 0.1% SDS) at 30 milli amps for 4-6 hours, or until the dye front was within 3cm of the bottom of the gel. Protein was transferred from the gel to a membrane (High bond C, Amersham) overnight in an electro-blotter (Biorad) in blot buffer (192mM glycine, 20% (v/v) methanol, 25mM Tris-HCl pH 8).

The membrane was then probed on a shaking platform at room temperature as follows: Non-specific sites were blocked by incubation in block buffer (5% Marvel (skimmed

milk powder), 0.1% Tween 20 in PBS) for 1 hour. The primary antibody was then added (diluted in 4% Marvel, 0.1% Tween 20 in PBS) and incubated for 1-2 hours. The membrane was then washed in wash buffer (1% Marvel, 1% Tween 20 in PBS) for 5 minutes, and the wash was repeated. Secondary antibody (HrP conjugate, in 4% Marvel, 0.1% Tween 20 in PBS) was incubated for 1 hour. The membrane was then washed in wash buffer 4 times for 5 minutes per wash and a final 5 minutes wash was carried out in 1% Tween 20 in PBS.

Enhanced Chemiluminescence (ECL, Amersham) was used according to manufacturers instructions to visualise labelled bands by exposure to photographic film. The membrane was stripped in 1M glycine pH 2.9 for 30 minutes and re-probed with an antibody to actin to check for equal protein loading. Bands were quantified by densitometry on a Biorad GS-670 Densitometer.

Antibodies and conditions for use are shown in table 2.7.1.

Table 2.7.1 Antibodies used in Western blotting.

Antibody	Species raised against	Species raised in	Species recognised	Primary antibody dilution	Secondary antibody	Size of band	Source
Hsp90	Achlyia ambisexualis	Mouse monoclonal	Human, mouse, rat, rabbit etc.	1/500	Horseradish peroxidase (HrP) conjugated anti mouse, 1/1000, (DAKO)	90kDa	A kind gift from D.O. Toft, Rochester, USA.
Hps70 inducible	Human	Mouse monoclonal	Human, mouse, rat, etc.	1/1000	Anti-mouse HrP, 1/1000, (DAKO)	70kDa	StressGen Biotechnologies, York, U.K.
Hsp56	Rabbit	Mouse monoclonal	Rabbit, human,	1/1000	Anti-mouse HrP, 1/1000, (DAKO)	59kDa	StressGen Biotechnologies, York, U.K.
Hsp56 UP30	Human	Rabbit polyclonal	Human, rat, etc.	1/200	Anti-rabbit HrP, 1/1000, (DAKO)	59kDa	A kind gift from Dr K. Leach, Upjohn Co., kalamazoo, MI, USA.
Hsp27	Chinese Hamster	Mouse monoclonal	Human, rat, mouse etc.	1/1000	Anti-mouse HrP, 1/1000, (DAKO)	27kDa	A kind gift from J. Landry, Quebec, Canada.
Actin	Human	Goat polyclonal	Human, rat, mouse etc.	1/1000	Anti-goat HrP, 1/1000 (DAKO)	46kDa	Santa Cruz Biotechnology, California, USA.

2.8.0 Assessment of promoter activity

Cells were washed twice in PBS and excess liquid taken off with an aspirator. 100µl of 1x reporter lysis buffer (Promega) was added to each well (based on 6 well plate), cells scraped with a rubber policeman and transferred to sterile 1.5ml microcentrifuge tubes. Samples were incubated at room temperature for 10 minutes to allow lysis of cells, centrifuged at 12000g for 1 minute to pellet cellular debris and the supernatant was transferred to sterile 1.5ml microcentrifuge tubes. Cell extracts were stored at –20°C and used to measure reporter construct activity.

2.8.1 Chloramphenicol acetyl transferase (CAT) assay

Protein was extracted (as above) from cells transfected with CAT reporter constructs. The amount of CAT produced by the reporter gene can be measured by its ability to acetylated [^{14}C]-chloramphenicol and the amount of product determined by thin layer chromatography. CAT assays were carried out according to Invitrogen technical information.

A reaction was set up containing:
- 50µl cell extract
- 2µl 200µCi/ml [^{14}C]-chloramphenicol
- 20µl 40mM acetyl CoA
- 32.5µl 1M Tris-Cl pH 7.5,
- 45.5µl dd$H_2$0.

The reaction was incubated at 37°C for 2 hours, when 1ml ethylacetate was added. The tube was vortexed for 1 minute, centrifuged at 12000g for 1 minute and the top layer transferred to a sterile 1.5ml microcentrifuge tube. The sample was dessicated in a speed vac for 45 minutes and then resuspended in 30µl ethylacetate. The sample was spotted onto the bottom of a thin layer chromatography plate run for 1 hour in 19:1 chloroform: methanol. The plate was then exposed to film for several days. Results were quantified by densitometry and the % acetylated of total chloramphenicol calculated.

2.8.2 β-galactosidase (β-gal) assay

The amount of β-gal enzyme produced from a transfected cmv promoter driven lac-z reporter gene was measured with a Galacto-Light™ assay kit (TROPIX Inc. Massachusetts, USA.) according to manufacturers instructions.

Galacton substrate was diluted 1/100 with Galacto-Light reaction buffer diluent to make reaction buffer. 20µl cell extract was placed in a luminometer tube. 50µl reaction buffer was added to the cell extract, mixed and incubated at room temperature for 30 minutes. 30µl of accelerator was added, mixed, and the sample read on a luminometer for 45 seconds after a 5 second delay. Mock transfected cells were used as a negative control. The amount of light produced by the reaction and measured by the luminometer was proportional to the amount of β-gal enzyme in the sample.

2.8.3 Luciferase assay

The amount of luciferase produced by luciferase reporter constructs was quantified using a dual-luciferase™ reporter assay system (Promega), according to manufacturers instructions. This system quantifies both Firefly and *Renilla* luciferases in the same assay. A constitutively active thymidine kinase (TK) promoter driven *Renilla* luciferase construct was transfected as an internal control along side the experimental promoter driving expression of Firefly luciferase.

20µl cell extract was mixed with 100µl luciferase assay reagent II (LARII) and read for 10 seconds in a luminometer after a 2 second delay. This value represents the amount of Firefly luciferase present in the sample. 100µl Stop & Glow® reagent was then added, vortexed and a second reading taken from the luminometer. The second reading represents the amount of *Renilla* luciferase present in the sample. Each value for Firefly luciferase was then divided by the corresponding value for *Renilla* luciferase to standardise for transfection efficiency and cell number.

2.9.0 Measurement of Hypertrophy

2.9.1 Identification of β-gal positive cells by X-gal Staining

Media was removed from cells and 0.025% glutaraldehyde in PBS was added to fix cells for 10 minutes. Cells were washed twice in PBS and X-gal stain (5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM magnesium chloride and 250µg/ml 5-

Bromo-4-Chloro-3-tridolyl-β-D-galactopyranoside in PBS) was added, cells were stained overnight at 37°C. This stain labels transfected cells with a blue dye. The stain was removed, and 1ml 50% glycerol in PBS added for storage. Stained cells were stored at 4°C.

2.9.2 Staining Cells with Crystal Violet

Media was removed from cells and 0.025% glutaraldehyde in PBS was added to fix cells for 10 minutes. Cells were washed twice in PBS and crystal violet stain (Sigma) 0.1% in PBS was added for 30 minutes. Cells were then washed twice with PBS and 1ml 50% glycerol in PBS was added for storage.

2.9.3 Measurement of Planimetric area

Measurement of cell length and area was carried out as by (Wollert *et al.*, 1996). Cell length was measured as the longest axis of the cell. Cell area was measured by measuring planimetric area, that is the flat surface area of the cell when adhered to the plate. A Leitz Diavert microscope linked by a COHU high performance CCD video camera to a computer captured images of random views of cells. NIH (National institute of health) Image analysis software was used to take measurements from cells. A computer mouse was used to identify the long axis of the cell and to draw around the perimeter of cells so that the area could be calculated. Figures were exported into Microsoft excel where data analysis was carried out.

2.10.0 Assessment of Cell Death

Apoptotic cell death was assessed by TUNEL labelling and total cell death by trypan blue exclusion

2.10.1 TUNEL labelling

Apoptotic nuclei were assessed by end labelling of DNA 3' OH ends with FITC-dUTP as described by Gavrieli et al (Gavrieli *et al.*, 1992). Terminal transferase and Fluorescein-12-2'-deoxy-uridine-5'-triphosphate (FITC-dUTP) were purchased from Roche diagnostics, (Mannheim, Germany) and used according to the manufacturers instructions. Tunel positive cells were identified using a fluorescent microscope under which tunnel positive nuclei fluoresce green. Tunel labelled cells that were also stained blue with X-gal were counted and expressed as a % of the total number of blue stained

cells. Six fields of view were counted per treatment and the mean % of apoptotic-transfected cells was calculated.

2.10.2 Trypan blue exclusion

Cells were detached from the tissue culture plate by incubating in 10% trypsin in versine for 2 minutes at 37°C. When cells detached from the plate, 1ml foetal bovine serum was added per well to neutralize the trypsin. The cells were then transferred to 1.5ml microcentrifuge tubes and centrifuged at 145g for 2 minutes. The supernatant was discarded and the pellet resuspended in 500µl PBS and stored on ice. 20µl of cell suspension was then mixed with 20µl Trypan blue (0.4% solution, Sigma) and drawn onto a haemocytometer by capillary action. Cells were observed under phase contrast microscopy and blue cells, which had taken up dye were scored as dead and white cells which had not taken up dye were score as live. At least 3 separate counts of at least 100 cells each were made and the mean % of live cells was calculated.

2.11.0 Viral Infection

The viruses used in this study are Herpes simplex type 1 viral vectors, which were made by Marcus Wagstaff as part of a PhD project in the laboratory of Dr Rob Coffin. The viruses were made from the 17+ HSV-1 virus deleted for the essential gene ICP27 to make them unable to replicate in non-complementing cell lines. Transgene expression was driven by the human cytomeglovirus immediate early (cmv-IE) promoter, which is inserted into the virus immediately down stream of the viral LAT P1 and LAT P2 promoters. The viral LAT P1 and P2 promoters drive long-term transgene expression during viral latency.

All work involving viruses was carried out under Health and Safety Executive category 2 conditions.

Viral stocks were kindly grown up, purified and titrated by Dr Jill Smith. Cells to be infected with virus were incubated in 0.5ml DMEM containing 1% FBS for 2 hours prior to infection. Virus was then added to the cells at a multiplicity of infection (MOI) of 1, that is 1 plaque-forming unit per cell, and incubated for 1 hour. 1.5ml of full growth media was then over laid on to the cells and they were returned to the incubator for a further 5 hours. The media was then removed and 2mls of DMEM containing 1%

FBS added to cells. The cells were returned to the incubator overnight, and then harvested, or observed under a fluorescent microscope for green fluorescent protein expression.

2.12.0 Stastistical Analysis

Where only two comparisons were required in a data set, student's t-tests were used to test for significant difference between means with a p value of less than 0.05 taken as significant. Two sample t-tests assuming unequal variance were performed using Microsoft Excel.

Where multiple comparisons were required between treatment groups, single factor analysis of variance (ANOVA) was used to look for differences between treatments. Single factor ANOVA was carried out using Microsoft Excel and a p value of less than 0.05 was taken as significant.

When an ANOVA showed significant differences between groups, Bonferroni tests were performed post-hoc to test for significant difference between specific treatments. Significance was determined at the level of $p<0.05$. T-tests are not recommended for multiple comparisons as the risk of obtaining a type I error (i.e. falsely rejecting the null hypothesis) is increased as more tests are performed. The Bonferroni test is a modified t-test where the p value is multiplied by the number of tests performed on the same data. This makes the chance of achieving significance less likely, and thus reduces the chance of type I errors.

When the data cannot be assumed to follow a normal distribution or when the n number is less than 10, non-parametric tests must be used. In chapter 3, comparisons are made between scanned values from western blots. The n numbers are low and the analysis is comparing pairs of data, i.e. treated and untreated. Wilcoxon signed rank tests are used because this test is non-parametric and is designed to test whether there is an increase or decrease in each pair of data. Tests were carried out by hand according to (Ostle, 1963).

CHAPTER 3

THE EFFECT OF CT-1 ON HSP EXPRESSION AND INTERACTION WITH STRESSFUL STIMULI

3.1.0 Introduction

Cardiotrophin-1 (CT-1) has well documented protective effects in the heart (Sheng *et al.*, 1996; Sheng *et al.*, 1997). CT-1 has also been shown to increase the level of cardioprotective heat shock proteins (hsps) in the heart (Stephanou *et al.*, 1998a). In a clinical setting the heart would be subject to stress such as ischaemia reperfusion injury. It is therefore important to know how the heart responds to CT-1 in the presence of stressful stimuli. It is also of interest to discover whether the cardioprotective effects of CT-1 are mediated by hsp induction. In view of the known cardioprotective role of hsps in the heart, an investigation of the mechanisms involved in hsp induction by CT-1 may yield novel ways of increasing hsp levels in the heart.

3.1.1 CT-1 increases hsp90 level in BW and Clem cells.

In initial studies cardiac cell lines were used to investigate the effect of CT-1 on hsp levels. Cardiac cell lines are a useful model as they are cheap, readily available, easy to culture in large quantities, and do not require animals to be sacrificed. BW and Clem cells are derived from foetal rat cardiac myocytes and are immortalized so can be maintained in culture (Engelmann *et al.*, 1993).

In order to observe the effect of CT-1 treatment on hsp levels in BW and Clem cells, cells were either left untreated, treated with 2ng/ml CT-1 or 10ng/ml CT-1 for 24 hours. Cells were then harvested and extracts subjected to western blotting. CT-1 caused an increase in hsp90 levels in both BW and Clem cells (figure 3.1.1). No hsp70 or hsp27 was detected in untreated or treated cells.

To compare the response of BW and Clem cells to CT-1, cells were treated with different doses of CT-1 in parallel. Clem cells showed increased hsp90 in response to 0.5ng/ml CT-1 while BW cells did not respond to this dose. In BW cells, a dose of 2ng/ml CT-1 was required to increase hsp90 levels. 10ng/ml CT-1 was sufficient to cause a maximal increase of hsp90 in both BW and Clem cells. No hsp70 was produced in response to CT-1 at any of the doses tested (Figure 3.1.2).

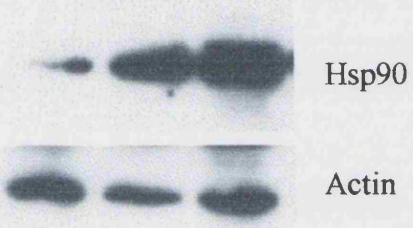
Results shown are representative western blots from at least 3 separate experiments showing similar results. Clem cells were more sensitive to CT-1 than BW cells and produced a more consistent induction of hsp90 levels. Western blots from at least 4

experiments in Clem cells were quantified by densitometry and the mean fold induction calculated. This data is summarised in table 3.3.1.

Figure 3.1.1 The effect of CT-1 treatment on hsp90 levels in BW and Clem cells.

BW Cells

CT-1: - 2ng 10ng



Clem Cells

CT-1: - 2ng 10ng

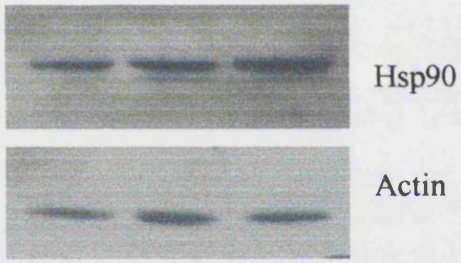


Figure 3.1.1

BW and Clem cells were either left untreated (-) or treated with CT-1 2ng/ml or 10ng/ml for 24 hours. Cells were then harvested and extracts subjected to western blotting. Membranes were probed with antibodies to hsp90, hsp70, hsp27 and actin. Membranes were stripped before each re-probe. No hsp70 or hsp27 was detected on these blots. CT-1 caused a dose dependent increase in hsp90 in BW and Clem cells.

Figure 3.1.2 CT-1 dose response.

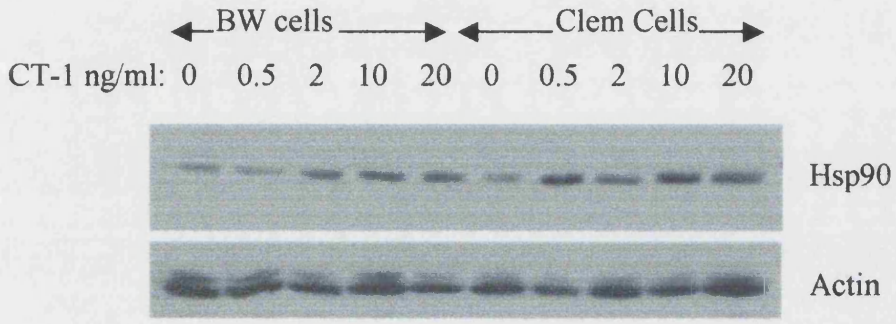


Figure 3.1.2.

BW cells and Clem cells were left untreated (0) or treated with CT-1 at 0.5ng/ml, 2ng/ml, 10ng/ml, or 20ng/ml for 24 hours. Cells were then harvested and subjected to western blotting. Membranes were probed with hsp90 and hsp70 antibodies then stripped and reprobed for actin. CT-1 caused a dose dependent increase in hsp90 levels. No hsp70 was detected.

3.1.2 CT-1 Protects BW and Clem cells against lethal heat shock

Due to the known protective role of hsp induction, it was necessary to discover whether BW or Clem cells could be protected by CT-1 under the same conditions where the level of hsp90 was increased. Thus, Clem or BW cells were left untreated or treated with CT-1 10ng/ml. 24 hours later cells were either maintained at 37°C or subjected to heat shock at 43°C for 2 hours. Following heat shock cells were trypsinised to detach them from culture dishes, pelleted and resuspended in PBS. Live and dead cells were then counted, based on the live cells ability to exclude trypan blue.

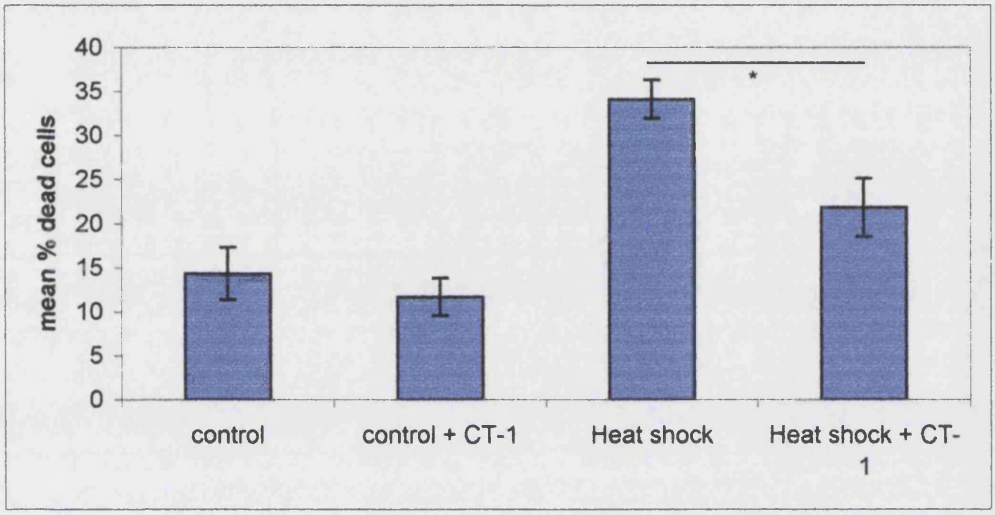
In BW cells CT-1 treatment caused a slight, but not significant reduction in the percentage of dead cells in the absence of heat shock. There were 14% dead in untreated cells compared to 12% dead in CT-1 treated cells. When cells were subjected to a lethal heat shock, CT-1 pre-treatment caused a significant reduction in cell death from 34% without CT-1 to 22% with CT-1 (t-test, $p=0.02$) (figure 3.1.3).

A similar pattern was seen in Clem cells. No significant difference was observed in percentage cell death in unstressed cells, with untreated cells having a mean of 8% dead and CT-1 treated 6% dead. When cells were subjected to lethal heat shock, 46% of cells were dead and this was significantly reduced to 28% by CT-1 treatment (t-test, $p=0.002$) (figure 3.1.3).

Clem cells were protected more than BW cells by the same dose of CT-1. This could be because Clem cells are more sensitive to CT-1 (as in the dose response in figure 3.1.2). Additionally there was more death in the Clem cells in response to heat shock so there would be more capacity for protection.

Figure 3.1.3 CT-1 protects BW and Clem cells against heat stress.

BW cells



Clem Cells

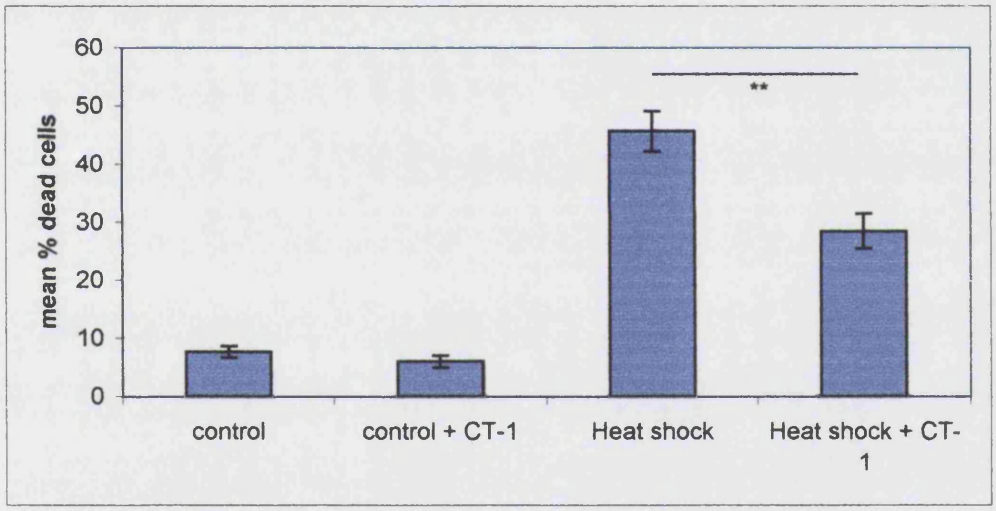


Figure 3.1.3 BW and Clem cells were left untreated (control) or treated with CT-1 10ng/ml for 24 hours. Cells were then either maintained at 37°C or subjected to heat shock at 43°C for 2 hours. Directly after heat shock all cells were harvested by gentle trypsinisation, resuspended in PBS and counted in trypan blue. At least 6 fields of view were counted per condition, mean % dead cells calculated and plotted +/- standard error. Students t-tests were performed to test for significant differences between treated and untreated cells. CT-1 treatment caused a significant reduction in cell death in the presence of heat shock ($p=0.02$ BW and $p=0.002$ Clem).

3.2.0 The effect of CT-1 treatment on hsp induction by heat shock

Due to the finding that CT-1 increases levels of hsp90 in cardiac cells and protects these cells against lethal heat shock, it was necessary to investigate the effect of CT-1 on hsp levels in conjunction with stressful stimuli. This is important, as if CT-1 were to be used as a cardioprotective agent in the clinical setting, the heart would already be subject to stressful stimuli such as ischaemia.

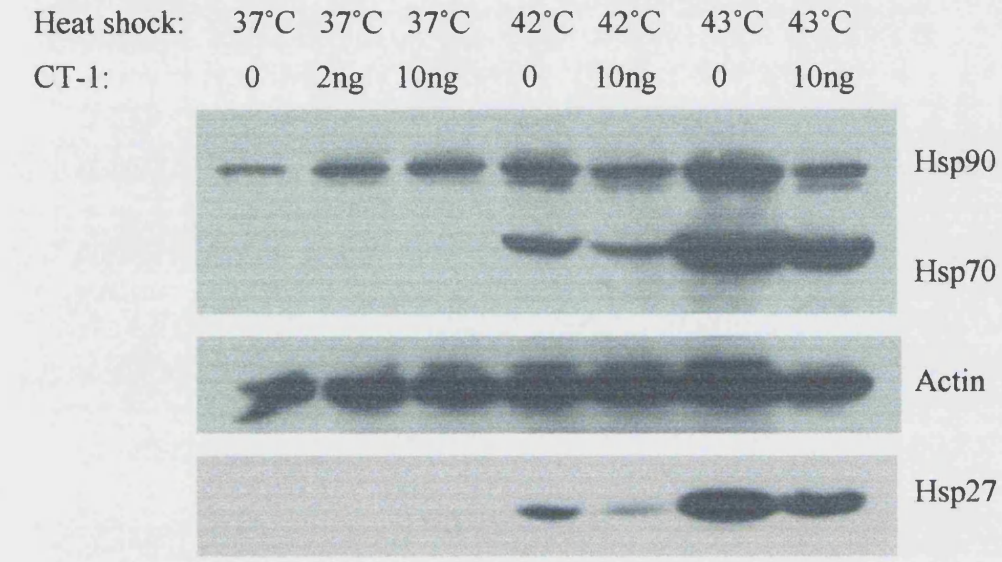
BW and Clem cells were left untreated, or treated with CT-1 at 2ng/ml or 10ng/ml. Six hours later cells were either maintained at 37°C, heat shocked at 42°C for 30 minutes or heat shocked at 43°C for 30 minutes. Cells were returned to 37°C incubator for 18 hours and then harvested for western blotting. Membranes were probed with hsp90 and hsp70 antibodies then stripped and reprobed for hsp27. Membranes were stripped again and re-probed with an antibody for actin.

An increase in hsp90 levels was observed in response to CT-1 treatment in unstressed cells. Heat shock caused a temperature dependent increase in hsp90, hsp70 and hsp27 levels. Pre-treatment with CT-1 reduced the amount of hsp90, hsp70 and hsp27 produced in response to heat shock at both temperatures (Figure 3.2.1). In BW cells, hsp70 and hsp27 expression in response to heat shock was completely abolished by CT-1 pre-treatment. This effect was less dramatic in Clem cells but a consistent reduction in hsp levels in response to heat shock in the presence of CT-1 was still observed (Figure 3.2.1).

These findings reveal a novel antagonistic effect of CT-1 on stress induced hsp production. These results also imply that the protective effect of CT-1 is not due to an increase in hsp90 levels.

Figure 3.2.1 The effect of CT-1 on stress induced hsp production.

Clem Cells



BW cells

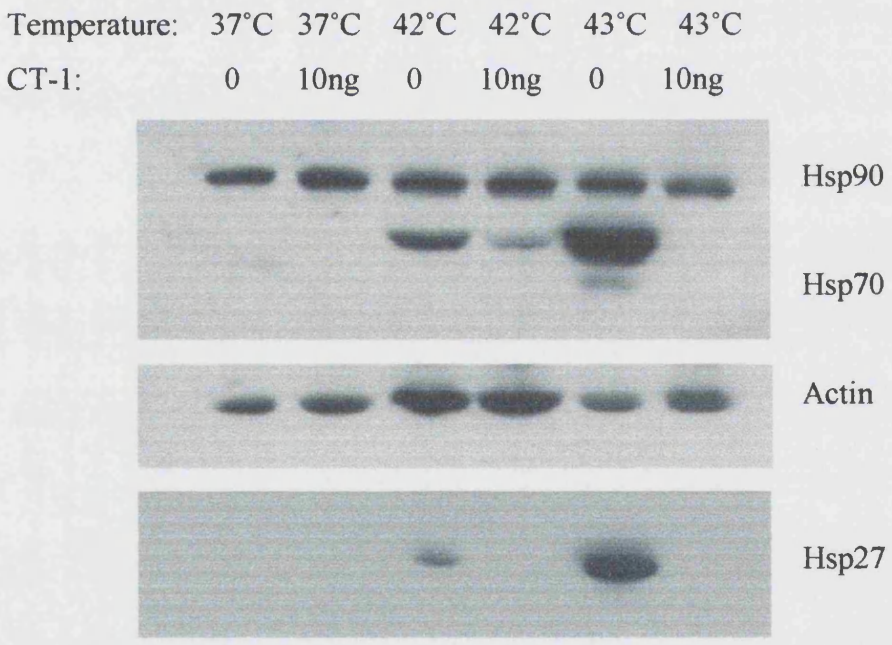


Figure 3.2.1 BW and Clem cells were left untreated or treated with CT-1 at 2ng/ml or 10ng/ml for 6 hours. Cells were then incubated at 37°C or heat shocked at 42°C or 43°C for 30 minutes. 18 hours later cells were harvested for western blotting. CT-1 pre-treatment reduced heat induced hsp90, hsp70 and hsp27 levels.

3.2.2 Effects of CT-1 in primary rat cardiac myocytes

The use of cell lines can sometimes be unreliable due to changing characteristics of cells in long term culture. For this reason, the results presented so far were confirmed by experiments in primary neonatal rat cardiac myocyte cultures (primary cells), which are a well established model system for the heart.

Primary neonatal rat cardiac myocytes were prepared (see methods) and maintained in maintenance medium for 48 hours. Cells were then left untreated or treated with CT-1 at 5ng/ml for 6 hours. Cells were then maintained at 37°C or heat shocked at 43°C for 30 minutes. Cells were then returned to the incubator for 18 hours and then harvested for western blotting.

CT-1 treatment caused an increase in hsp90 and hsp70 levels. Low levels of hsp70 could be detected in untreated cells. This contrasts with observations in BW and Clem cells where no hsp70 was detected in response to CT-1 and hsp70 could only be detected in heat shocked cells. This slight elevation of hsp70 in primary cells may be due to the fact that cells may have been stressed during their isolation from the heart. The increase seen in hsp90 levels supports that seen in BW and Clem cells but is not as dramatic. This could be due to the high basal level of hsp90 in unstimulated cells (Figure 3.2.2).

Heat shock caused a large increase in hsp70 levels but only a small increase in hsp90 levels. Pre-treatment with CT-1 reduced the amount of hsp90 and hsp70 produced in response to heat shock (Figure 3.2.2). This supports the results for BW and Clem cells and again indicates an antagonistic response of CT-1 on heat induced hsp90 and hsp70 production.

Western blots presented here are representatives of at least 3 experiments showing similar results. Western blots were quantified by densitometry and the mean fold induction presented in figure 3.3.1.

Figure 3.2.2. The effect of CT-1 on stress induced hsp production in primary cells

Primary neonatal rat cardiac myocytes.

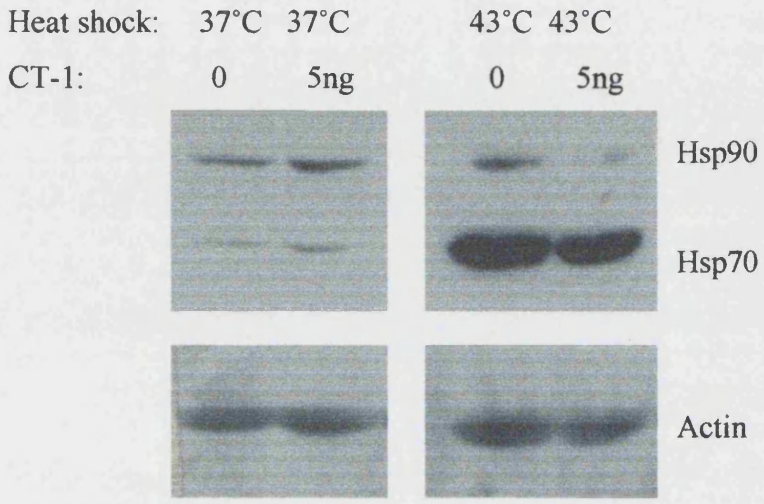


Figure 3.2.2 Primary neonatal rat cardiac myocytes were either left untreated (0), or treated with CT-1 at 5ng/ml for 6 hours. Cells were then maintained at 37°C or heat shocked at 43°C for 30 minutes. Cells were subsequently returned to the incubator for 18 hours and then harvested for western blotting. Membranes were probed with antibodies for hsp90 and hsp70, then stripped and re-probed for actin. CT-1 caused an increase in hsp70 and hsp90 levels. Pre-treatment with CT-1 reduced the amount of hsp90 and hsp70 produced in response to heat shock.

3.3.0 Comparison of effects of CT-1 and heat shock

Results presented as western blots in the previous sections are representatives from at least four experiments. In this section several experiments in Clem and primary cells were quantified by densitometric scanning and the fold induction of hsp90 in response to CT-1 treatment was calculated. Hsp70 expression was not detected in Clem cells in response to CT-1 treatment and rarely seen in primary cells. For this reason, hsp70 levels were only compared in the presence of stressful stimuli.

Experiments were carried out as described in sections 3.2.1 and 3.2.2 and western blots from at least four experiments were quantified by densitometry. Representative western blots were shown in sections 3.2.1 and 3.2.2.

Wilcoxon's signed rank tests were used to test for significant differences between pairs of data. This test was used as it looks for an increase or decrease in response to treatment but is not affected by the variablility in the amount of increase or decrease. This is important, as results obtained on western blots can be difficult to compare directly between blots, as the intensity of a band depends on many factors such as duration of film exposure, efficiency of transfer etc. These variables, however, do not affect comparisons within the same blot and a degree of standardization can be achieved using the actin band.

CT-1 treatment of CLEM cells caused a 4 fold (mean, $n=4$, $p<0.05$) induction in hsp90 levels. Heat shock for 30 mins at 43°C caused a 20 fold (mean, $n=4$, $p<0.05$) induction in hsp90 levels. When cells were pre-treated with CT-1, the induction in hsp90 levels caused by heat shock at 43°C for 30 mins was reduced from 20 fold (mean, $n=4$, $p<0.05$) with heat shock alone to 6 fold (mean, $n=4$) above control when pre-treated with CT-1. Hsp70 protein was only detected in cells subjected to stressful stimuli. Pre-treatment with CT-1 reduced the amount of hsp70 detected after heat stress to half the amount seen with heat shock alone (mean fold induction = 0.5, $n=6$, $p<0.05$) (Table 3.3.1).

A 2.9 fold (mean, $n=4$, $p<0.05$) increase in hsp90 levels was seen in CT-1 treated primary cardiac myocytes compared to untreated cells. Heat shock caused a 4.0 fold

(mean, n=4, $p<0.05$) increase in hsp90 levels above control. To investigate the effect of CT-1 in the presence of a heat stress, cells were pre-treated with CT-1 before heat shock; here the induction was reduced from 4.0 fold to 3.0 fold above control. A small increase in hsp70 levels could be detected in primary cells in response to CT-1 treatment in some cases. In untreated cells, hsp70 levels increase dramatically in response to heat shock. As is the case for hsp90, reduced induction of hsp70 was observed with CT-1 and heat shock compared to heat shock alone. When pre-treated with CT-1, the amount of hsp70 detected after heat shock was reduced by half (mean fold induction=0.5, n=7, $p<0.05$) (Table 3.3.1).

These results demonstrate that pre-treatment with CT-1 causes a reduction in the amount of hsp70 and hsp90 produced in response to heat stress.

Table 3.3.1. The effect of CT-1 and heat shock on hsp90 and hsp70 levels

Mean fold induction over control

Cell Type	Heat Shock Protein	Control	CT-1	Heat Shock	CT-1 + Heat Shock
CLEM (n=4)	90	1.0	4.0	20.0	6.0
CLEM (n=6)	70			1.0	0.5
Primary (n=4)	90	1.0	2.9	4.0	3.0
Primary (n=7)	70			1.0	0.5

Table 3.3.1.

Western blots probed with hsp70 and hsp90, then re-probed with actin were quantified using densitometry. The value for the hsp was divided by the corresponding actin band to equalise for protein loading. To calculate the fold induction, the equalised value for each treatment was divided by the control (untreated) value. The mean fold induction was then calculated from several experiments. No hsp70 was detected in unstressed cells, the fold induction for hsp70 was calculated by dividing the value for CT-1 + heat shock by that for heat shock alone.

3.4.0 Time course of hsp expression in response to CT-1 or Heat shock

In order to further investigate the induction of hsp90 by CT-1 and by heat shock, time course experiments were carried out.

Primary cells were left untreated, or treated with CT-1 10ng/ml for 10 minutes, 30 minutes, 1 hour, or 48 hours. Cells were then harvested and extracts subjected to western blotting. Hsp90 levels were slightly elevated after 10 minutes and reached maximal levels after 30 minutes. Increased hsp90 was still observed 48 hours after treatment with CT-1. Hsp70 was not detected in any treatment (Figure 3.4.1).

To compare the time course of heat shock protein induction after heat shock, primary cells were maintained at 37°C or heat shocked at 43°C for 30 minutes. Cells were harvested directly after heat shock, after 1 hour, 2 hours, 3 hours, 5 hours, 18 hours or 24 hours. Extracts were then subjected to western blotting. Hsp90 levels were increased after 10 minutes and reached maximal levels after 3 hours. Hsp90 levels began to fall after 18 hours but were still slightly elevated at 24 hours. Hsp70 was not detected until 2 hours after heat shock and reached maximal levels after 3 hours. Levels of hsp70 reduced after 18 hours but a considerable amount was still present after 24 hours (Figure 3.4.2).

This shows that hsp90 levels are increased rapidly in response to CT-1 or heat shock and the elevation of hsp90 in response to CT-1 is more sustained than that produced by heat shock. Production of hsp70 in response to heat shock is a slower process, taking 2 hours but lasting for over 24 hours.

Figure 3.4.1 Time course of hsp90 expression after CT-1 treatment.

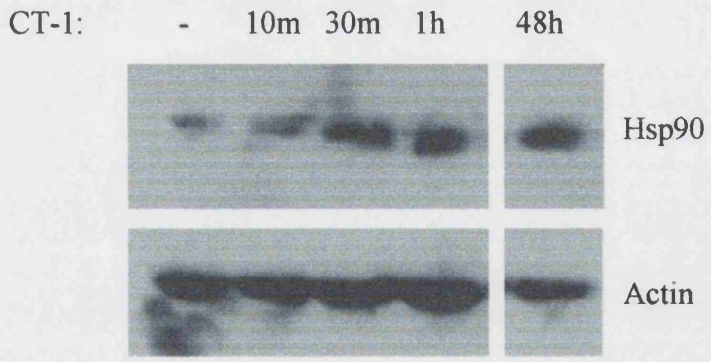


Figure 3.4.1

Primary neonatal rat cardiac myocytes were left untreated, or treated with 10ng/ml CT-1 and harvested after 10 minutes (10m), 30 minutes (30m), one hour (1h) or 48 hours (48h). Extracts were subjected to western blotting and probed with hsp90 and hsp70, then stripped and re-probed for actin. Hsp90 levels were increased after 10 minutes and reached a maximal level after 30 minutes. Elevated hsp90 was still observed after 48 hours. No hsp70 was detected.

Figure 3.4.2 Time course of hsp90 and hsp70 expression after heat shock.

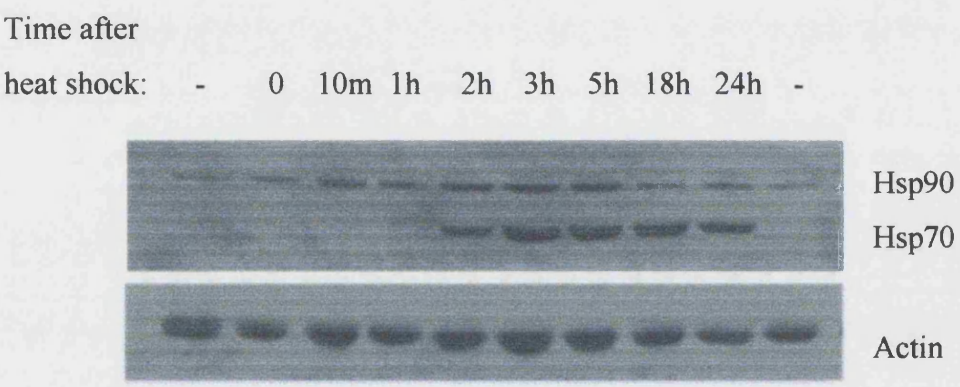


Figure 3.4.2 Primary neonatal rat cardiac myoctes were incubated at 37°C (-) or heat shocked at 43°C for 30 minutes. Cells were then returned to the incubator and harvested; immediately (0), after 10 minutes (10m), after 1 hour (1h), after 2 hours (2h), after 3 hours (3h), after 5 hours (5h), after 18 hours (18h), or after 24 hours (24h). Extracts were subjected to western blotting and probed for hsp90 and hsp70, then stripped and reprobed for actin. Hsp90 levels were elevated 10 minutes after heat shock, reached maximal levels after 3 hours and declined after 18 hours. Hsp70 was detected 2 hours after heat shock, reached maximal levels after 3 hours and had declined slightly but was still present after 24 hours.

3.5.0 CT-1 increases hsp90 levels independently from transcription.

Following the observed rapid increase in hsp90 levels in response to CT-1 or heat shock it was necessary to determine whether this increase was occurring at the transcriptional level. This was done using the RNA synthesis inhibitor actinomycin D to block transcription.

Primary cells were left untreated or treated with actinomycin D at 5μg/ml or 25μg/ml. One hour later some cells were treated with CT-1 at 10ng/ml. One hour later untreated or CT-1 treated cells were maintained at 37°C or heat shocked at 43°C for 30 minutes. Cells were harvested 5hrs later and extracts subjected to western blotting.

CT-1 caused an increase in hsp90 levels, which was reduced, but not abolished by actinomycin D. Actinomycin D reduced the basal level of hsp90, but there was still an increase in hsp90 in response to CT-1. Hsp90 and hsp70 levels in heat shocked cells were reduced by pre-treatment with CT-1 and completely abolished by actinomycin D (Figure 3.5.1). This shows that CT-1 is increasing hsp90 at least partly by a post-transcriptional mechanism, which is resistant to actinomycin D. Heat shock, however is acting entirely at the transcriptional level to increase hsp90 and hsp70.

Figure 3.5.1 CT-1 mediated hsp90 induction is independent of transcription but hsp90 and hsp70 induction by heat stress requires transcription.

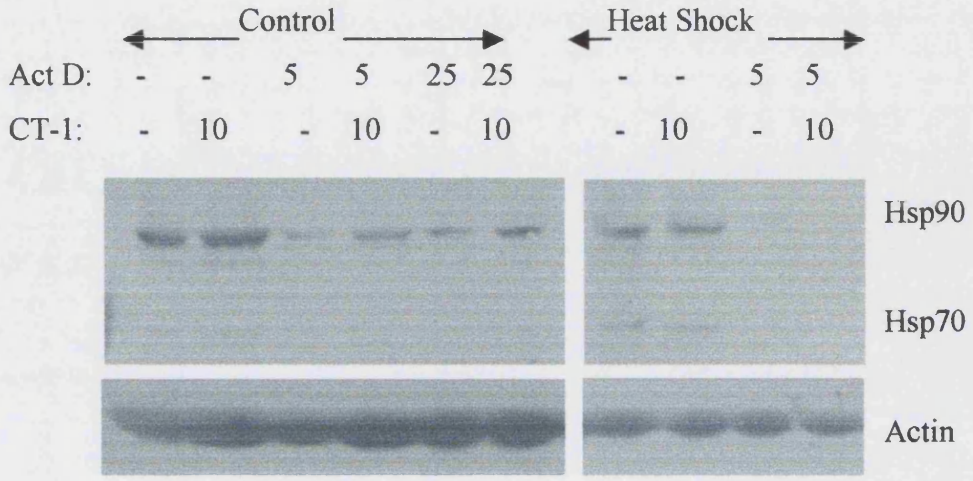


Figure 3.5.1 Primary cells were left untreated or treated with Actinomycin D at 5µg/ml or 25µg/ml. One hour later some cells were treated with CT-1 at 10ng/ml. One hour later, untreated or CT-1 treated cells were maintained at 37°C or heat shocked at 43°C for 30 minutes. Cells were harvested 5hrs later and extracts subjected to western blotting. The membrane was probed with hsp90 and hsp70, then stripped and re-probed for actin. CT-1 was still able to produce an increase in hsp90 in the presence of acinomycin D, while heat shock was not. The heat shocked samples appear to have less hsp90 than non-heat shocked samples. This is due to lower levels of total protein in the heat shocked lanes (see actin blot).

3.6.0 CT-1 does not activate the hsp90 promoter

In the light of results demonstrating that CT-1 increases levels of hsp90, the hsp90 promoter was studied for CT-1 responsiveness. This is important, as even though a post-transcriptional mechanism has been implicated, this may not be totally responsible for the increase in hsp90 seen with CT-1.

Hsp90α and hsp90β reporter constructs were transfected into primary cells (see methods for information on reporter constructs). Cells were then left untreated or stimulated with CT-1 for 6 hours and then maintained at 37°C or heat shocked at 43°C for 30 minutes. 18 hours later cells were harvested and CAT assays were performed to measure promoter activity. Cells were co-transfected with a cytomeglovirus (cmv) promoter driven β-galactosidase expression vector to allow correction for transfection efficiency.

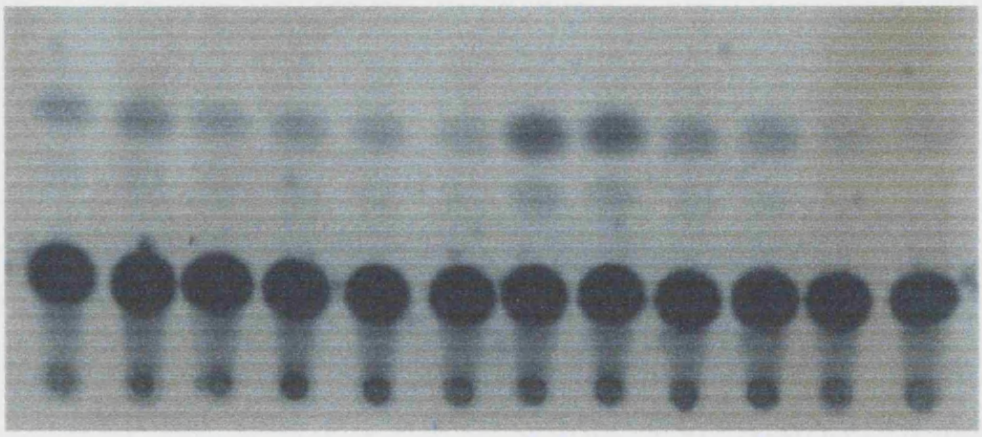
No consistent activation of any promoter construct tested was detected in response to CT-1. The hsp90α and hsp90β-B reporter constructs were activated by heat shock but not by CT-1. The hsp90β-C construct is truncated and does not contain any heat shock elements, as expected, it did not respond to CT-1 or heat shock (Figure 3.6.1). The increase in activity of the hsp90α promoter in response to heat shock was greater than that of the hsp90β promoter. Hsp90α is known to be more inducible than hsp90β with hsp90β being expressed at greater levels constitutively (Csermely *et al.*, 1998). CT-1 pre-treatment slightly decreased the amount of hsp90α promoter activity after heat shock.

A reporter construct containing a heat shock element driving expression of a CAT reporter gene was also tested for responses to CT-1 or heat shock. CT-1 was unable to activate this construct despite strong activation by heat shock (Figure 3.6.1).

These results show that CT-1 was unable to activate the hsp90α or hsp90β promoter constructs tested but these constructs were activated by heat shock. An antagonistic effect of CT-1 pre-treatment on hsp90α activity in response to heat shock was also seen. This shows that the antagonistic effect of CT-1 on hsp90 production by heat shock may be occurring at the transcriptional level.

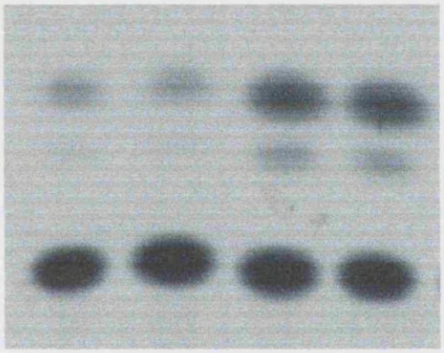
β-galactosidase values were approximately equal in each experiment showing that the transfection efficiency was constant (data not shown).

Figure 3.6.1 The effect of CT-1 on the hsp90 promoter.



Promoter:	Hsp90α		Hsp90β-B		Hsp90β-C		Hsp90α		Hsp90β-B		Hsp90β-C	
CT-1:	-	10	-	10	-	10	-	10	-	10	-	10
Heat shock:	-	-	-	-	-	-	43°	43°	43°	43°	43°	43°

Heat Shock element



Promoter:	← HSE →			
CT-1:	-	10	-	10
Heat Shock:	-	-	43°	43°

Figure 3.6.1

Primary cells were transfected with the following reporter constructs: hsp90α, hsp90β-B (full length), hsp90β-C (truncated), or heat shock element (HSE). In all cases a β-galactosidase expression vector was co-transfected to allow measurement of transfection efficiency. Cells were then left untreated (-) or treated with CT-1 10ng/ml for 24 hours. 6 hours after addition of CT-1 some cells were heat shocked at 43°C for 30 minutes and then returned to the incubator for 18 hours. Cells were then harvested and promoter activities assessed by CAT assay. A β-galactosidase assay was also performed to check for variations in transfection efficiency. β-galactosidase values were constant in each experiment.

3.6.3 CT-1 does not activate the hsp90 promoter, despite increasing hsp90 protein levels in the same extracts.

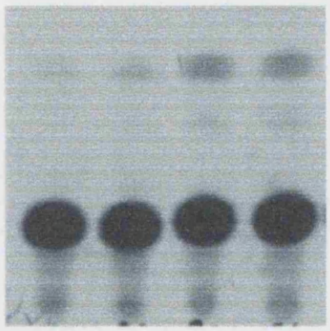
It was possible that the inability of CT-1 to activate the hsp90 promoter was due to reduced activity of CT-1 in transfected cells. To investigate this, primary cells were transfected with hsp90 reporter constructs and stimulated with CT-1 as described in 3.6.2. A CAT assay was performed to assess promoter activity and western blots were carried out on the same cell extracts to monitor hsp90 protein levels.

As reported in section 3.6.2, CT-1 did not activate the hsp90α or hsp90β promoter, but elevated levels of hsp90 protein were detected in response to CT-1 in the same extracts (Figure 3.6.3). This shows that hsp90 is increasing at the protein level without a corresponding increase in promoter activity in response to CT-1. It is possible, however that CT-1 responsive sites may be located outside of the promoter constructs used here.

These results, together with data showing that the increase in hsp90 in response to CT-1 is resistant to actinomycin D, imply that CT-1 is acting post-transcriptionally to increase hsp90 levels.

Figure 3.6.3 CT-1 increases hsp90 protein levels but not promoter activity

CAT assay



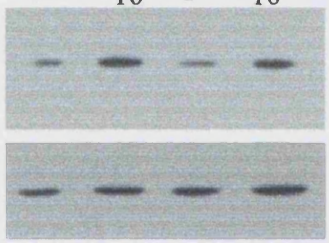
Promoter: Hsp90α | Hsp90β-β

CT-1: - 10 - 10

Western Blot

Promoter: Hsp90α | Hsp90β-β

CT-1: - 10 - 10



Hsp90

Actin

Figure 3.6.3 Primary cells were transfected with hsp90α or hsp90β-B reporter constructs and co-transfected with a β-galactosidase expression vector. Cells were left untreated or treated with CT-1 at 10ng/ml for 24 hours. Cells were then harvested and extracts subjected to CAT assay, western blot and β-galactosidase assay. The CAT assay showed that neither promoter construct was activated by CT-1. β-galactosidase values remained constant showing equal transfection efficiency. The western blot showed that hsp90 levels increased in response to CT-1 treatment.

3.7.0 The effect of CT-1 on hsp mRNA levels

Northern blots were carried out to study the effect of CT-1 on hsp mRNA levels. Primary cells were left untreated or treated with 10ng/ml CT-1 for 6 hours, some cells were then heat shocked at 43°C for 30 minutes. Cells were harvested 18 hours later and RNA isolated for Northern Blotting.

No increase in hsp90 or hsp70 mRNA levels was detected in response to CT-1 treatment. A large increase in both hsp70 and hsp90 mRNA levels was observed in heat shocked cells. Treatment with CT-1 prior to heat shock slightly decreased the levels of hsp70 and hsp90 produced in response to heat shock (Figure 3.7.1). This is difficult to see for hsp70 due to the intensity of the bands.

These results show that CT-1 does not increase hsp90 mRNA levels after 24 hours of treatment despite a clear induction of hsp90 protein (Figure 3.2.2) at this time. This could be because the hsp90 mRNA level increases rapidly after CT-1 treatment and has dropped back to basal levels after 24 hours. This will be investigated with a short time point in the next section. Alternatively, the lack of an increase in hsp90 mRNA level in response to CT-1 may be because CT-1 is acting via a post-transcriptional mechanism to increase hsp90 levels. Such a mechanism could involve increasing the translational efficiency of hsp90 mRNA already present in the cell, or increasing the stability of the hsp90 protein.

3.7.1 Effect of CT-1 on hsp mRNA levels

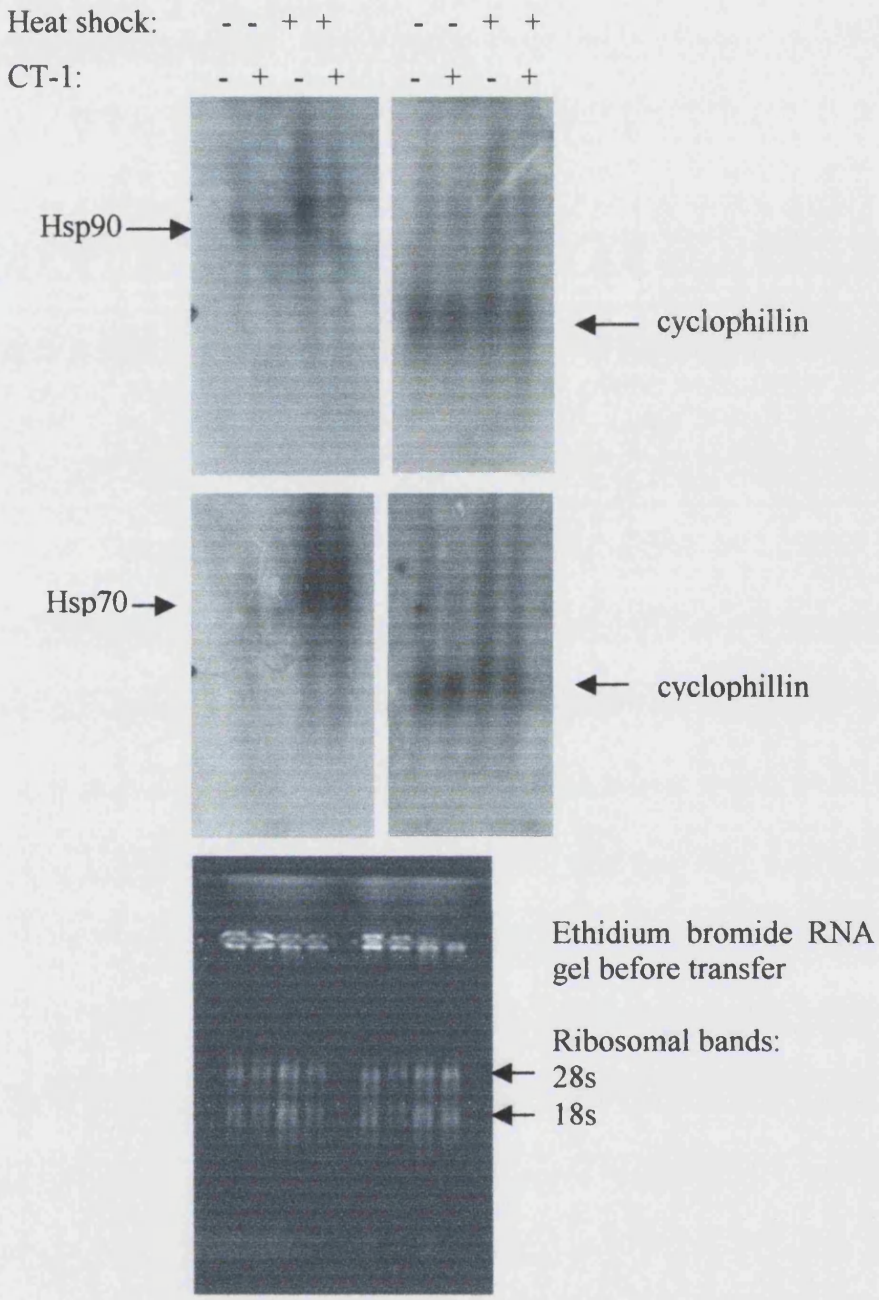


Figure 3.7.1 Primary cells were either left untreated (-) or treated with 10ng/ml CT-1 for 6 hours (+), some cells were then heat shocked at 43°C for 30 minutes (+). Cells were harvested 18 hours later and RNA isolated for Northern Blotting. RNA gels were run in duplicate, photographed, transferred and probed for hsp70 and hsp90. Membranes were then stripped and reprobed for cyclophillin to check for equal loading.

3.8.0 RNA Slot Blot

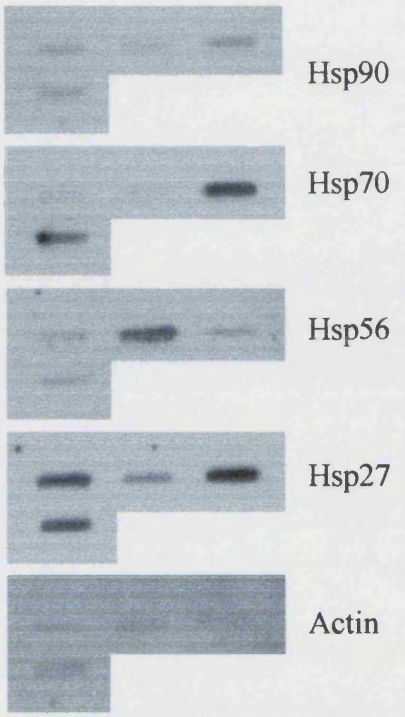
To further investigate how CT-1 mediates its effects on hsp levels, mRNA levels were monitored by RNA slot blot. In this experiment a shorter time period after the stimuli was used in case any changes in mRNA level were no longer visable after 24 hours. Primary cardiocytes were either left untreated, treated with 10ng/ml CT-1 for 1 hour, heat shocked at 43°C for 30 minutes and allowed to recover for 15 minutes or treated with CT-1 for 15 minutes, heat shocked at 43°C for 30 minutes and allowed to recover for 15 minutes. Isolated RNA was applied to membranes with a slot blotting apparatus and fixed by UV cross-linking, RNA was probed with hsp90, hsp70 hsp56, hsp27, or actin cDNA probes.

Hsp90 and hsp70 mRNA levels did not change in CT-1 treated cells compared with untreated cells. In contrast, a large increase was seen in both hsp90 and hsp70 mRNA levels in heat shocked cells (Figure 3.8.1). As already shown in figure 3.4.1, a large increase in hsp90 protein can be detected following CT-1 treatment under the same conditions used here, when no increase in hsp90 mRNA is seen. This implies that the increase in protein is not entirely due to enhanced transcription in response to CT-1, as this would give rise to an increase in mRNA. The level of hsp27 mRNA decreased in response to CT-1 treatment, but increased in response to heat shock. A large increase in hsp56 mRNA was observed in CT-1 treated cells but no change was seen in response to heat shock. In cells pre-treated with CT-1 and subsequently heat shocked, less hsp90, hsp70 and hsp27 mRNA was seen than with heat shock alone (Figure 3.8.1). This agrees with results demonstrated at the protein level, and suggests that the antagonistic effect of CT-1 on heat shock is likely to be occurring at the transcriptional level, and certainly at a different level to the induction of hsp90 by CT-1.

These results show that CT-1 does not increase hsp90, hsp70 or hsp27 mRNA levels under these conditions. Surprisingly, a large increase in hsp56 mRNA was observed in response to CT-1 treatment.

This experiment was repeated twice with the same result.

Figure 3.8.1 RNA Slot blot.



Each band represents the following treatment:

Control | CT-1 | HS | CT-1 + HS

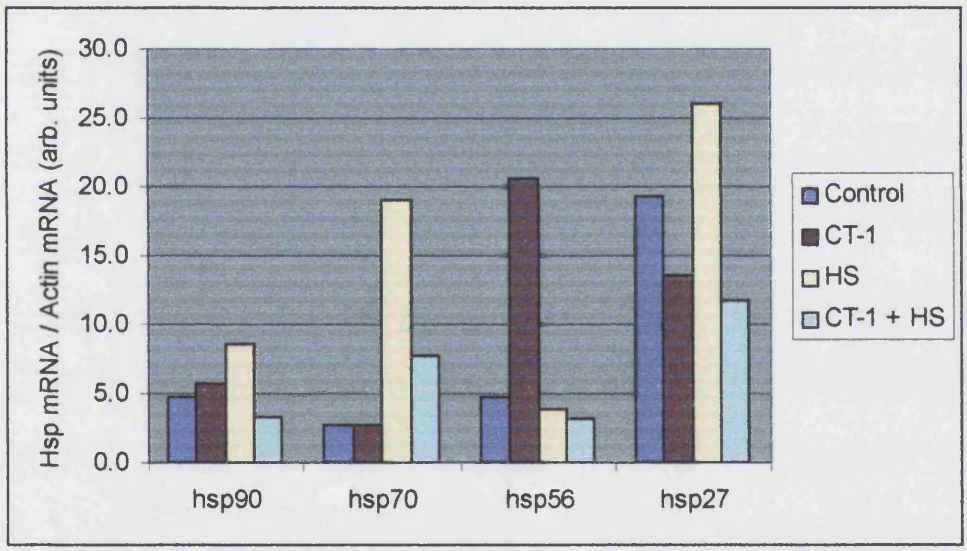


Figure 3.8.1 Primary cardiac myocytes were either left untreated, treated with 10ng/ml CT-1 for 1 hour, heat shocked at 43°C for 30 minutes and allowed to recover for 15 minutes or treated with CT-1 for 15 minutes, heat shocked at 43°C for 30 minutes and allowed to recover for 15 minutes. Isolated RNA was applied to membranes with a Slot blotting apparatus and fixed by UV crosslinking, RNA was probed with hsp27, hsp56, hsp70, hsp90 or actin cDNA probes. Bands were quantified using densitometry and each band was divided by its corresponding actin value, and plotted on a graph.

3.8.2 CT-1 causes an increase in hsp56 mRNA and Protein

It was shown in figure 3.8.1 that CT-1 treatment caused a large increase in hsp56 mRNA level in an RNA slot blot. It was therefore necessary to investigate whether CT-1 treatment increased hsp56 protein levels. In order to do this, an antibody (UP30 serum) that recognised the rat form of hsp56 was obtained, a generous gift from Karen Leach (Pharmacia Inc.). The Corticotrophin releasing hormone (CRH) family hormone urocortin was also investigated for any effect on hsp56 levels, as like CT-1, urocortin is known to increase hsp90 protein levels (Brar *et al.*, unpublished observations).

Neonatal rat cardiac myocytes were left untreated or treated with CT-1 (10ng/ml) or urocortin ($1x10^{-7}M$) for 1 minute, 10 minutes, 30 minutes, 1 hour, 2 hours, 6 hours, or 24 hours. Cells were then harvested and extracts subjected to western blotting. Membranes were probed with UP30 rabbit serum (for hsp56), then stripped and re-probed for actin. The doublet of bands shown for hsp56 is in accordance with results found previously, and may represent isoforms of hsp56 (Ruff *et al.*, 1992).

Hsp56 levels started to increase after 2 hours of CT-1 treatment and further increased after 6 and 24 hours treatment (Figure 3.8.2). This shows that CT-1 treatment causes an increase in hsp56 levels and supports the increase in hsp56 mRNA observed previously (Figure 3.8.1).

Treatment with urocortin caused an increase in hsp56 levels after 2 hours, levels further increased to a maximal level at 2 hours after treatment and then slightly decreased after 6 and 24 hours (Figure 3.8.2). This shows that treatment with urocortin causes an increase in hsp56 levels which is more rapid and less sustained than that caused by CT-1 treatment.

Basal levels of hsp56 appear higher in the western blot with urocortin treatment than CT-1 treatment. This is due to the exposure time of the film on the western blot as the samples contained the same amount of total cell extract.

Figure 3.8.2 CT-1 and urocortin increase hsp56 protein levels.

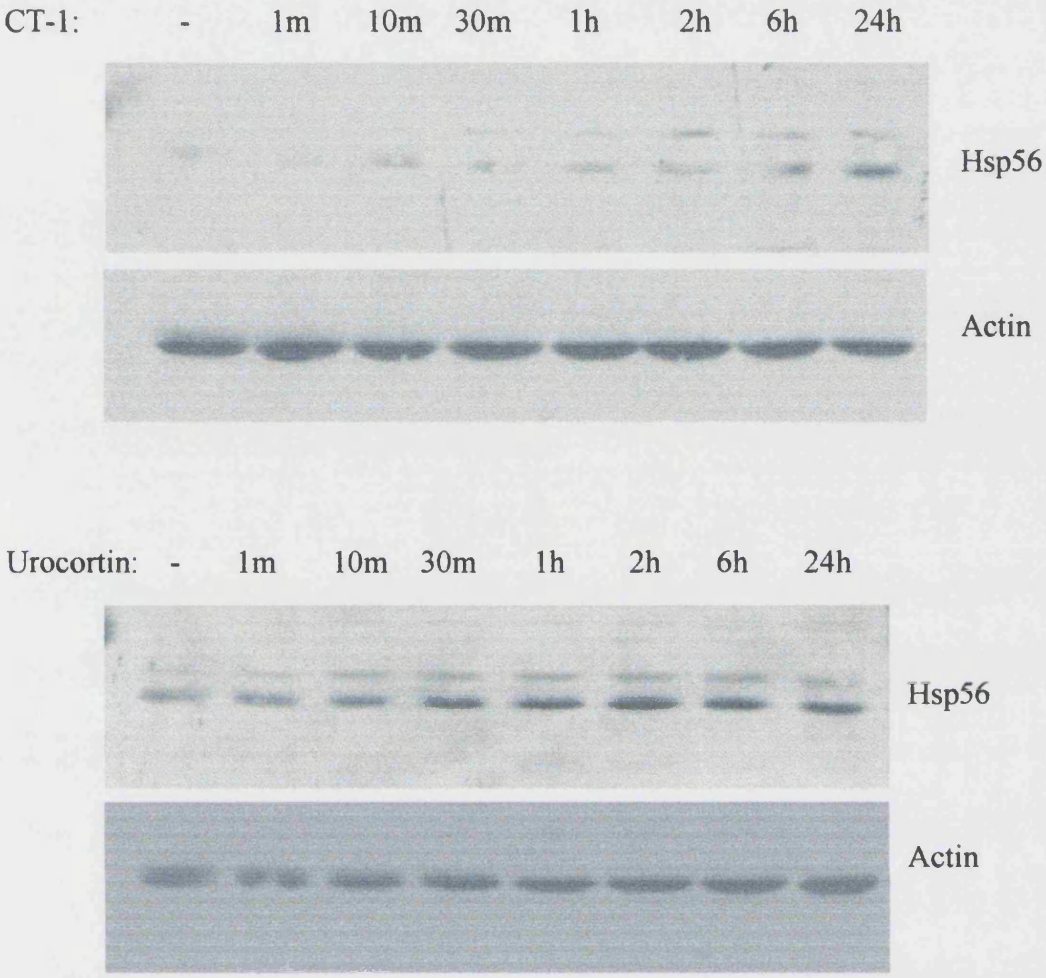


Figure 3.8.2 Neonatal rat cardiac myocytes were left untreated (-) or treated with CT-1 (10ng/ml) or urocortin ($1x10^{-7}$M) for 1 minute (1m), 10 minutes (10m), 30 minutes (30m), 1 hour (1h), 2 hours (2h), 6 hours (6h), or 24 hours (24h). Cells were then harvested and extracts subjected to western blotting. Membranes were probed with UP30 rabbit serum (anti- hsp56), then stripped and re-probed for actin.

3.9.0 Discussion

The data presented in this chapter show that CT-1 treatment combined with heat shock causes a reduction in hsp90 levels below that seen with heat shock alone. When CT-1 is administered in the absence of heat shock, hsp90 levels are rapidly elevated at the protein level, but no increase can be detected at the promoter or mRNA level. Furthermore, actinomycin D failed to block the increase in hsp90 in response to CT-1, indicating that the increase in protein is not due to an increase in transcription of the gene. Together, these results indicate that the increase in hsp90 in response to CT-1 observed previously (Stephanou *et al.*, 1998a) is occurring at the post-transcriptional level. The antagonism between CT-1 and heat shock on hsp27, hsp70 and hsp90 levels can be demonstrated at both the protein and mRNA levels, so is likely to be occurring at the transcriptional level. Promoter studies also showed a slight reduction in hsp90α promoter activity in cells treated with CT-1 and subsequently heat shocked compared to untreated heat shocked cells. This also supports a transcriptional mechanism for the antagonistic effect of CT-1 on hsp90 production after heat shock. In order to confirm these findings it would be necessary to perform nuclear run on analysis to directly measure the rate of transcription of the hsp90 genes in the presence and absence of CT-1 and / or heat shock. Nuclear run on analysis was attempted in this study but the results remained inconclusive. Further attempts at this complex technique should be made in future studies to clarify the involvement of transcriptional regulation in the induction of hsp90 by CT-1 and heat shock.

IL-6, which shares the common receptor component gp130 with CT-1, is known to increase hsp90 levels and to activate the hsp90β promoter. This activation is mediated by STAT3 and Nuclear Factor- IL-6 (NFIL6) transcription factors binding to sequences overlapping heat shock elements (HSE) in the hsp90β promoter (Stephanou *et al.*, 1997). One of the many characterised effects of the p42/p44 MAPK pathway is the activation of NFIL6 (Nakajima *et al.*, 1993). CT-1 is known to activate the p42/p44MAPK pathway and to phosphorylate STAT3 (Robledo *et al.*, 1997b). It is therefore possible that CT-1 could increase hsp90 levels by the same mechanism as IL-6. The results presented here make this unlikely, as CT-1 failed to activate hsp90β promoter constructs that are readily activated by IL-6 (Stephanou *et al.*, 1997; Sheng *et al.*, 1997). Additionally, induction of hsp90 by STAT3 and NFIL-6 would be a

transcriptional event that would be blocked by actinomycin D, which failed to block induction of hsp90 by CT-1.

In the experiments presented here, heat shock is used as a stressful stimulus to cause induction of hsps. The mechanism for this induction is known to be the activation of Heat Shock Factor 1 (HSF-1), a transcription factor that is present in the cytoplasm as an inactive monomer. On activation it forms an active trimer, which translocates to the nucleus and binds to HSEs in hsp promoters causing transcription of these genes (Sarge *et al.*, 1993). NFIL6 acts synergistically with HSF-1 to increase hsp90 gene transcription, conversely, STAT3 is antagonistic with HSF-1, causing a reduction in transcription compared with the effect of either stimulus alone (Stephanou *et al.*, 1998b).

A possible mechanism for the reduction in hsp production seen in cells pre-treated with CT-1 and subsequently heat shocked, would therefore be antagonism between STAT3 and HSF-1 (Stephanou *et al.*, 1998b). STAT3 and HSF-1 both bind to the same region in the hsp gene promoters and when activated simultaneously, have been shown to have an antagonistic effect on transcription of the gene (Stephanou *et al.*, 1998b). In cells subjected to stressful stimuli, HSF-1 is activated. When STAT3 is also activated as a result of CT-1 stimulation, the two transcription factors may compete for DNA binding, resulting in less activation by HSF-1. This hypothesis could explain the reduction in heat inducibility of hsp90 observed following treatment with CT-1.

The RNA synthesis inhibitor Actinomycin D did not block the CT-1 mediated increase in hsp90 levels. Actinomycin D did, however, abolish the heat induced increase in hsp90 levels and reduces constitutive expression. Furthermore, no activation of the hsp90 promoter could be detected in response to CT-1 while clear activation was seen in response to heat shock. This confirms that stressful stimuli activate transcription of hsp genes and that constitutive expression of hsp90 is under transcriptional control. These results further indicate that CT-1 may be acting at least partly through a post-transcriptional mechanism to increase hsp90 levels. Thus CT-1 alone would enhance hsp levels post-transcriptionally but transcriptional antagonism would explain the inhibitory interaction between CT-1 and heat shock.

Post-transcriptional regulation could occur at several levels. Modulation of mRNA stability, translational initiation and elongation, as well as post-translational mechanisms such as protein folding, maturation and stability can all be involved in regulation of protein levels.

There are a number of examples of mRNA stability as an important factor in hsp regulation: Hsp70 mRNA has a short half life in unstressed cells and this is increased by up to ten times in heat shocked cells (Theodorakis & Morimoto, 1987). This shows that post-transcriptional regulation is also involved in stress induced hsp production, although the initial induction is dependent on transcription (Panniers, 1994). Additionally, regulation of hsp30 genes occurs partly by modulation of mRNA stability in Xenopus embryos, resulting in varying inducibility of different isoforms in different stages of development (Ohan & Heikkila, 1995). Long term elevation of hsp expression in response to chronic hyperthermia is due to continued translation of stable hsp messages and occurs independently of transcription. These hsp messages are degraded on return to normal temperatures (DiDomenico *et al.*, 1982). There are also many examples of non heat shock genes regulated by mRNA stability. Vascular endothelial growth factor (VEGF) levels are increased by hypoxia, this increase is mediated by an increase in the half life of its mRNA from 30-45 minutes up to 6-8 hours (Shima *et al.*, 1995). Elevated calcium inhibits IL-1 induced nitric oxide release by reducing iNOS (nitric oxide synthase) mRNA stability (Geng & Lotz, 1995). This is an example of negative regulation of a protein by a reduction in mRNA stability.

mRNA stability has not been directly investigated in this study, however, an increase in mRNA level would be expected in response to CT-1 treatment if this was the mechanism for the induction of hsp90. No increase in hsp90 mRNA was observed in response to CT-1 treatment but it is possible that the techniques used here were not quantitative enough to measure a small change, or that an increase would be observed at time points other than those observed here. An assessment of hsp90 mRNA half life in the presence and absence of CT-1 would be required to determine whether CT-1 affects RNA stability.

In addition to changes in mRNA stability, translational or post-translational regulation may be involved in CT-1 mediated hsp90 induction. In contrast to other hsp genes, the

genes for hsp90α and hsp90β contain introns. mRNA splicing occurs prior to translation and this could be a possible point of regulation, but this is yet to be investigated. If hsp90 were regulated at the level of mRNA splicing, an accumulation of un-spliced mRNA would be expected under conditions where hsp90 protein levels are low. The northern blot presented in figure 3.7.1 shows a slightly larger band for hsp90 mRNA in heat shocked cells but no larger bands in non heat shocked, CT-1 treated or untreated cells. The larger band seen after heat shock may represent hsp90 mRNA that has been transcribed in response to heat shock but has not yet been spliced.

Hsp90 proteins are phosphorylated and form dimers as a pre-requisite to function (Minami *et al.*, 1994). The regulation of phosphorylation and dimerization is poorly understood, but it is possible that monomeric hsp90 is unstable so dimerization may be an important step in the regulation of hsp90 levels. The final point for regulation of hsp90 levels is protein stability and the rate of degradation. Chicken hsp23 can be increased in the absence of transcription in response to heat shock. An increase in the half life of hsp23 protein from 2 hours in unstressed cells to 13 hours in heat shocked cells suggests that an increase in protein stability is responsible for the induction of hsp23 by heat shock (Edington & Hightower, 1990). Hsp70 levels are elevated in patients with Systemic Lupus Erythematosus and this elevation of hsp70 cannot be correlated with increased transcription or increased mRNA levels (Twomey *et al.*, 1993). This is an example where translational or post-translational mechanisms are likely to be responsible for elevated hsp70 levels. Further work will be required to identify the exact mechanism involved in the induction of hsp90 by CT-1 and it is likely that several regulatory steps are involved.

These results are important as hsps have well documented cardioprotective effects but there are few ways to induce hsps, which do not themselves, cause damage to the cell. CT-1 is a cardioprotective agent and it is thought that the induction of hsps may contribute to this protective effect (Stephanou *et al.*, 1998a). However, when administered in combination with a stressful stimulus, CT-1 attenuates the heat shock response. When cardioprotection is studied *in-vitro*, the protective agent is administered a given time before a lethal stress is administered. According to these results, treatment with CT-1 before a stressful stimulus would result in less hsps being produced than with the stressful stimuli alone. This suggests that the protective effect

of CT-1 may not be due to induction of hsp90. It is unlikely, however, that hsps induced by a stressful stimulus protect against that stressful stimulus, as they are not produced quickly enough. Induction of hsp90 by CT-1 prior to a stressful stimulus may be one of the mechanisms by which CT-1 protects cardiac myocytes. The reduction in stress induced hsp production caused by CT-1 pre-treatment is unlikely to affect the protective effect of hsps present before the stress. Hsps have many protective functions and their induction by a seemingly novel mechanism could prove important in future work.

The antagonistic effect of CT-1 on the heat shock response is an important finding as it is the first time that a cytokine has been shown to have an inhibitory effect on the heat shock response. In addition to having important protective functions, heat shock proteins are elevated in several diseases and in such cases are thought to have a deleterious effect. Examples include rheumatoid arthritis (Schett *et al.*, 1998) and systemic lupus erythematosus (Dhillon *et al.*, 1993; Stephanou *et al.*, 1998c) where elevated levels of hsps are thought to be correlated with disease severity. In rheumatoid arthritis the increase in hsp90 is thought to be due to an increase in HSF-1 (Schett *et al.*, 1998), here, a treatment that could antagonise the induction of hsps by HSF-1 could be of therapeutic interest.

A large, and unexpected increase in hsp56 mRNA was observed in response to CT-1 treatment. This increase was confirmed at the protein level and urocortin was also shown to cause an elevation of hsp56. The induction of hsp56 by CT-1 is rapid and is likely to be occurring at the transcriptional level due to the fact that increased mRNA can be observed. It would be interesting to test the hsp56 promoter for responsiveness to CT-1 but this construct is not currently available. A possible role for hsp56 in the hypertrophic response to CT-1 will be discussed in chapter 6.

CHAPTER 4

DEVELOPMENT OF A METHOD TO ALLOW INVESTIGATION OF THE SIGNALLING PATHWAYS INVOLVED IN THE HYPERTROPHIC EFFECT OF CT-1.

4.1.0 Introduction

CT-1 was isolated as a cardiac hypertrophic agent (Pennica *et al.* 1995a) and since then a number of studies have investigated its hypertrophic response. CT-1 is said to cause a type of hypertrophy resembling that seen in volume overload with increased myocyte planimetric area attributable to an increase in cell length (Wollert *et al.*, 1996). The increase in cell length in response to CT-1 treatment is due to accumulation of sarcomeres in series, and this sarcomeric organisation is also seen in volume overload hypertrophy. Other hypertrophic agents such as phenylephrine cause hypertrophy by an overall increase in cell size without accumulation of sarcomeres, or a specific increase in cell length (Wollert *et al.*, 1996).

The aim of this study is to identify the signalling pathways involved in the hypertrophic effect of CT-1. In order to do this, a technique must be established to effectively measure the hypertrophic effect of CT-1 in primary neonatal rat cardiac myocytes. To allow genetic manipulation of signalling pathways, the specific measurement of transfected cells should be made possible.

CT-1 is known to increase ANF secretion from cardiac myocytes (Pennica *et al.* 1995a; Wollert *et al.*, 1996), regulation of the ANF promoter will also be investigated as a possible parameter for the measurement of hypertrophy in response to CT-1.

4.2.1 Measurement of thinly plated cells

It was thought that accurate measurement of cell size would be best achieved on thinly plated cells where the perimeter of each cell can be clearly identified. A study by Wollert *et al.* showed that CT-1 caused a significant increase in cell length but no significant increase in cell width (Wollert *et al.*, 1996). Thus cell length was measured to quantify the hypertrophic effect of CT-1 in cardiac myocytes. Cells were fixed, and stained with crystal violet to increase contrast and allow identification of the edge of the cell. Primary neonatal rat cardiac myocytes (primary cells) were used as they do not divide in culture after the first 24 hours. It is important to use non-dividing cells for the measurement of hypertrophy as a hypertrophic stimulus may cause a non-terminally differentiated cell to divide instead of undergo hypertrophy.

Primary cells were plated at $1x10^3$ cells/ml in 6 well plates. 48 hours after plating cells were left untreated, or treated with CT-1 at 10ng/ml. Cells were returned to the incubator for a further 48 hours then fixed and stained with crystal violet. Cell length was then measured by identifying and measuring the longest axis of the cell. At least 50 cells were measured from random fields of view and mean cell length determined.

The mean cell length in control cells was 70.3μm, which increased to 74.4μm in CT-1 treated cells. This increase was not significant in a t-test ($p=0.23$). The increase in cell length observed after CT-1 treatment was much smaller than expected and was not significant, even when over 100 cells were measured per treatment.

It was observed that thinly plated cells seemed larger than cells in a confluent monolayer. For this reason an experiment was carried out to determine the effect of plating density on cell size with the hypothesis being that thinly plated cells may have already undergone hypertrophy, and therefore did not respond significantly to CT-1.

Primary cells were plated at $5x10^3$, $1x10^4$, or $5x10^4$ cells per ml in 6 well plates. After 3 days cells were fixed, stained with crystal violet and the cell length of at least 50 cells per condition measured. One way ANOVA was carried out and showed significant differences in mean cell length at different plating densities ($p=0.004$). Post-hoc Bonferroni tests were carried out to test for significant differences between particular plating densities.

The mean cell length of cells plated at $5x10^3$ cells/ml was 74.9μm which decreased to 68.1μm in cells plated at $1x10^4$ cells/ml (p=0.1), and further decreased to 63.4μm in cells plated at $5x10^4$ cells/ml (p=0.001) (Figure 4.2.1). P values are for Bonferroni tests versus cells plated at $5x10^3$ cells/ml.

Figure 4.2.1 The effect of cell plating density on cell length

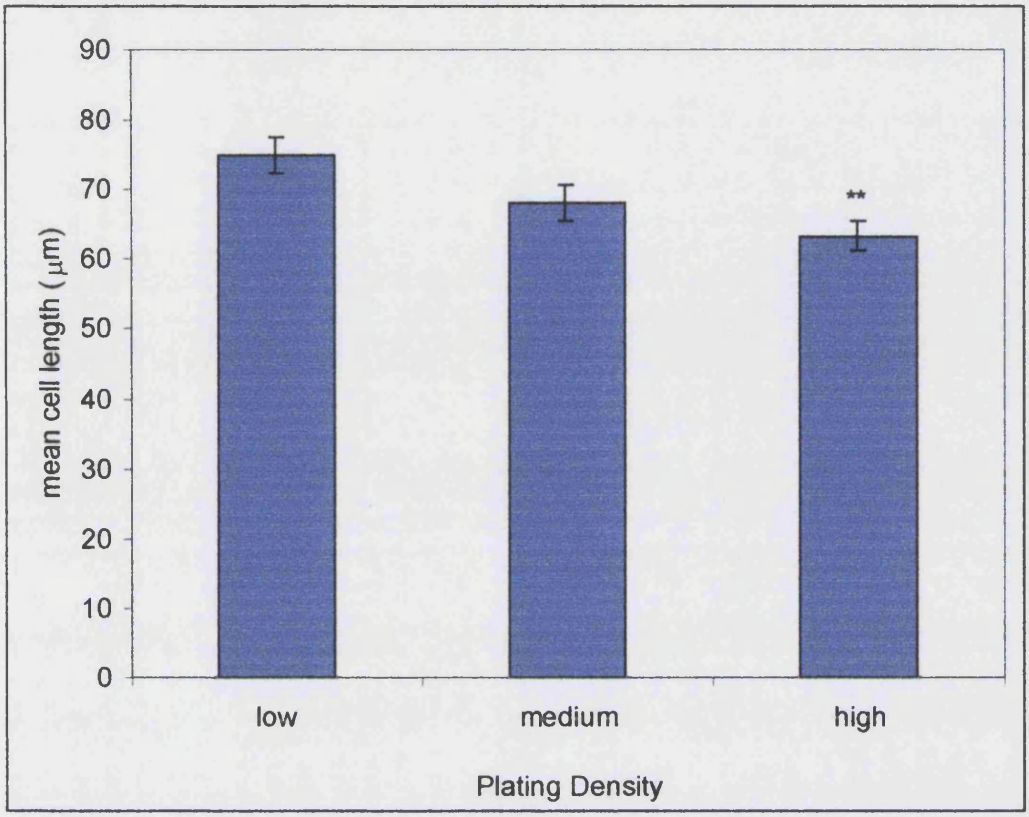


Figure 4.1.1 Primary cells were plated at $5x10^3$ cells/ml (low), $1x10^4$ cells/ml (medium) or $5x10^4$ cells/ml (high) and incubated for 3 days. Cells were then fixed, crystal violet stained and the cell length measured from at least 50 cells per condition. Mean cell length was calculated and plotted on a graph +/- standard error. One way ANOVA was carried out and showed significant differences between plating densities ($p=0.004$). Bonferroni tests were carried out to compare mean cell length at each plating density against the length of cells plated at $5x10^3$ cells/ml (** = $p<0.001$).

4.2.2 Measurement of hypertrophy in confluent cells

Results presented in the previous section suggest that less confluent cells increase in size and that the lower the plating density, the larger the cell. Heart cells in-vivo are surrounded by other cells and form tight junctions with adjacent cells. It is therefore more accurate to take measurements from cells forming a confluent monolayer. This may be a closer cell culture based model of the in-vivo situation.

Cells plated at high density form a confluent monolayer and it can be difficult to identify the edge of each cell, even when stained with crystal violet. For this reason, cells were stained with an antibody to actin to stain cells more clearly and allow more accurate measurement of cell length.

Cells were plated at $5x10^5$ cells/ml, which is a density known to produce a confluent monolayer. 48 hours after plating cells were either left untreated, or treated with CT-1 at 10ng/ml or phenylephrine (PE) at 50μM for a further 48 hours. Phenylephrine is an α_1-adrenergic agonist known to cause hypertrophy in the heart (King *et al.*, 1998). Cells were then fixed and stained with an antibody to actin and a fluorescent secondary antibody. Cell length was measured using a confocal microscope, and mean cell length calculated. ANOVA was used to test for significant differences in mean cell length and post-hoc Bonferroni tests used to test for significant differences between treated and untreated cells. The ANOVA showed significant differences in cell length ($p<0.0001$), p values for Bonferroni tests are shown below for each comparison made.

Untreated cells had a mean cell length of 53.3μm, which increased to 71.5μm after CT-1 treatment ($p<0.0001$). Treatment with PE caused an increase to 59.5μm but this was not significant ($p=0.3$) (Figure 4.2.2).

These results show that CT-1 causes a highly significant increase in cell length when added to primary cells in a confluent monolayer. PE did not cause a significant increase in cell length but cells did appear larger. This is as expected as PE causes hypertrophy by a uniform increase in cell size while CT-1 has been reported to mediate its hypertrophy by an increase in cell length (Wollert *et al.*, 1996). In order to measure hypertrophy caused by agents other that CT-1, cell area was also measured in subsequent experiments.

Figure 4.2.2 The effect of CT-1 and PE on cell length.

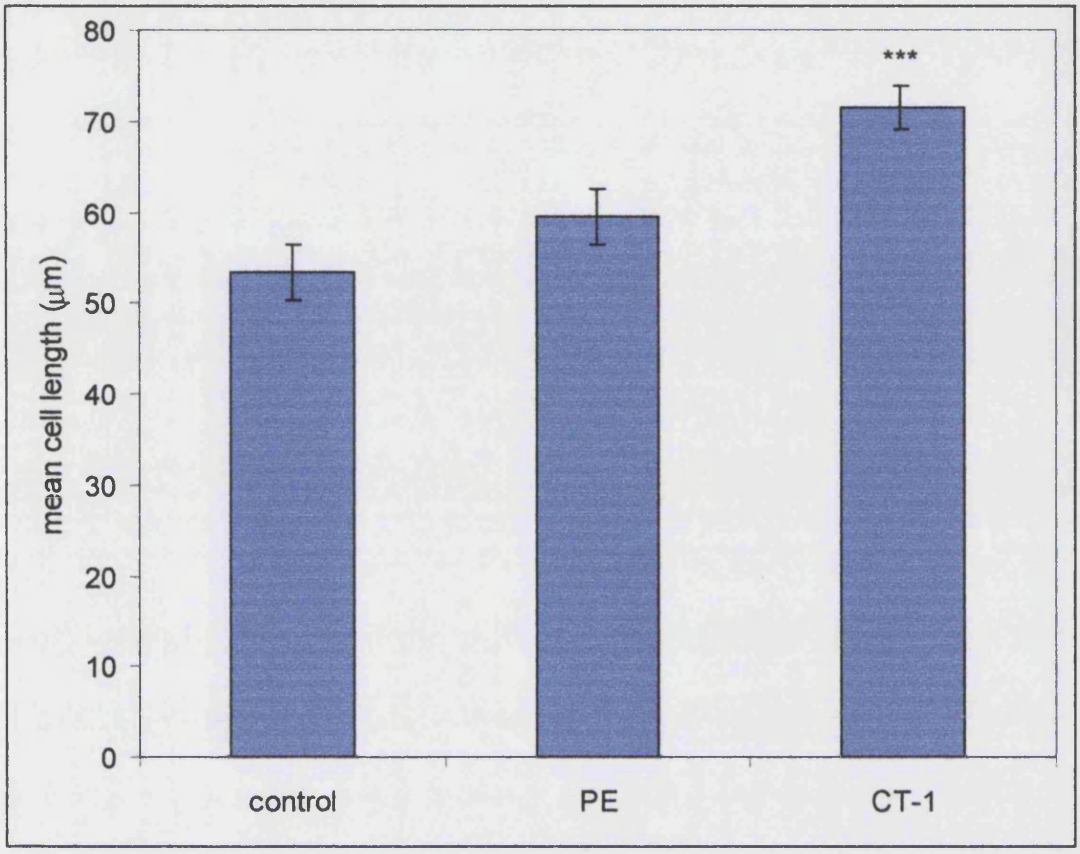


Figure 4.2.2 Primary cells were plated at $5x10^5$ cells/ml. 48 hours after plating cells were either, left untreated, treated with CT-1 10ng/ml or phenylephrine (PE) 50μM for a further 48 hours. Cells were then fixed and stained with an antibody to actin with a fluorescent secondary antibody. Cell length was measured using a confocal microscope, and mean cell length calculated. ANOVA showed significant differences in mean cell length ($p<0.0001$). Bonferroni tests were used to test for significant differences between treated and untreated cells. P values shown are for treated versus untreated (control) cells (*** = $p<0.0001$).

4.3.1 Measurement of transfected cells.

To allow investigation of the signalling pathways involved in the hypertrophic effect of CT-1, genetic inhibitors are used to block specific signalling pathways. The transfection efficiency achieved in primary cells is relatively low (1-5%) so it is important to specifically measure only successfully transfected cells. For this reason cells were transfected with a cytomeglovirus (cmv) promoter driven green fluorescent protein (gfp) expression vector. Transfected cells fluoresced green under a fluorescent microscope and could be specifically measured.

Primary cells were plated at $5x10^5$ cells/ml or $1x10^3$ cells/ml. 24 hours after plating cells were transfected with a gfp expression vector using the calcium phosphate method. 24 hours later cells were left untreated or treated with CT-1 at 10ng/ml for a further 48 hours. Cells were then immediately observed and measured using a confocal microscope.

Transfected control cells plated at $1x10^3$ cells/ml had a mean length of 86.5μm, which was increased to 99.0μm by CT-1 treatment. This increase was not significant in a t-test ($p=0.17$). Transfected cells plated at $5x10^5$ cells/ml had a mean cell length of 48.8μm and were significantly smaller than cells plated at $1x10^3$ cells/ml ($p<0.0001$). CT-1 treatment caused a significant increase in cell length of cells plated at $5x10^5$ cells/ml to 65.8μm ($p=0.002$) (Table 4.3.1).

These results show that hypertrophy occurs and can be measured in transfected cells. As before, cells plated more densely hypertrophied more than thinly plated cells and thinly plated cells were larger than confluent cells. This appears to be because the cells are extending processes to make contact with other cells when they are thinly plated.

Cells transfected with gfp must be measured within 1 day of fixation as the fluorescence is short lived. Measuring cells is time consuming so it would be useful to have a cellular marker that could be preserved over longer periods of time. For this reason a cmv-βgal expression vector was used to express the enzyme β-galactosidase in transfected cells. These cells can be stained with X-gal stain, which stains transfected cells blue and lasts indefinitely in fixed cells when stored at 4°C.

It was now important to check whether transfection of cells with gfp or β-gal expression vectors affects their ability to undergo hypertrophy. Cells were plated at $5x10^5$ cells/ml and either transfected with a cmv-gfp expression vector, a cmv-β-gal expression vector, or not transfected. 24 hours later, cells were either left untreated or treated with CT-1 at 10ng/ml. 48 hours later un-transfected cells were fixed, un-transfected cells were stained with crystal violet, cells transfected with gfp were observed under a fluorescent microscope and cells transfected with β-gal were stained with X-gal. Cells were then measured, in gfp transfected cells, only green fluorescent cells were measured and in β-gal transfected cells only blue stained cells were measured.

Un-transfected control cells had a mean cell length of 57.0μm, which increased significantly to 72.9μm after CT-1 treatment ($p<0.0001$) (Table 4.3.2).

Cells transfected with gfp had a mean length of 57.1μm, which increased significantly to 72.1μm after CT-1 treatment ($p=0.04$). Cells transfected with β-gal had a mean cell length of 56.8μm, which increased to 73.8μm after CT-1 treatment ($p<0.001$) (Table 4.3.2).

Therefore, un-transfected cells increased in length by 28% in response to CT-1, cells transfected with gfp increased in length by 26%, and cells transfected with β-gal showed a 30% increase in mean cell length in response to CT-1 treatment. There was no significant difference between transfected and un-transfected cell length in CT-1 treated or untreated cells in either case.

This shows that transfecting cells with a gfp or β-gal expression vector does not affect their size or their ability to undergo hypertrophy.

Table 4.3.1 Measurement of transfected cells.

Plating density (cells/ml)	Control mean cell length	CT-1 treated mean cell length	% increase	P value
$1x10^3$	86.5	99.0	14%	0.17
$5x10^5$	48.8	65.8	35%	0.002

Table 4.3.1 Primary cells were plated at $5x10^5$ cells/ml or $1x10^3$ cells/ml. 24 hours after plating cells were transfected with a gfp expression vector using the calcium phosphate method. 24 hours later cells were left untreated or treated with CT-1 at 10ng/ml for a further 48 hours. Transfected cells were then identified by their green fluorescence and at least 20 cells were measured per condition. P values are from t-tests comparing treated to untreated cells at each plating density.

Table 4.3.2 Comparison of hypertrophy in transfected and un-transfected cells.

	Control mean cell length	CT-1 treated mean cell length	% increase	P value
Un-transfected	57.0	72.9	28%	<0.0001
Transfected with gfp	57.1	72.1	26%	0.04
Transfected with β-gal	56.8	73.8	30%	<0.001

Table 4.3.2 Primary cells were plated at $5x10^5$ cells/ml and either transfected with a gfp expression vector, a β-gal expression vector, or not transfected. 24 hours later, cells were either left untreated or treated with CT-1 at 10ng/ml. 48 hours later un-transfected cells were fixed and stained with crystal violet. Both sets of cells were measured, in gfp transfected cells, only green fluorescent cells were measured. β-gal transfected cells were stained with X-gal and blue stained cells were measured. P values are from t-tests comparing CT-1 treated and untreated cells in transfected and un-transfected cells.

4.4.1 Measurement of ANF promoter activity

Although cell size is the main determinate of hypertrophy, the induction of foetal genes is also often measured. Atrial natriuretic factor (ANF) is a foetal gene that is switched back on in many forms of hypertrophy (Chien *et al.*, 1991). Measurement of ANF promoter activity would provide an additional parameter by which to quantify the hypertrophic effect of CT-1 and urocortin, as compared to other hypertrophic agents. ANF promoter activity can be quantified by transfection of a reporter construct consisting of 3kb of the ANF promoter driving expression of a firefly luciferase gene (Knowlton *et al.*, 1991). The amount of luciferase produced can be quantified in a luciferase assay and is directly related to promoter activity. Co-transfection of a *Renilla* luciferase expression vector driven by the constitutively active viral thymidine kinase (TK) promoter allows correction for transfection efficiency.

Primary cells were transfected with the ANF-luciferase reporter construct and the TK-*Renilla* luciferase expression vector. 24 hours later cells were left untreated or were treated with CT-1 (10ng/ml), CT-1 (50ng/ml), PE (50μM), or urocortin ($1x10^{-7}$M) for 24 hours. Cells were then harvested and extracts subjected to a dual luciferase assay (Promega). This assay measures Firefly luciferase to quantify activity of the ANF promoter and *Renilla* luciferase to equalize for transfection efficiency. Each value for Firefly luciferase was divided by the corresponding value for *Renilla* luciferase and plotted on a graph. The experiment was repeated three times with the same result.

Treatment with CT-1 or urocortin did not activate the ANF promoter. However, treatment with PE caused a 2.5 fold induction in ANF promoter activity (Figure 4.4.1).

These results show that CT-1 and urocortin are unable to activate this region of the ANF promoter, but this promoter construct did respond to PE, in agreement with previous reports (Wollert *et al.*, 1996).

Figure 4.4.1 ANF promoter activity

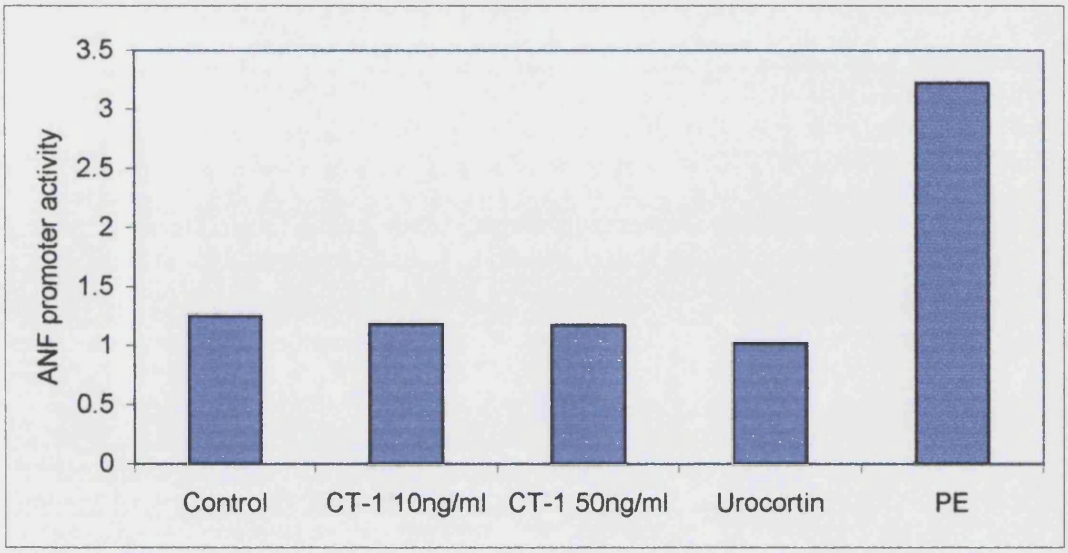


Figure 4.4.1 Primary cells were co-transfected with an ANF-luciferase reporter construct and a TK-*Renilla* luciferase expression vector. 24 hours later cells were left untreated or treated with CT-1 (10ng/ml), CT-1 (50ng/ml), PE (50μM), or urocortin ($1x10^{-7}$M) for 24 hours. Cells were then harvested and extracts subjected to a dual luciferase assay. Each value for the ANF promoter driven Firefly luciferase was divided by the corresponding value for *Renilla* luciferase and plotted on a graph. The experiment was repeated three times with similar results.

4.5.0 Discussion

The aim of this chapter was to establish a method for the measurement of hypertrophy to allow investigation of the signalling pathways involved.

Cardiac cells increased in size when plated at a sub-confluent density. This was probably because cells expand until they make contact with other cells. Sub-confluent cardiac myocyte cultures have, however, been used in other studies in the assessment of hypertrophy. In these studies, cells were serum starved for at least 24 hours before treatment with CT-1 (Wollert *et al.*, 1996). Serum starvation may prevent the expansion in cell size so allowing maximum hypertrophy to occur in response to CT-1. In serum starved cells, addition of 5% serum is sufficient to cause significant hypertrophy (Simpson *et al.*, 1982a). In-vivo, cells are constantly bathed in plasma but still undergo hypertrophy in response to CT-1. In this study, cells were not serum starved and were maintained in medium supplemented with 1% serum throughout experiments. It is thought that this more closely resembles the in-vivo situation and that serum starvation may lead to an artificial hypertrophic response caused by deprivation of factors essential to cell growth prior to stimulation. It is also thought that cells in a confluent monolayer are also the most accurate representation of the in-vivo situation.

When cells are in a confluent monolayer it can be difficult to identify the boundary of each cell, so measurement becomes difficult. One way to visualize individual cells is the transfection of a reporter gene such as gfp or β-gal, which allows transfected cells to be clearly seen. A random 1-5% of cells are successfully transfected, and these cells can be measured. This facilitates both the random selection of cells to be measured and clearly stains the whole cell to allow accurate measurement. The main advantage of measuring transfected cells is that other constructs can be co-transfected, which allows specific manipulation of signalling pathways.

The fluorescence of gfp transfected cells is short lived and cells must be measured within one day of fixation. Measuring cells is very time consuming so it was decided that a longer lasting marker should be used. For this reason a β-galactosidase (β-gal) expression vector was used instead of gfp to mark transfected cells. β-gal positive cells can be stained blue by X-gal stain (see methods), and this stain lasts indefinitely in fixed cells. Additionally, X-gal stained cells can be measured under phase contrast

microscopy, removing the need for fluorescence microscopy. It was also shown that transfection with gfp or β-gal expression vectors had no effect on cell size or the ability of cells to undergo hypertrophy in response to CT-1. This indicates that measurement of transfected cells should provide an accurate representation of the hypertrophic response in cultured neonatal cardiac myocytes.

ANF promoter activity was assessed as a possible additional parameter for the quantification of hypertrophy. A reporter construct containing a 5' proximal 3 kilobase pair region of the ANF promoter was used to measure promoter activity. This construct was activated by PE but was not activated by CT-1 or urocortin. CT-1 and urocortin have both been shown to increase ANF secretion in cardiac myocytes (Wollert *et al.*, 1996; Ikeda *et al.*, 1998) but neither has been shown to activate the ANF promoter. In a previous study using the same ANF reporter construct as used here, it was also shown that PE activated the promoter but CT-1 did not (Wollert *et al.*, 1996). Additionally, no activation of a BNP reporter construct was detected in response to CT-1, despite activation of this construct by endothelin-1 (Kuwahara *et al.*, 1998). This shows that either the CT-1 responsive sites are absent from all the promoter constructs used or that CT-1 is increasing ANF and BNP levels by a post-transcriptional mechanism. This would support results presented in chapter 3, where CT-1 failed to activate the hsp90 promoter despite increasing hsp90 protein levels under the same conditions, implying that the induction of hsp90 by CT-1 may be occurring by a post-transcriptional mechanism. There has, however been one demonstration of transcriptional activation of ANF by CT-1 in a nuclear run on, but again, no activation of the promoter could be demonstrated (Wollert *et al.*, 1996).

Due to the inability of CT-1 to activate the ANF promoter, the construct used here will not be further used as a measure of hypertrophy. Measurement of ANF release would be a good alternative but this would not allow specific measurement from transfected cells. It has therefore been decided that quantification of ANF is not a good parameter for the measurement of hypertrophy in this system.

Therefore, the method used to investigate the signalling pathways involved in cardiac hypertrophy will be as follows: Cells will be transfected with a β-gal expression vector, either alone or co-transfected with various constructs to modulate specific signalling

pathways. 48 hours after treatment, cells will be fixed and stained with X-gal. Blue stained, transfected cells will then be measured, with cell area and cell length being measured for at least 30 cells per condition.

CHAPTER 5

AN INVESTIGATION OF THE SIGNALLING PATHWAYS INVOLVED IN THE HYPERTROPHIC EFFECTS OF CT-1 AND UROCORTIN

5.1.0 Introduction

CT-1 is known to activate the Jak-STAT and p42/p44 MAPK signalling pathways through binding to its gp130 / LIFRβ receptor (Robledo *et al.*, 1997b; Sheng *et al.*, 1997). The cardioprotective effect of CT-1 is dependent on the p42/p44 MAPK pathway but the hypertrophic effect was shown to be independent from this pathway (Sheng *et al.*, 1997). This indicated divergence in the signalling pathways for the protective and hypertrophic effects of CT-1. Such a divergence in signalling is interesting as cardioprotection is a desirable outcome in the heart, but hypertrophy is potentially harmful. If the hypertrophic effects of CT-1 could be blocked it could potentially be used as a cardioprotective therapeutic agent.

In this chapter the signalling pathways involved in the hypertrophic effect of CT-1 will be studied. The first pathways to be investigated are the p42/p44 MAPK and Jak-STAT pathways as these are known to be activated by CT-1. The PI3 kinase pathway will also be studied as activation of this pathway has recently been demonstrated in response to CT-1 treatment (Kuwahara *et al.*, 2000).

Urocortin is a peptide hormone of the corticotrophin releasing hormone family (CRH). It is cardioprotective by the same pathway as CT-1 (Brar *et al.*, 2000) and a possible hypertrophic effect has been suggested by its ability to induce release of ANP from cardiac myocytes (Ikeda *et al.*, 1998). In this study urocortin will be studied for a possible hypertrophic effect and any such effect compared to that of CT-1. Comparison will also be made with the known hypertrophic agent phenylephrine (PE), which is an α_1-adrenergic receptor agonist.

5.2.0 Urocortin causes hypertrophy in neonatal cardiac myocytes.

24 hours after plating, primary cells were transfected with a cmv promoter driven β-gal expression vector. 24 hours later cells were either left untreated, treated with CT-1 (10ng/ml), urocortin ($1x10^{-7}$M) or phenylephrine (PE) (50μM) for 48 hours. Cells were then fixed and stained with X-gal to allow blue transfected cells to be identified and measured. Mean cell area and mean cell length were calculated. Single factor ANOVA was used to test for significant differences between groups, and then Bonferroni tests were performed to test for differences between each treatment and control. The ANOVA showed highly significant differences between groups ($p<0.0001$) both for cell length and cell area. P values for Bonferroni tests are shown as each treatment is discussed.

Control cells had a mean cell length of 59.2μm, this increased by 20% to 70.9μm after CT-1 treatment ($p=0.1$). Treatment with urocortin also caused a 20% increase in mean cell length to 70.9μm ($p=0.08$). Treatment with PE caused a 29% increase in mean cell length to 76.2μm ($p=0.03$) (Figure 5.1.1).

Cell area was also measured, control cells had a mean area of 886μm^2 this increased by 28% to 1133 μm^2 after CT-1 treatment ($p=0.05$). Treatment with urocortin also caused an increase in cell area to 1446μm^2, which is a 63% increase from control cell area ($p<0.0001$). Treatment with PE caused a 71% increase in mean cell area to 1520μm^2 ($p=0.0005$) (Figure 5.1.2).

This shows that urocortin is acting as a hypertrophic agent to increase the size of cardiac myocytes. The hypertrophic effect of urocortin is greater than that of CT-1. The strongest hypertrophic effect was caused by PE

The increases seen in cell area due to any of the hypertrophic agents tested were greater, and more significant than those seen in cell length. This may be because cell area is a more accurate and more consistent measure of cell size than cell length. Results for cell length are more variable as the long axis of the cell can be difficult to identify due to the asymmetric shape of the cell.

In this study, treatment with PE caused a significant increase in cell area and cell length. This conflicts with results presented in chapter 4 where PE did not cause a significant increase in cell length. The significant increase in cell area observed here after PE treatment is probably due to the large (71%) increase in cell area. Any increase in cell area will produce a corresponding increase in cell length. Whether or not the increase is significant, depends on the degree of hypertrophy observed in that experiment.

It is however clear that PE causes a much greater increase in cell area than cell length (71% compared to 29%), while CT-1 causes only a slightly greater increase in area than length (28% compared to 20%). This supports the finding that CT-1 mediates its hypertrophic effect predominantly by an increase in cell length while PE causes a uniform increase in cell size.

The novel hypertrophic effect of urocortin causes a substantially greater increase in cell area than cell length (63% compared to 20%). This demonstrates that urocortin is causing an overall increase in cell size and does not seem to be specifically increasing cell length.

Micrographs of randomly selected fields of view of control, CT-1 treated, urocortin treated and PE treated cells are shown in figure 5.1.3. The blue cells are transfected and it is these cells that were measured.

Figure 5.1.1 The effect of CT-1, urocortin and PE treatment on cell length

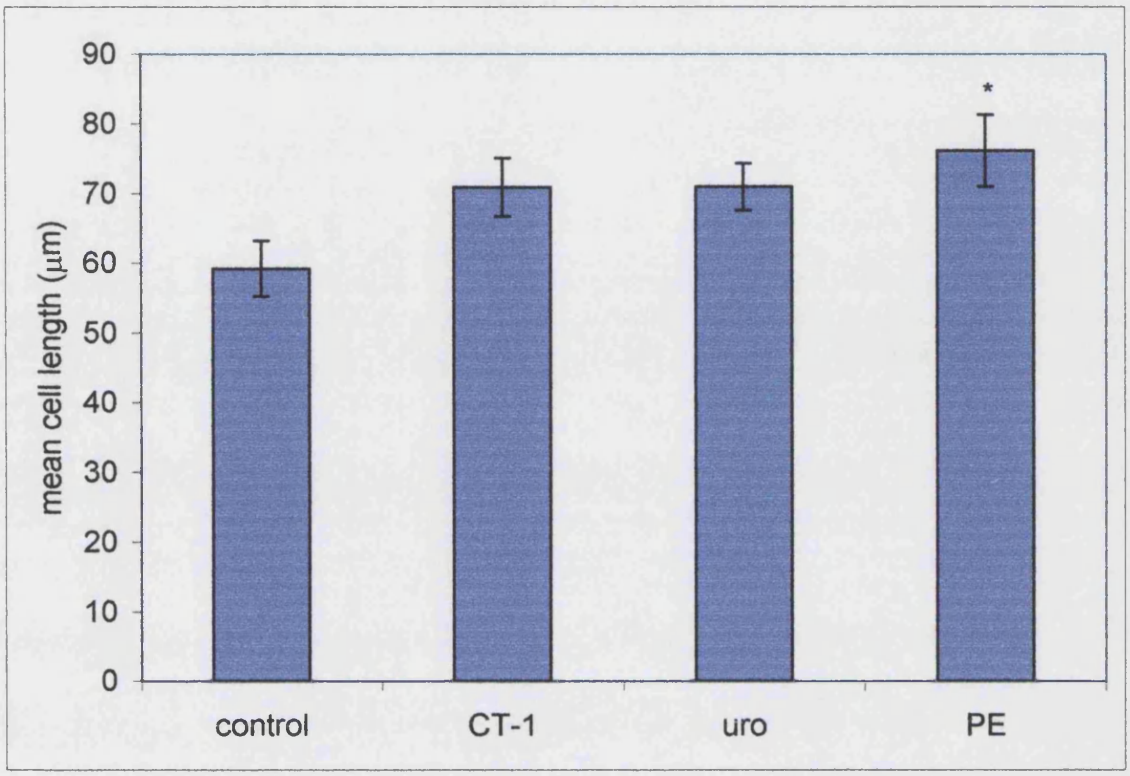


Figure 5.1.1 Neonatal cardiac myocytes were transfected with a cmv-β-gal expression vector. 24 hours later cells were either left untreated (control), treated with CT-1 10ng/ml 48 hrs, treated with urocortin $1x10^{-7}$M 48 hrs, or phenylephrine (PE) 50μM 48 hrs. Cell length was measured, and mean cell length plotted +/- standard error. Single factor ANOVA showed significant differences between groups, $p<0.0001$. Post-hoc Bonferroni tests were performed to compare each treatment to the relevant control (* = $p<0.05$). At least 30 cells were measured per condition, and the experiment was repeated 3 times with similar results.

Figure 5.1.2 The effect of CT-1, urocortin and PE treatment on cell area

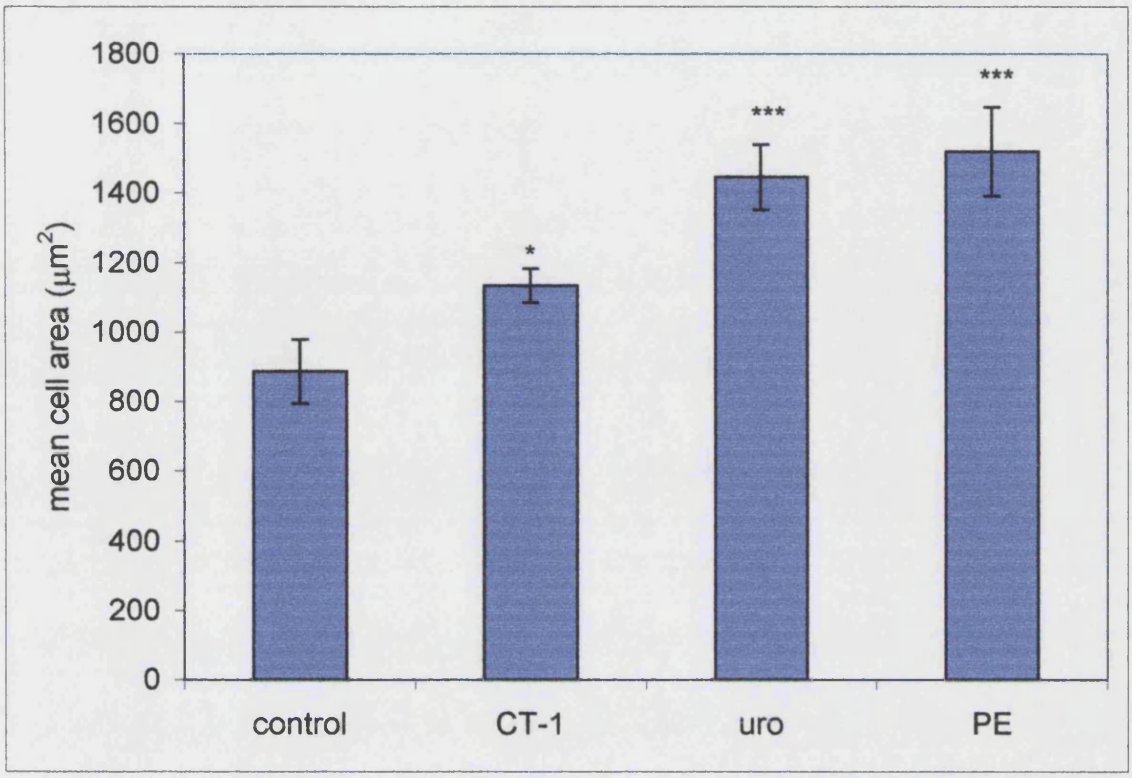


Figure 5.1.2 Neonatal cardiac myocytes were transfected with a cmv-β-gal expression vector. 24 hours later cells were either left untreated (control), treated with CT-1 10ng/ml 48 hrs, treated with urocortin $1x10^{-7}$M 48 hrs, or phenylephrine (PE) 50μM 48 hrs. Cell area was measured, and mean cell area plotted +/- standard error. Single factor ANOVA showed significant differences between groups, $p<0.0001$. Post-hoc Bonferroni tests were performed to compare each treatment to control with significance levels: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). At least 30 cells were measured per condition, at the experiment was repeated 3 times with similar results.

Figure 5.1.3 Primary neonatal cardiac myocytes

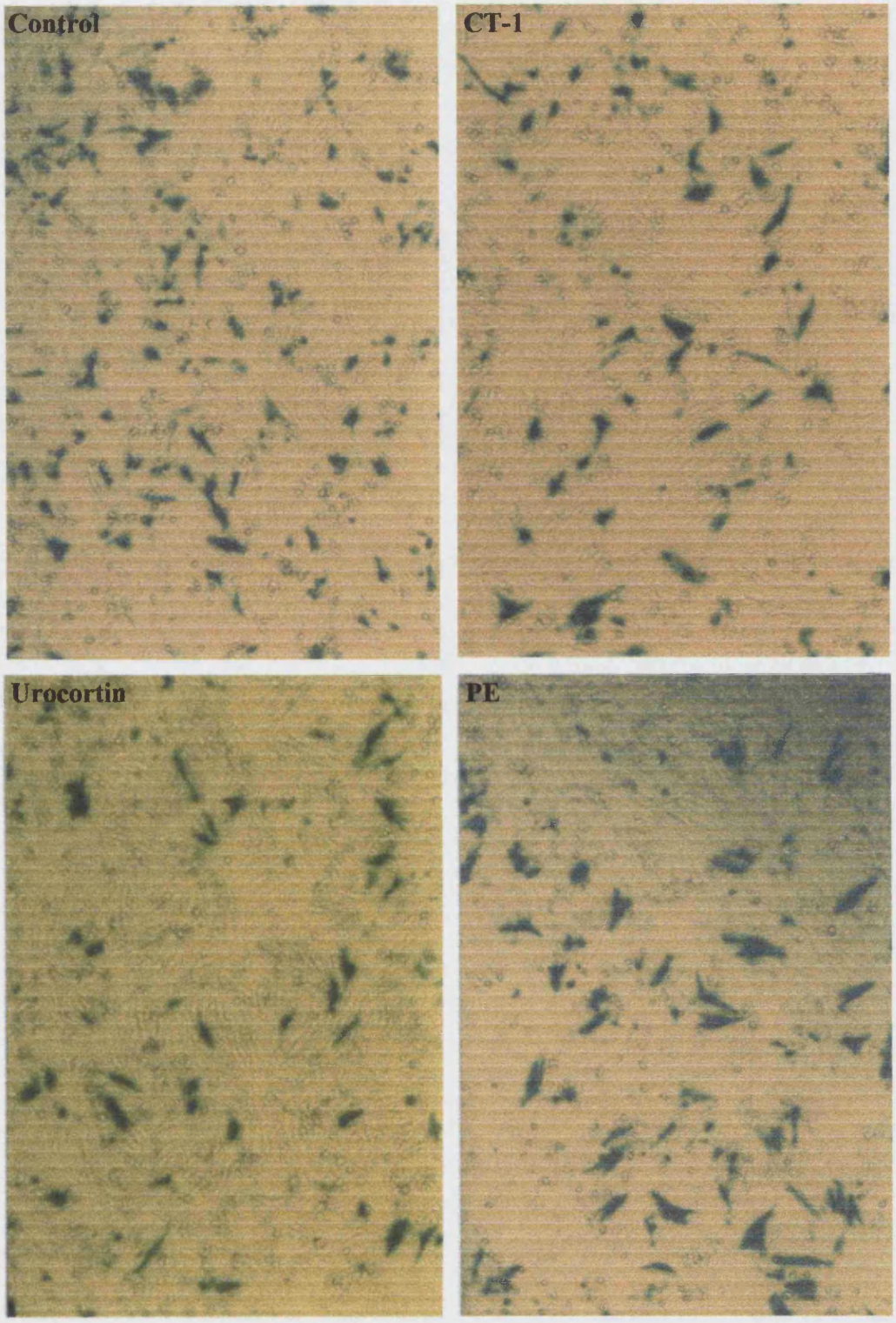


Figure 5.1.3 Micrographs showing random field of view of cells transfected with cmv-βgal then left untreated, treated with CT-1 (10ng/ml), urocortin (1x10-7M), or PE (50μM) for 48 hours. Cells were then fixed and stained with X-gal to stain transfected cells blue for easy measurements. Magnification = x10.

5.2.1 CT-1 and urocortin cause hypertrophy in adult rat cardiac myocytes.

Adult rat cardiac myocytes are a better model for the human heart than neonatal cells as their morphology more closely resembles that of adult human cardiac myocytes. Adult cardiac myocytes appear as distinct rods with visable myofilaments and rarely make contacts with other cells in culture. Because the cells exist individually in culture and are not part of a monolayer they are easy to measure, even when unstained. However, neither CT-1 nor urocortin has previously been tested for any hypertrophic effect on adult as opposed to neonatal cardiac myocytes.

Adult rat cardiac myocytes were left untreated (control), treated with CT-1 (5ng/ml), CT-1 (50ng/ml), or urocortin ($1x10^{-7}$M) for 48 hours, fixed and measured. Mean cell area and mean cell length were calculated and ANOVA was used to test for significant differences in area and length. The ANOVA for area showed significant differences mean cell area ($p=0.03$). The ANOVA for cell length showed no significant differences in cell length ($p=0.6$). Post-hoc Bonferroni tests were used to test for significant differences between each treatment and control. P values for Bonferroni tests are shown below as each comparison is discussed.

Treatment with 5ng/ml CT-1 caused an 11.2% increase in mean cell area but this was not statistically significant. 50ng/ml CT-1 resulted in a significant 18.6% increase in cell area ($p=0.02$). Urocortin caused a significant 16.5% increase in cell area ($p=0.05$) (Figure 5.2.1).

Cell length was also measured; untreated cells had a mean cell length of 107μm, which was increased to 109μm by 5ng/ml CT-1 and 113μm by 50μg/ml CT-1. Urocortin caused no change in cell length. None of the changes in cell length were significant in Bonferroni tests. This supports data from neonatal cardiac myocytes presented in the previous section showing that cell area is a more consistent measure of hypertrophy than cell length.

These results demonstrate for the first time that CT-1 and urocortin are hypertrophic in adult cardiac myocytes.

Figure 5.2.1 The effect of CT-1 and urocortin on cell area in adult rat cardiac myocytes.

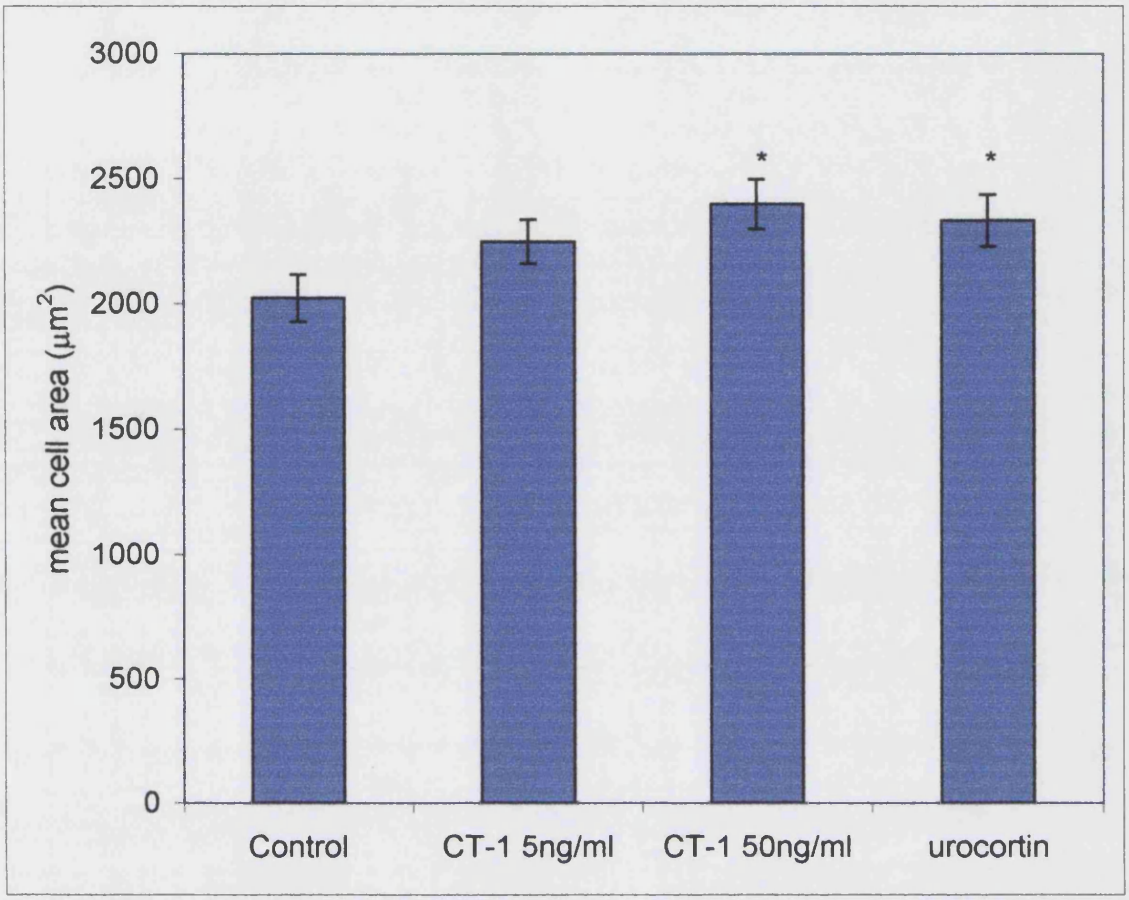


Figure 5.2.1 Adult rat cardiac myocytes were left untreated (control), treated with CT-1 (5ng/ml), CT-1 (50ng/ml), or urocortin ($1x10^{-7}M$) for 48 hours, fixed and measured. Mean cell area was calculated and plotted on a graph + /- standard error. Single factor ANOVA showed significant differences between groups ($p<0.05$). Bonferroni tests were performed to test for significant differences between treated and untreated cells (* = $p<0.05$). At least 30 cells were measured per condition and the experiment was repeated 3 times with similar results.

Figure 5.2.2 The effect of CT-1 and urocortin on cell length in adult rat cardiac myocytes.

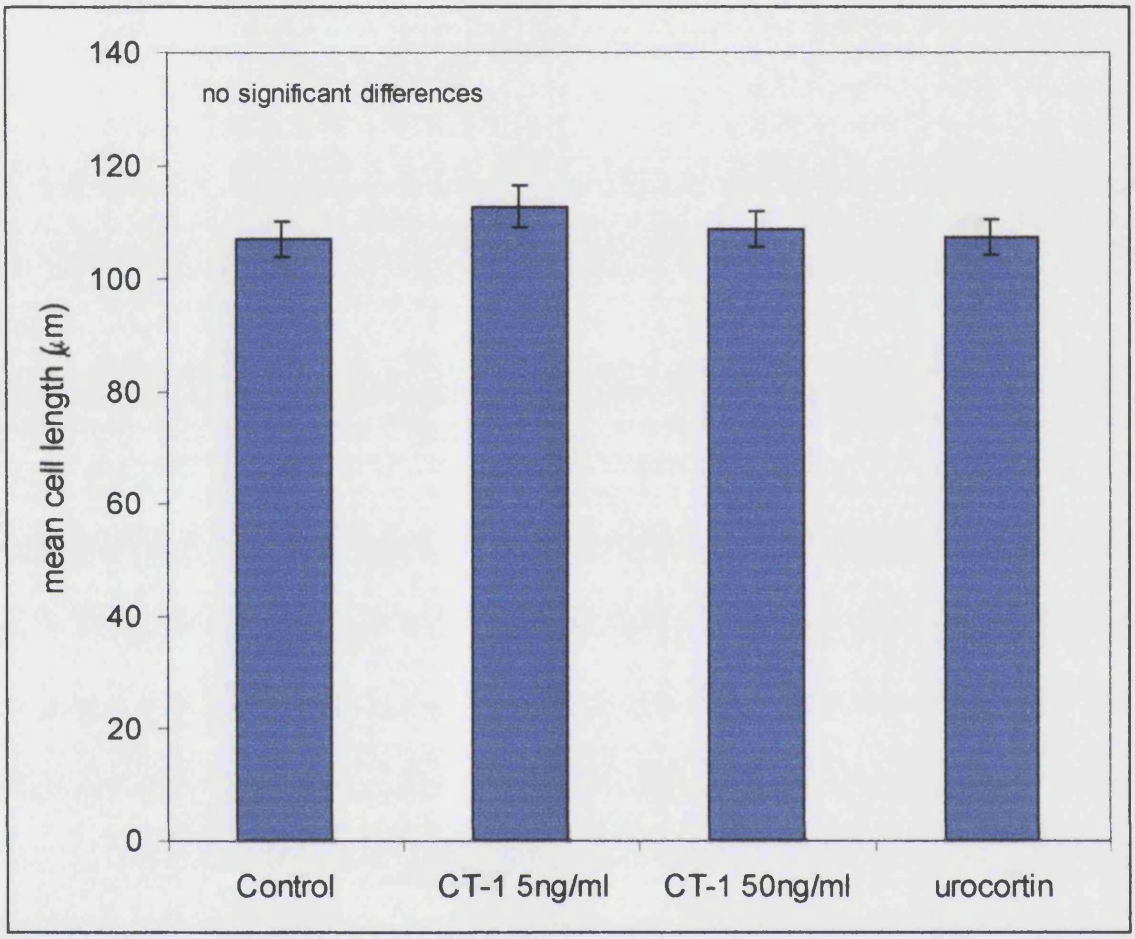


Figure 5.2.2 Adult rat cardiac myocytes were left untreated (control), treated with CT-1 (5ng/ml), CT-1 (50ng/ml), or urocortin ($1x10^{-7}$M) for 48 hours, fixed and measured. Mean cell length was calculated and plotted on a graph + /- standard error. Single factor ANOVA showed no significant differences between groups (p=0.6). Bonferroni tests were performed to test for significant differences between treated and untreated cells. No significant differences were observed. At least 30 cells were measured per condition and experiment was repeated 3 times with similar results.

5.3.0 Signalling pathways involved in the hypertrophic response to CT-1 and urocortin.

To investigate the signalling pathways involved in CT-1 and urocortin mediated hypertrophy, specific signalling pathways were inhibited, by transfecting appropriate expression plasmids. Adult cardiac cells could not be effectively transfected, so studies of the signalling pathways involved in hypertrophy were carried out in neonatal rat cardiac myocytes.

To investigate the role of STAT1 and STAT3 signalling in CT-1 mediated hypertrophy, STAT3 signalling was inhibited by transient transfection of a construct encoding a specific protein inhibitor of activated STAT3 (PIAS3). PIAS1, a similar construct encoding a protein inhibitor of activated STAT1 was also used to see whether the effects were specific to STAT3. PIAS3 specifically blocks the DNA binding activity of phosphorylated STAT3 (Chung *et al.*, 1997). Likewise, PIAS1 specifically blocks the DNA binding activity of phosphorylated STAT1 (Liu *et al.*, 1998). To study the role of the p42/p44MAPK pathway in hypertrophy, transfection of dominant negative MEK-1 (dn MEK-1) was used. MEK-1 is a MAPK kinase upstream from p42/p44MAPK and inhibition of MEK-1 has been shown to inhibit this pathway.

Neonatal cardiac myocytes (primary cells) were transfected with a cmv-β-gal expression vector, with either control plasmid lacking an insert, PIAS1, PIAS3 or dn MEK-1. 24 hours later cells were either left untreated, treated with CT-1 (10ng/ml), or urocortin ($1x10^{-7}$M) for 48 hours. Cells were then fixed, stained with X-gal and blue transfected cells were measured. Single factor ANOVA showed significant differences between groups ($p<0.0001$) and Bonferroni tests were used to test for differences between treated and un-treated cells in the presence of each transfected construct.

In cells transfected with control plasmid, CT-1 caused a 21% increase in mean cell area ($p=0.05$) and urocortin caused a 33% increase in mean cell area ($p=0.001$) (Figure 5.3.1).

In cells transfected with dn MEK-1, CT-1 caused a 29% increase in mean cell area ($p=0.008$) and urocortin a 26% increase ($p=0.01$) compared to untreated cells transfected with dn MEK-1. Inhibition of the p42/p44MAPK pathway therefore does

not block the hypertrophic effect of CT-1 or urocortin. Transfection of dn MEK-1 alone had no significant effect on the size of untreated cells (Figure 5.3.1).

In cells transfected with PIAS1 to inhibit the STAT1 pathway, CT-1 caused a 68% increase in mean cell area ($p<0.0001$) and urocortin a 30% increase ($p=0.02$) compared to untreated cells transfected with PIAS1 (Figure 5.3.1). Transfection with PIAS1 caused a significant ($p=0.002$) decrease in mean cell area compared to cells transfected with control plasmid. This decrease in size makes the hypertrophic effect of CT-1 appear larger in the presence of PIAS1. PIAS1, however, had no significant effect on the size of CT-1 treated cells when compared to CT-1 treated cells transfected with control plasmid. The hypertrophic effect of urocortin was attenuated by transfection with PIAS1. However, this was only a partial effect as urocortin still produced a significant ($p=0.02$) increase in cell size in the presence of PIAS1. Therefore, inhibition of STAT1 signalling causes a reduction in size of untreated cells, does not effect the hypertrophic effect of CT-1 and partially inhibits the hypertrophic effect of urocortin. However, both CT-1 and urocortin can still induce hypertrophy when STAT1 signalling is blocked.

In cells transfected with PIAS3 to inhibit the STAT3 pathway CT-1 caused no significant increase in cell size, showing that the hypertrophic effect of CT-1 is dependent on the STAT-3 pathway. In the presence of PIAS3, urocortin caused a 65% increase in cell size ($p<0.0001$) compared with untreated cells transfected with PIAS3. This shows that unlike CT-1, urocortin does not require STAT-3 signalling to produce its hypertrophic effect. Like PIAS1, transfection with PIAS3 caused a significant ($p=0.002$) decrease in untreated cell area compared to cells transfected with control plasmid (Figure 5.3.1). This implies that both STAT1 and STAT3 are involved in the basal maintenance of cell size.

Cell length was also measured and showed the same pattern as cell area (Figure 5.3.2).

Figure 5.3.1 Signalling pathways and hypertrophy – effect on cell area

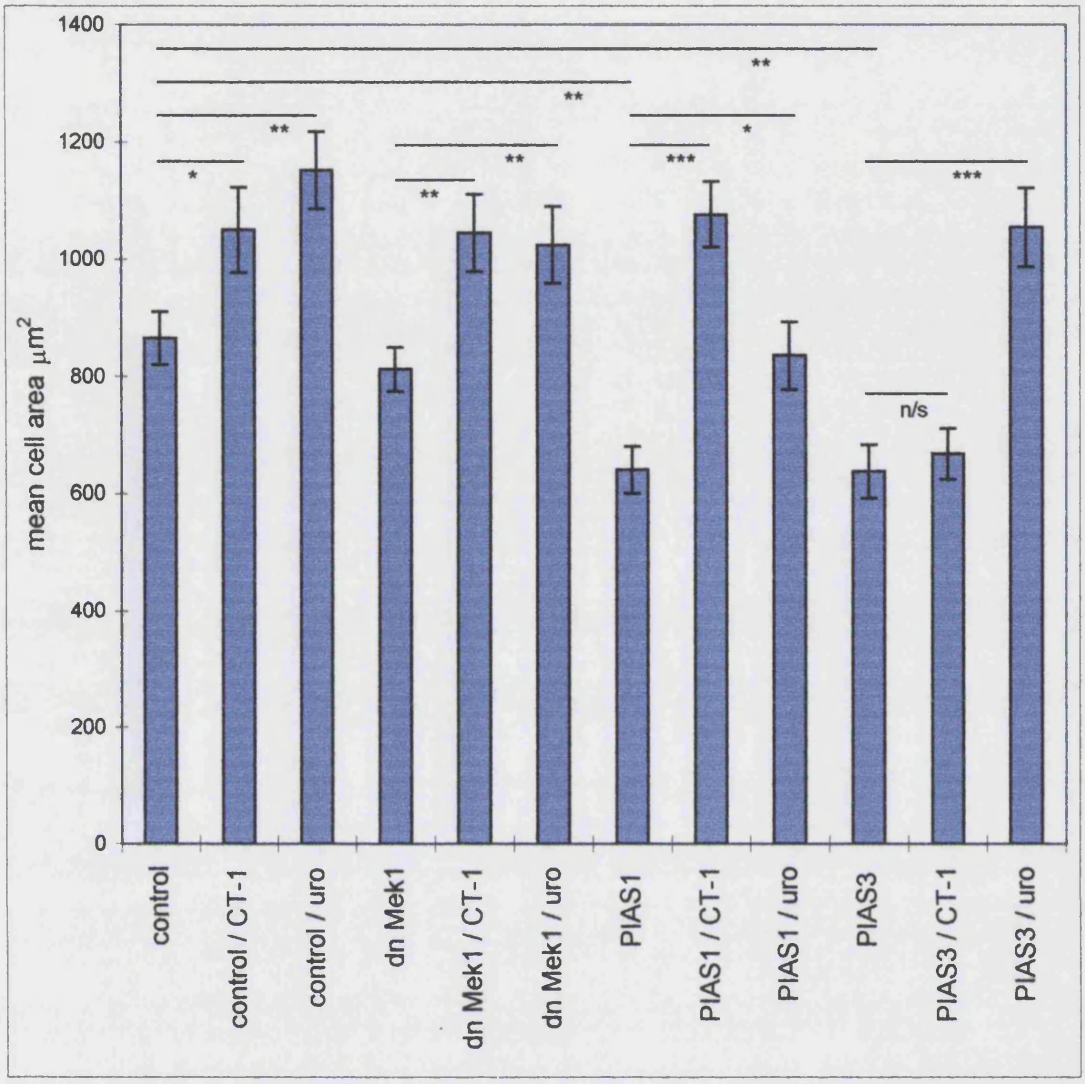


Figure 5.3.1 Neonatal cardiac myocytes were transfected with a cmv-β-gal expression vector with either control plasmid lacking an insert (control), PIAS1 (STAT1 inhibitor), PIAS3 (STAT3 inhibitor), or dominant negative MEK-1 (dn Mek1). Cells were either left untreated, treated with CT-1 10ng/ml or urocortin (uro) $1x10^{-7}$M 48 hrs. Cell area was measured by planimetry. Mean cell area +/- standard error was plotted, n=30 for each treatment. Single factor ANOVA showed significant differences between groups, $p<0.0001$. Post-hoc Bonferroni tests were performed to compare each treatment to the relevant control. Horizontal lines between bars represent Bonferroni tests between pairs of data, with significance levels depicted by stars as follows: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) or no significance (n/s).

Figure 5.3.2 Signalling pathways and hypertrophy – effect on cell length.

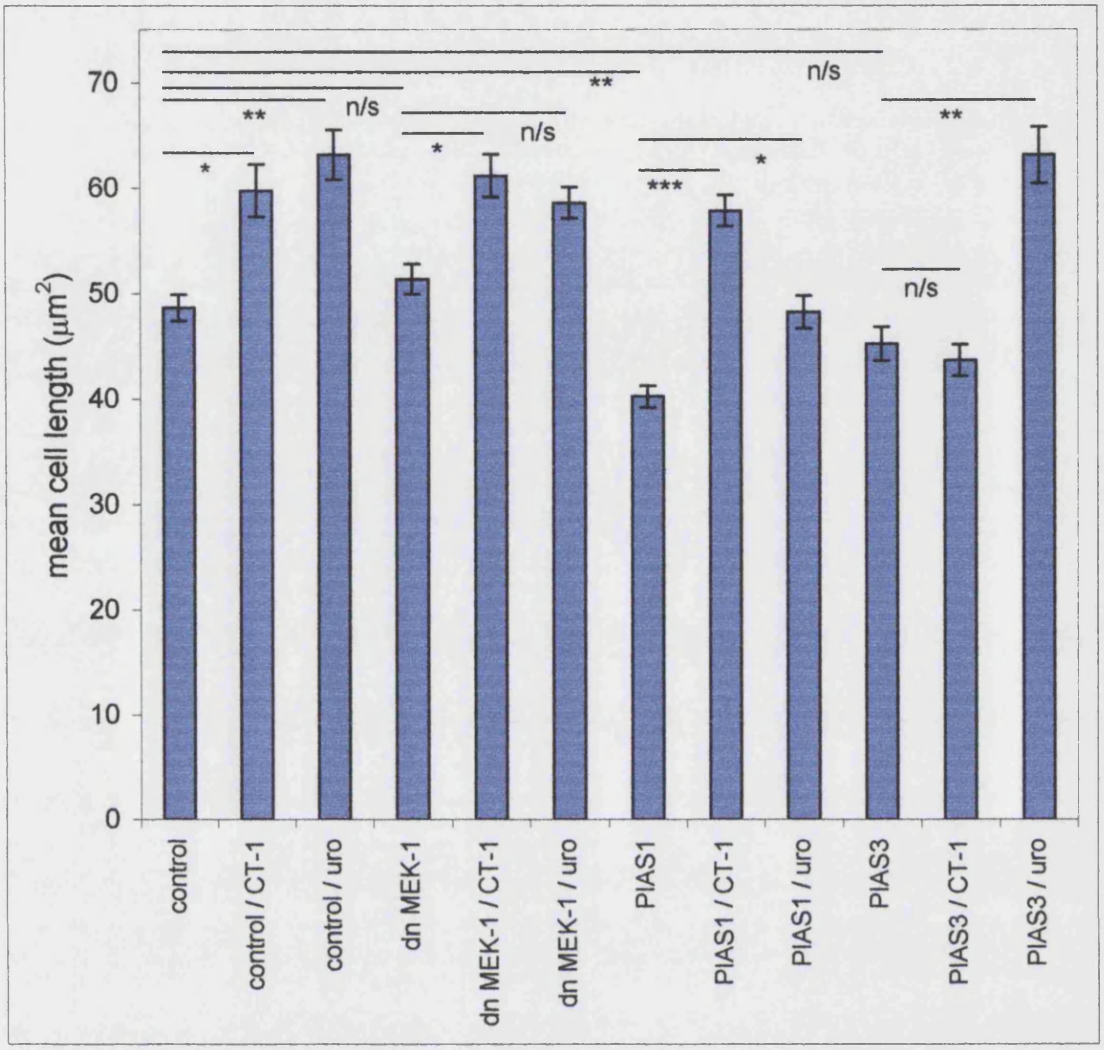


Figure 5.3.1 Neonatal cardiac myocytes transfected with cmv-β-gal expression vector with either control plasmid lacking an insert (control), PIAS1 (STAT1 inhibitor), PIAS3 (STAT3 inhibitor), or dominant negative MEK-1 (dn Mek1). Cells were either left untreated, treated with CT-1 10ng/ml or urocortin (uro) $1x10^{-7}$M 48 hrs. Cell length was measured, and mean cell length +/- standard error was plotted, n=30 for each treatment. Single factor ANOVA showed significant differences between groups, $p<0.0001$. Post-hoc Bonferroni tests were performed to compare each treatment to the relevant control. Horizontal lines between bars represent Bonferroni tests between pairs of data, with significance levels depicted by stars as follows: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) or no significance (n/s).

5.4.0 The protective effect of CT-1 and urocortin is dependent on the p42/p44 MAPK signalling pathway and independent from the STAT3 pathway.

We have shown that the hypertrophic effect of CT-1 is dependent on the STAT3 pathway, whereas the hypertrophic effect of urocortin is not. It is therefore important to study the effect of inhibition of the STAT3 pathway on the protective effects of CT-1 and urocortin when given at reperfusion as carried out by Brar et al (unpublished observations) and (Brar *et al.*, 2000). Using the same method as for the previous experiment, cells were transfected with control plasmid, dn MEK-1, PIAS1 or PIAS3. Cells were then either incubated under control conditions or subjected to a hypoxic stress. At the point of re-oxygenation cells were left untreated, treated with CT-1 or urocortin and incubated for 48 hrs. Cells were then fixed, X-gal stained and TUNEL labelled so that the percentage of apoptotic-transfected cells could be calculated.

The mean % of apoptotic cells was calculated and ANOVA carried out to test for significant differences between treatments. Post-hoc Bonferroni tests were carried out to test for significant differences between control and treated cells in the presence of each transfected construct. The ANOVA showed significant differences in mean % apoptotic cells ($p<0.0001$). P values for Bonferroni tests are shown below, as each comparison is discussed.

In cells transfected with control plasmid and subjected to hypoxia, 31% of transfected cells were TUNEL positive and therefore apoptotic. This was reduced to 12.6% ($P=0.05$) by CT-1 treatment and to 12.2% ($P=0.05$) by urocortin (Figure 5.4.1).

In the presence of dn MEK-1, 34% of cells were apoptotic after hypoxic stress. Treatment with CT-1 or urocortin caused no significant protective effect in the presence of dn MEK-1. Although the attenuation of the protective effect was more significant with CT-1 than urocortin as previously shown (Brar *et al.*, 2000; Stephanou *et al.*, 1998), there was no significant difference between control and urocortin treated cells in the presence of dn MEK-1. This supports previous evidence stating that the protective effect of CT-1 (Sheng *et al.*, 1997) and urocortin (Brar *et al.*, 2000) are dependent on the p42/p44 MAPK pathway (Figure 5.4.1).

In cells transfected with PIAS1 to inhibit STAT1 activity, cell death was reduced to 12.2% after hypoxia compared to 31% in cells transfected with control plasmid ($p=0.08$). This shows that inhibition of STAT1 signalling is protecting the cells against hypoxic injury and agrees with previous work in our laboratory indicating that activation of the STAT1 pathway has a damaging effect (Stephanou *et al.*, 2000). There were no significant differences seen after CT-1 or urocortin treatment in the presence of PIAS1, possibly because the cells transfected with PIAS1 were already protected (Figure 5.4.1).

In cells transfected with PIAS3 and subjected to hypoxia, 38.3% of cells were apoptotic. Treatment with CT-1 reduced this to 21% ($P=0.05$) and treatment with urocortin reduced the amount of apoptosis to 21.6% ($P=0.001$). This shows that although STAT3 inhibition can block the hypertrophic effect of CT-1, under the same conditions there is no significant effect of STAT3 inhibition on the protective effects of CT-1 or urocortin (Figure 5.4.1).

No significant differences in cell survival were observed in transfected cells that were not subjected to hypoxic stress.

These results support previous findings (Sheng *et al.*, 1997) in showing that the protective effect of CT-1 is dependent on the p42/p44 MAPK pathway. It is also shown that inhibition of the STAT3 pathway has no effect on the protective effect of CT-1. The effect of inhibition of STAT1 or STAT3 signalling on the protective effect of CT-1 had not previously been tested. It was therefore important to verify that inhibition of the STAT3 pathway did not affect the protective effect of CT-1.

Figure 5.4.1 Signalling pathways – effect on protection.

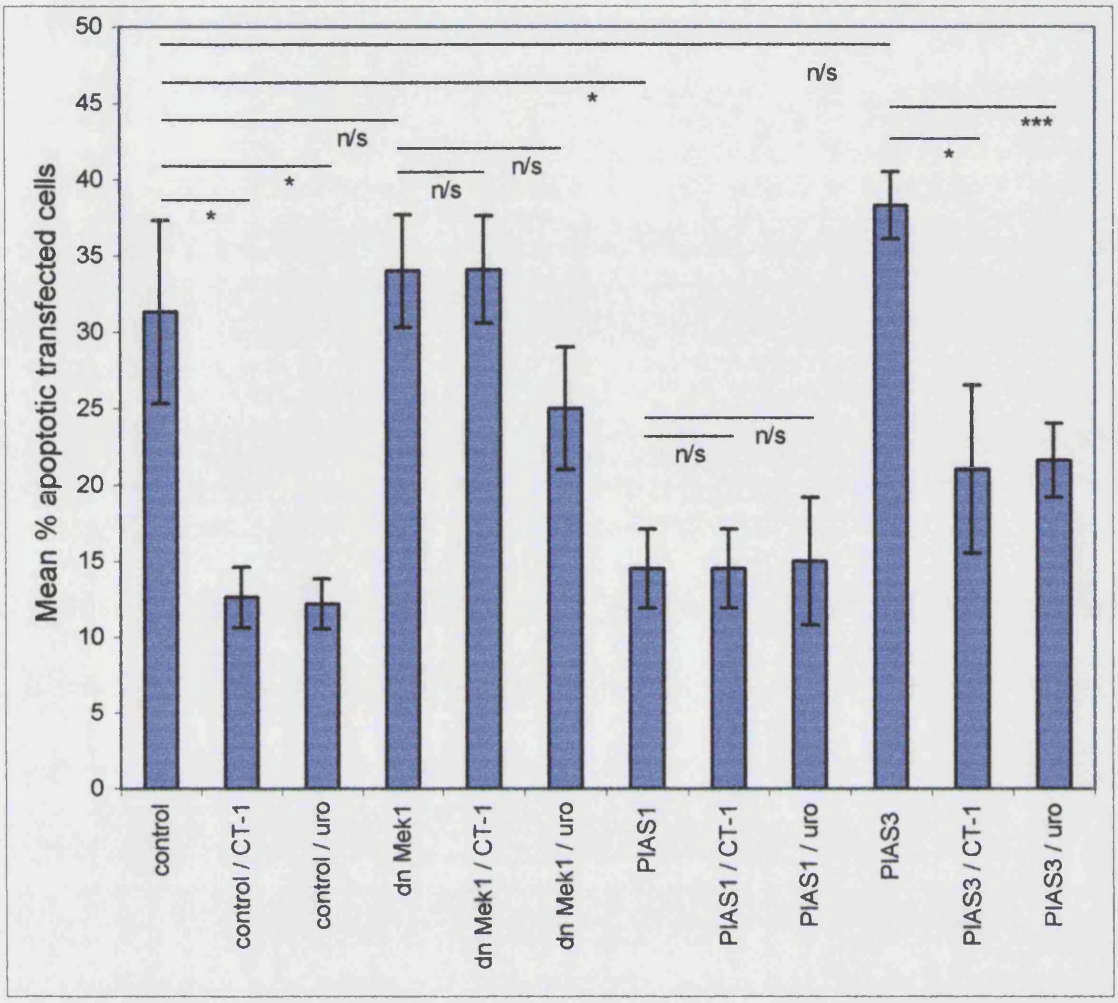


Figure 5.4.1 Neonatal cardiac myocytes were transfected with cmv-β-gal expression vector with either control plasmid lacking an insert (control), PIAS1 (STAT1 inhibitor), PIAS3 (STAT3 inhibitor), or dominant negative MEK-1 (dn Mek1). 24 hours later cells were left untreated, treated with CT-1 (10ng/ml) or urocortin (uro) ($1x10^{-7}$M) for 48 hrs. Transfected cells were stained blue with X-gal and TUNEL labelling was used to identify apoptotic cells. Mean % apoptotic transfected cells out of total transfected cells +/- standard error was plotted. Single factor ANOVA showed significant differences between groups ($p<0.0001$). Post-hoc Bonferroni tests were performed using Microsoft excel to compare each treatment to the relevant control. Horizontal lines between bars represent Bonferroni tests between pairs of data, with significance levels depicted by stars as follows: * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) or n/s (no significance).

5.5.0 Use of chemical inhibitors to investigate the role of other signalling pathways in cardiac hypertrophy.

In addition to genetic manipulation of signalling pathways, chemical inhibitors were also used to investigate the role of different signalling pathways in the hypertrophic effect of CT-1 and urocortin. PD98059 (PD) blocks the p42/p44 MAPK pathway at the level of MEK-1. SB202190 (SB) blocks the P38 MAPK pathway. Wortmanin (Wort) inhibits tyrosine kinases but specifically inhibits PI3 kinase at a dose of 50nM.

Primary cells were transfected with a cmv-βgal expression vector to allow easy visualisation, and random selection of cells to be measured. 24 hours later cells were either left untreated, treated with PD (50μM), SB (20μM), or Wort (50nM). One hour later cells were either treated with CT-1 (10ng/ml), urocortin ($1x10^{-7}$ M) or left untreated. 48 hours later cells were fixed, X-gal stained and blue cells measured. Single factor ANOVA showed significant differences between groups in both cell area and cell length ($p<0.0001$). Post-hoc Bonferroni tests were then performed to test for significant differences between treated and untreated cells.

In the absence of inhibitor, CT-1 caused a 64% increase in mean cell area, which was highly significant ($p<0.0001$) in a t-test. Urocortin caused a 43% increase in mean cell area ($p=0.004$) (Figure 5.5.1).

In cells pre-treated with PD, CT-1 caused a 49% increase ($p<0.0001$) in mean area and urocortin a 59% increase ($p<0.0001$). PD treatment caused no significant change in cell area in untreated cells (Figure 5.5.1). This shows that PD does not significantly inhibit the hypertrophic effect of CT-1 or urocortin. This supports the data presented in figure 5.3.1 where a dominant negative MEK-1 construct did not inhibit the hypertrophic effect of CT-1 or urocortin. PD also inhibits MEK-1, so both these results show that the hypertrophic effect of CT-1 and urocortin occurs independently from the p42/p44 MAPK pathway.

Treatment with SB caused an increase in mean cell area compared to untreated cells, but this was not significant in a Bonferroni test ($p=0.1$). Treatment with CT-1 or urocortin in the presence of SB caused a 19% increase in cell size, but this was not significant in

either case. There was no significant difference between CT-1 treated cell area in the presence or absence of SB (Figure 5.5.1). The lack of a significant increase in cell area in response to CT-1 or urocortin in the presence of SB is due to the enlargement of control cells treated with SB. Inhibition of the P38 MAPK pathway with SB caused hypertrophy, treatment with CT-1 or urocortin caused a small, but not significant additional increase in cell size. There was no significant difference between CT-1 or urocortin treated cells in the presence or absence of SB. It therefore does not appear that SB is blocking the hypertrophic effect of CT-1 or urocortin but it is impossible to be sure due to the increase in control cell size caused by SB alone.

In cells pre-treated with wortmanin, CT-1 caused a 54% increase in cell area ($p=0.0001$) and urocortin a 38 % increase ($p=0.005$). Wortmanin treatment itself caused no significant change in cell area (Figure 5.5.1). This shows that inhibition of the PI3 kinase pathway by wortmanin has no effect on the hypertrophic effect of CT-1 or urocortin.

Cell length was also measured and showed the same pattern as for cell area. As shown previously, results for cell length were more variable and less significant than results for area (Figure 5.5.2).

Figure 5.5.1 The effect of chemical inhibitors on cell area.

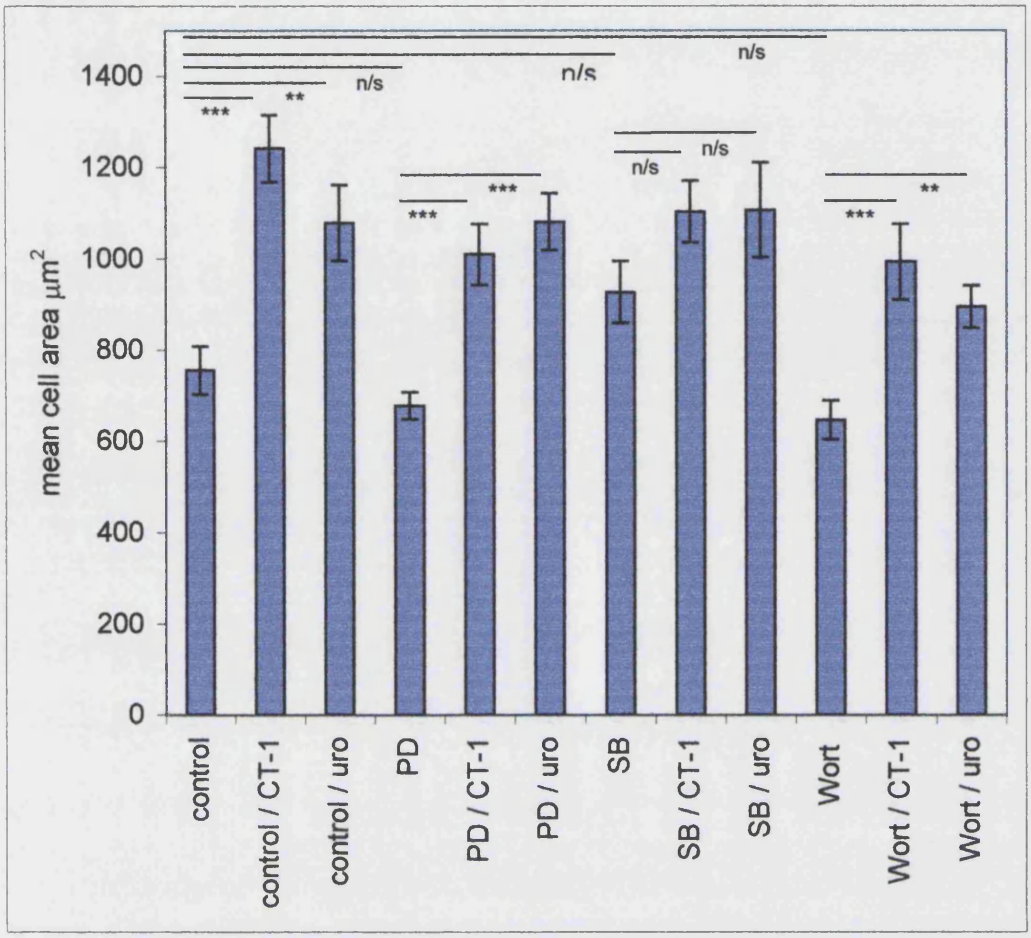


Figure 5.5.1 Primary cells were transfected with a cmv-βgal expression vector. 24 hours later cells were either left untreated, treated with PD (50µM), SB (20µM), or Wort (50nM). One hour later cells were either treated with CT-1 (10ng/ml), urocortin ($1x10^{-7}$ M) or left untreated. 48 hours later cells were fixed, X-gal stained and blue cells measured. Single factor ANOVA showed significant differences between groups in cell area ($p<0.0001$). Mean cell area was plotted +/- standard error. Bonferroni tests were then performed to test for significant differences between treated and untreated cells, as shown by horizontal bars, with significance levels depicted by stars as follows: * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) or n/s (no significance).

Figure 5.5.2 The effect of chemical inhibitors on cell length

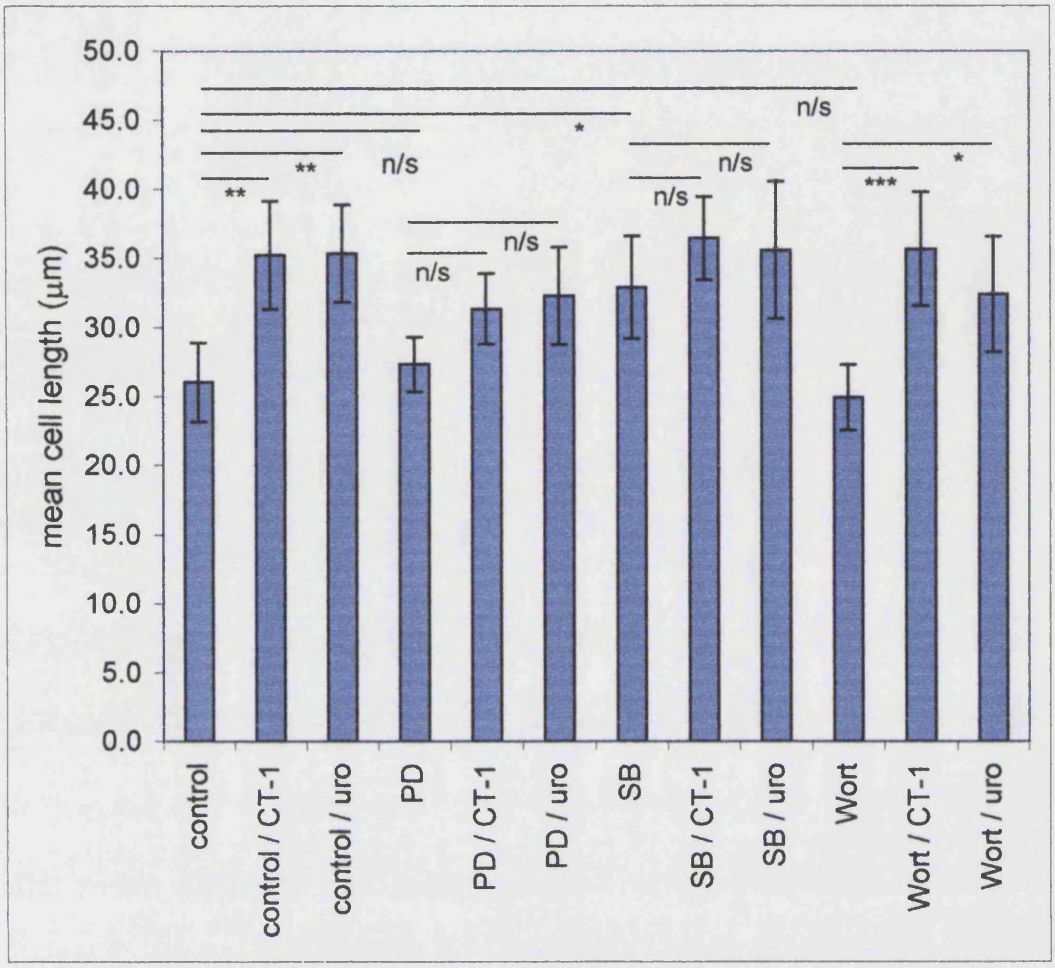


Figure 5.5.2 Primary cells were transfected with a cmv-βgal expression vector. 24 hours later cells were either left untreated, treated with PD (50µM), SB (10µM), or Wort (50nM). One hour later cells were either treated with CT-1 (10ng/ml), urocortin ($1x10^{-7}$ M) or left untreated. 48 hours later cells were fixed, X-gal stained and blue cells measured. Single factor ANOVA showed significant differences between groups in cell length ($p<0.0001$). Mean cell length was plotted +/- standard error. Bonferroni tests were then performed to test for significant differences between treated and untreated cells, as shown by horizontal bars, with significance levels depicted by stars as follows: * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) or n/s (no significance).

4.5.0 Discussion

The hypertrophic response is one of the most important adaptive mechanisms in the heart. It is initially compensatory and maintains normal cardiac function in response to an increased demand on the heart, for example in hypertension or valvular insufficiency. Chronic hypertrophy, however, can lead to an irreversible loss of function and heart failure (Johnson & Dell'Italia, 1996). It is therefore important to investigate the signalling pathways involved in cardiac hypertrophy and to identify the mediators of this response.

It is shown here that urocortin causes hypertrophy in neonatal rat cardiac myocytes. This finding introduces a new candidate ligand in the search to find possible causative agents of hypertrophy in a clinical setting. The knowledge that urocortin is hypertrophic will be an important consideration in any future therapeutic uses of urocortin as a cardioprotective agent. Urocortin has clearly beneficial protective functions in the heart (Brar *et al.*, 1999a; Brar *et al.*, 2000) but these beneficial effects may be compromised by potentially detrimental hypertrophic effects. It is therefore important to investigate the signalling pathways involved in the hypertrophic response, to see if the hypertrophic effect can be dissected away from the beneficial protective effect of urocortin.

It is also shown that urocortin and CT-1 are hypertrophic in adult rat cardiac myocytes. Adult rat cardiac myocytes are morphologically more similar to adult human cardiac myocytes than are neonatal cells. Adult cells are seen as distinct rods, whereas neonatal cardiac myocytes form a monolayer of asymmetric cells. The increase in cell size in response to hypertrophic stimuli is much greater in neonatal cells than that observed in adult cardiac myocytes. This is likely to be because neonatal cells retain an increased capacity for growth and survive better in culture.

The hypertrophic effect observed in neonatal cardiac myocytes is also greater than that observed in vivo. In vivo administration of CT-1 for 14 days in mice caused an increase in mean ventricular weight: body weight ratio of 8% (Jin *et al.*, 1996). This hypertrophic effect was not specific to the heart and increases in spleen, liver and kidney weights were also observed. Experimental congestive heart failure in dogs lead to a 16% increase in left ventricular mass index with a positive correlation to CT-1 gene

expression (Jougasaki *et al.*, 2000). It is important to note that an in vivo system also contains non-cardiac cells, which may not undergo hypertrophy and thus may account for the smaller increase in size.

Both the p42/p44 MAPK and STAT3 pathways are known to be important in cardiac hypertrophy. Transgenic mice with constitutively active ras, an upstream activator of the p42/p44 MAPK pathway, develop ventricular hypertrophy (Hunter *et al.*, 1995). Additionally, inhibition of this pathway by antisense oligonucleotides to p42 and p44 MAPKs down regulates the phenylephrine (PE) induced hypertrophic response (Glennon *et al.*, 1996). Sheng et al (Sheng *et al.*, 1997), however, reported that activation of the p42/p44 MAPK pathway is not required for CT-1 mediated cardiac hypertrophy, indicating that the alternative STAT3 pathway may be involved. Phosphorylation (activation) of STAT3 occurs in the heart in response to hypertrophic stimuli such as pressure overload (Pan *et al.*, 1997) and mechanical stretch (Pan *et al.*, 1999). In both these cases, STAT3 phosphorylation was mediated by gp130 (Pan *et al.*, 1998; Pan *et al.*, 1999). Additionally, STAT3 has been shown to activate the angiotensinogen promoter in cardiac cells and this is thought to be a cellular signal for hypertrophy in cardiac muscle (Mascareno *et al.*, 1998). More direct evidence for the role of STAT3 signalling in CT-1 mediated cardiac hypertrophy comes from a study by Kunisada et al (Kunisada *et al.*, 1998), where replication-defective adenoviral vectors carrying wild type or mutant STAT3 were used to infect cardiac myocytes. The hypertrophic effect of the CT-1 related factor, LIF (as assayed by induction of c-fos, ANP and protein synthesis) was augmented by wild type STAT3 and attenuated by mutant STAT3. These effects occurred independently from any changes in MAPK activity. The receptors for LIF and CT-1 both have gp130 and LIFR components, so LIF and CT-1 have many parallel effects.

It is shown here that hypertrophy caused by CT-1 is directly dependent on the STAT3 pathway. Inhibition of STAT1 or p42/p44MAPK signalling did not block the hypertrophic effects of CT-1, whereas inhibition of the STAT3 pathway did block the hypertrophic effect. This shows that the STAT3 pathway is specifically required for this response. CT-1 is cardioprotective and its use therapeutically may depend on the ability to inhibit its hypertropic effects while maintaining cardioprotective functions.

Inhibition of the STAT1 pathway caused a reduction in the hypertrophic effect of urocortin. There is no evidence to date that urocortin activates the STAT1 pathway, but it is possible that STAT1 interacts with other factors induced by urocortin to augment its hypertrophic effect. Inhibition of STAT3 signalling had no effect on the hypertrophic effect of urocortin. This shows that urocortin is causing hypertrophy by a different pathway than CT-1, which is dependent on STAT3 signalling for its hypertrophic effects. The only signalling pathways known to be activated by urocortin are the p42/p44 MAPK pathway, which is necessary for its cardioprotective effects (Brar *et al.*, 2000), and the protein kinase A (PKA) pathway, which is required for the induction of ANP gene expression by urocortin (Ikeda *et al.*, 1998). Inhibition of the p42/p44MAPK pathway using dominant negative MEK-1 produced a small, but not significant decrease in the hypertrophic response to urocortin. It is likely that another, as yet unidentified pathway is important in mediating the hypertrophic response to urocortin. STAT1 and p42/p44MAPK may play a role in this signalling, but inhibition of these pathways only has a slight effect on the urocortin mediated hypertrophic response, which implies that other factors must be involved. In view of the role of the PKA pathway in the induction of ANP by urocortin, this pathway is the most likely candidate for mediating the hypertrophic effect of urocortin.

It is shown here that the protective effects of CT-1 and urocortin are inhibited by transfection with dominant negative MEK-1. This confirms studies showing that the protective effects of CT-1 (Sheng *et al.*, 1997) and urocortin (Brar *et al.*, 2000) are dependent on the p42/p44 MAPK pathway and indicates that the dn MEK-1 is effectively blocking this pathway despite the lack of any effect on the hypertrophic effect of CT-1 or urocortin. These previous studies both used the chemical inhibitor PD98059, which inhibits MEK-1, whereas a dominant negative signalling molecule was used here to confirm these findings.

Inhibition of the STAT3 pathway had no significant effect on the protective effects of CT-1 or urocortin. The involvement of the STAT3 pathway in the protective effect of CT-1 or urocortin has not previously been tested. The lack of chemical inhibitors for STAT3 has resulted in little investigation into the effects of this pathway. In this study, use of genetic inhibitors has enabled the effect of inhibition of the STAT3 and STAT1 pathways on the hypertrophic and protective effects of CT-1 and urocortin to be tested.

The results presented in this chapter reveal that the hypertrophic and protective effects of CT-1 are indeed mediated via distinct pathways and that it is possible to block one effect and to leave the other intact. Hence, inhibition of the STAT3 pathway blocks the hypertrophic effect of CT-1 but has no significant effect on protection. Inhibition of the P42/p44 MAPK pathway blocks the protective effect of CT-1 and urocortin but does not alter the hypertrophic effect. None of the signalling molecules tested had any significant effect on the hypertrophic effect of urocortin. Further studies will be required to identify the pathway involved in this effect.

Inhibition of the STAT1 pathway had no effect on hypertrophy mediated by CT-1 and a slight effect on hypertrophy caused by urocortin. The effect on protection was more significant; inhibition of the STAT1 pathway protected cells against death caused by hypoxic stress. This agrees with a previous study, which outlined a potential role for STAT1 in ischemia induced apoptosis in cardiac myocytes (Stephanou *et al.*, 2000). In the study by Stephanou *et al.*, STAT1 was activated by simulated ischemia, additionally, over expression of active STAT1 caused apoptosis in cardiac myocytes, and transfection of antisense STAT1 was shown to reduce cell death caused by ischemic injury. The results presented here, using transfected protein inhibitor of activated STAT1 (PIAS1) also show that inhibition of STAT1 signalling protects cardiac myocytes against hypoxic stress. Activated STAT1 has been shown to activate the promoter of the pro-apoptotic gene caspase 1 (Stephanou *et al.*, 2000). Activation of the caspase cascade is therefore a possible mechanism for the pro-apoptotic effect of activated STAT1.

Use of chemical inhibitors showed that the hypertrophic effects of CT-1 and urocortin are not dependent on the p42/p44 MAPK or PI3 kinase pathways. The results with PD, which inhibits the p42/p44 MAPK pathway at the level of MEK-1 support those seen with dominant negative MEK-1. The PI3 kinase pathway has recently been shown to be activated by CT-1 (Kuwahara *et al.*, 2000). Akt is a down stream target for P13 kinase and is also phosphorylated by CT-1 (Kuwahara *et al.*, 2000). Transfection of constitutively active Akt has been shown to increase cardiac myocyte surface area, protein content and ANF expression (Morisco *et al.*, 2000). In the same study, dominant negative Akt inhibited the increased expression of ANF caused by the β-

adrenergic agonist isoproterenol. This shows that the PI3 kinase pathway can mediate hypertrophy. However, it is shown here that the hypertrophic effects of CT-1 or urocortin are not mediated by this pathway. In this study, the PI3 kinase pathway was inhibited with wortmannin and this had no effect on the hypertrophic effect of CT-1 or urocortin. It is however possible that CT-1 or urocortin may be activating Akt by a PI3 kinase independent mechanism, and such an effect would not be blocked by wortmanin. It is therefore possible that Akt, activated by a PI3 kinase independent mechanism may be involved in the hypertrophic response to CT-1 or urocortin. There is, however, no evidence for such a pathway.

Inhibition of the P38 MAPK pathway with the chemical inhibitor SB202190 caused an increase in mean cell area. This may be due to a negative regulatory role for P38 MAPK on basal cell size. There are, however no previous studies to support such a role and several studies suggest a hypertrophic role for the P38 MAPK pathway. In cardiac myocytes, activation of P38 MAPK stimulated ANF expression and endothelin-1 mediated ANF expression was dependent on the P38 MAPK pathway (Nemoto *et al.*, 1998). Additionally, changes in hypertrophic morphology were observed in the presence of SB in response to endothelin-1, with cells becoming thinner and more elongated. However, no comparison was made between cell size in control versus SB treated cells in the study by Nemoto *et al.*

Chemical inhibitors are useful tools but they can have non-specific effects and sometimes may inhibit signalling pathways other than those to which they are specifically targeted. In this study chemical inhibitors have only been used to eliminate certain pathways, such as PI3 kinase and P38 MAPK from a possible role in the hypertrophic effects of CT-1 and urocortin. This data must be regarded as preliminary and the use of genetic inhibitors, as carried out for the p42/p44 MAPK, STAT1 and STAT3 pathways, would be required to further investigate the role of other pathways in hypertrophy

Further work will also be necessary to investigate the interaction of the hypertrophic STAT3 pathway and the protective p42/p44MAPK pathway in cardiac myocytes after CT-1 treatment. It is already clear however, that the CT-1 mediated induction of hypertrophy involves a distinct signalling pathway to its protective effect

The finding presented here that the hypertrophic effect of CT-1 could be blocked by inhibition of STAT3, while preserving the protective effect is of potential clinical relevance. It would also be of interest to discover whether other clinically relevant models of hypertrophy can also be blocked by inhibition of STAT3 signalling.

CHAPTER 6

HEAT SHOCK PROTEINS AND HYPERTROPHY

6.0.1 Introduction

CT-1 causes hypertrophy (Pennica *et al.* 1995a) and modulates hsp levels in cardiac myocytes (Stephanou *et al.*, 1998a). Heat shock proteins have many functions in the cell, but the effect of heat shock proteins on cell size or cardiac hypertrophy has not previously been investigated.

Elevated levels of heat shock proteins have been demonstrated in many situations where hypertrophy also occurs. Transcription of the hsp70 gene is increased by volume overload in the rat ventricle (Huang *et al.*, 1994). Elevated synthesis of hsp70, hsp68, and hsp56 was also observed in isolated adult rat cardiac myocytes 2-4 days after hemodynamic overload (Delcayre *et al.*, 1988). Both these studies show that hsps can be induced by hypertrophic stimuli. It is generally thought that the hsps are induced by stress and serve to protect the cells against stressful stimuli. No previous studies have investigated a potential role for hsps in the hypertrophic response.

The only documented link between hsps and hypertrophy is through the binding of hsp56 to the immunosuppressive drug FK506. There is a great deal of literature (for review see (Sugden, 1999)) showing that inhibition of calcineurin by FK506 modulates the hypertrophic response. There are, however, many contradictory reports. This chapter will therefore commence with an investigation of the effect of FK506 on hypertrophy.

6.1.1 The effect of FK506 on hypertrophy

FK506 is an immunosuppressive drug, which has been shown to bind to hsp56 (Yem *et al.*, 1992). Binding of FK506 to hsp56 (or another FK506 binding protein) forms a complex, which can then interact with and inhibit the calcium dependent phosphatase calcineurin (Liu *et al.*, 1991).

It has been shown that activation of calcineurin is both necessary, and sufficient to induce cardiac hypertrophy in cardiac myocytes (Molkentin *et al.*, 1998). In the same study, FK506 treatment has been shown to block the hypertrophic response to PE or Angiotensin II in cardiac myocytes. This work suggests that calcineurin is an important component of the hypertrophic response. Conversely, FK506 has been shown to cause cardiac hypertrophy in the clinical setting (Atkison *et al.*, 1995; Chang *et al.*, 1998).

The effect of FK506 on hypertrophy is therefore unclear as in some cases it prevents hypertrophy and in others seems to cause hypertrophy.

It was therefore decided to test whether the hypertrophic effects of CT-1 or urocortin are affected by FK506, and whether FK506 itself has any effect on cell size in this system.

Primary cells were transfected with a cmv-β-gal expression vector to allow random staining of individual cells. 24 hours later cells were left untreated, or treated with FK506 (250ng/ml). One hour later cells were left untreated, or treated with CT-1 (10ng/ml) or urocortin ($1x10^{-7}$M) for 48 hours. Cells were then fixed, X-gal stained and blue cells were measured. Mean cell area and mean cell length were calculated, single factor ANOVA was used to test for significant differences between groups, and showed significant differences in cell area ($p<0.0001$) and cell length ($p<0.0001$). Post-hoc Bonferroni tests were then performed to test for significant differences in cell size between each treatment and control cells. P values for Bonferroni tests are shown below as each treatment is discussed.

As seen previously, CT-1 and urocortin caused a significant increase in cell size. CT-1 caused a 50% increase in mean cell area ($p=0.006$) and urocortin a 34% increase ($p=0.04$). Cells treated with FK506 were not significantly different in size to untreated cells (Figure 6.1.1). This shows that FK506 does not affect cell size in this system.

Cells treated with CT-1 in the presence of FK506 were not significantly different in size compared to cells treated with CT-1 alone. CT-1 treatment caused a 34% increase in cell area in cells pre-treated with FK506 compared to cells treated with FK506 alone ($p=0.005$). Urocortin caused a 24% increase in mean cell area in the presence of FK506 ($p=0.02$). Additionally, cells treated with urocortin in the presence of FK506 were not significantly different in size to cells treated with urocortin alone (Figure 6.1.1). This shows that FK506 has no significant effect on the hypertrophic effect of CT-1 or urocortin.

Cell length was also measured and similar results obtained (Figure 6.1.2). The only difference in the results for cell length compared to area was that urocortin failed to cause a significant increase in cell length in the presence of FK506. There was,

however, a significant increase in area. FK506 treatment reduced the hypertrophic effect of both CT-1 and urocortin. CT-1 alone caused a 50% increase in cell area and a 47% increase in cell length, while CT-1 in the presence of FK506 caused a 34% increase in area and a 25% increase in length. Urocortin alone caused a 34% increase in cell area and a 25% increase in cell length, while urocortin in the presence of FK506 caused a 24% increase in area but no increase in length.

Although not statistically significant, the reduction in the hypertrophic effect of CT-1 and urocortin caused by FK506 treatment may indicate a role for calcineurin, or another factor affected by FK506 in the hypertrophic response to CT-1 and urocortin.

Figure 6.1.1 The effect of FK506 on cell area.

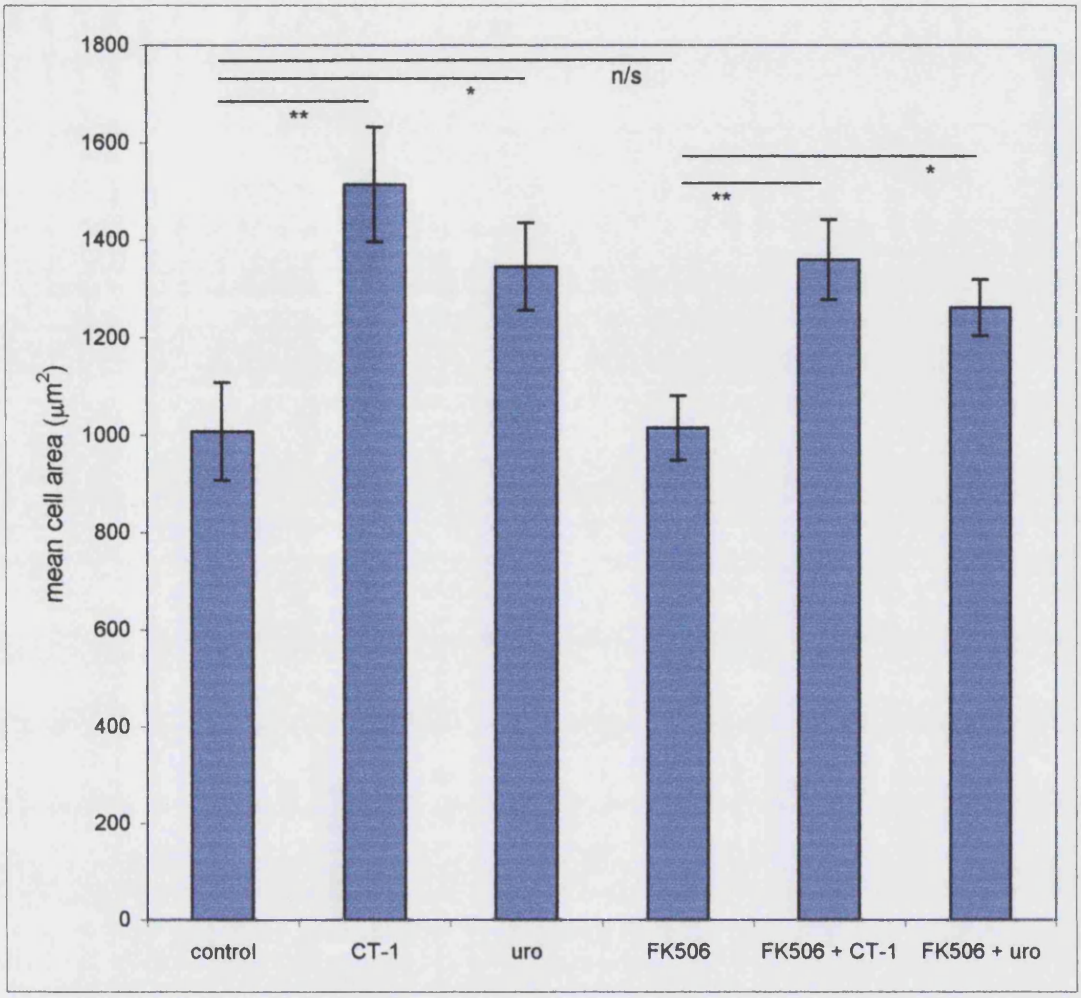


Figure 6.1.1 Primary cells were transfected with a cmv-β-gal expression vector to allow easy measurement of individual cells. 24 hours later cells were left untreated, or treated with FK506 (250ng/ml). One hour later cells were left untreated, or treated with CT-1 (10ng/ml) or urocortin ($1x10^{-7}$M) for 48 hours). Cells were then fixed, X-gal stained and blue cells were measured. Mean cell area was calculated and plotted +/- standard error, single factor ANOVA showed significant differences in mean cell area ($p<0.0001$). Post-hoc Bonferroni tests were then performed to test for significant differences in cell size between each treatment and control cells, as shown by horizontal bars. Significance levels are shown as follows: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), n/s (no significant difference).

Figure 6.1.2 The effect of FK506 on cell length

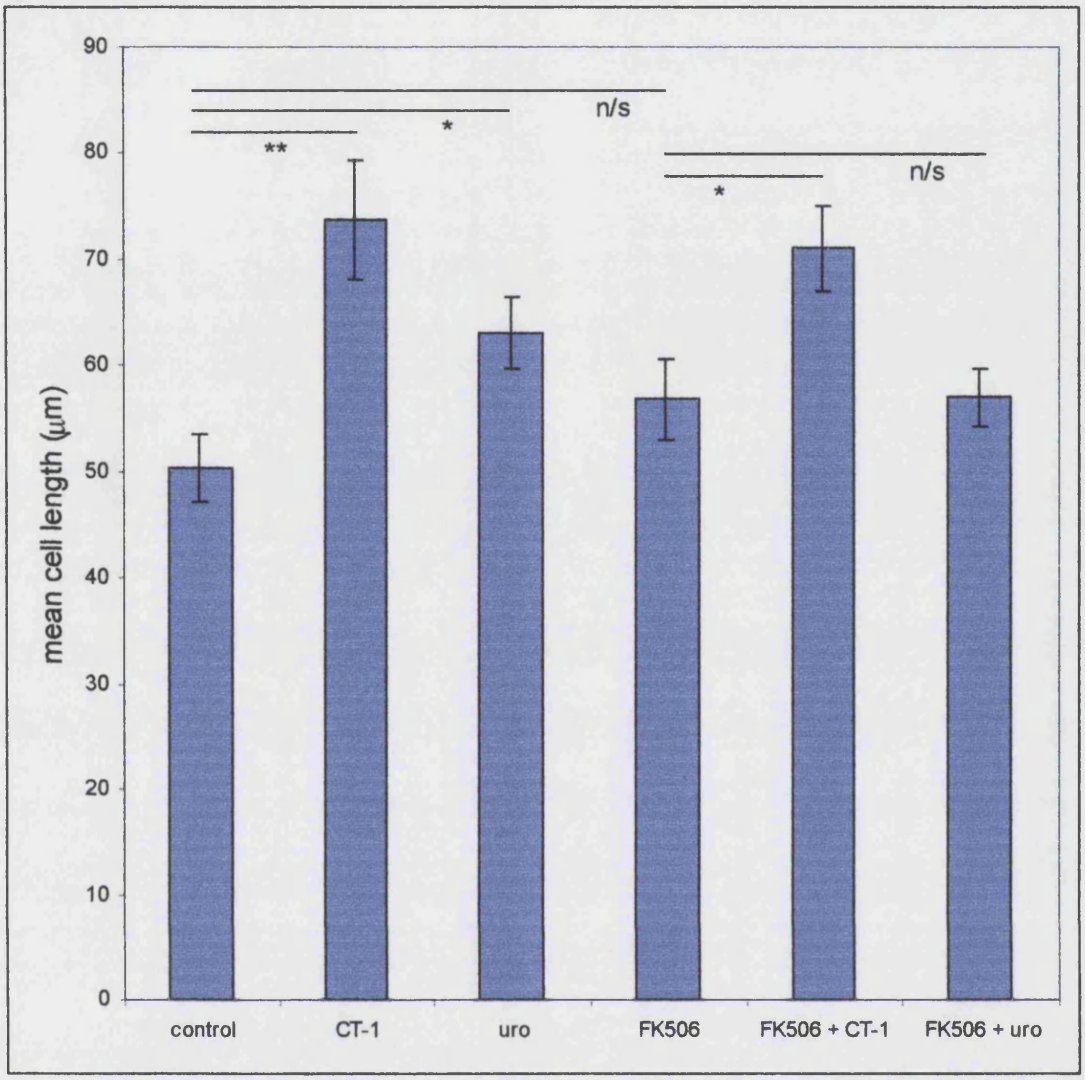


Figure 6.1.2 Primary cells were transfected with a cmv-β-gal expression vector to allow easy measurement of individual cells. 24 hours later cells were left untreated, or treated with FK506 (250ng/ml). One hour later cells were left untreated, or treated with CT-1 (10ng/ml) or urocortin ($1x10^{-7}$M) for 48 hours). Cells were then fixed, X-gal stained and blue cells were measured. Mean cell length was calculated and plotted +/- standard error, and single factor ANOVA showed differences in cell length ($p<0.0001$). Post-hoc Bonferroni tests were then performed to test for significant differences in cell length between each treatment and control cells, as shown by horizontal bars. Significance levels are shown as follows: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), n/s (no significant difference).

6.2.1 The effect of hsp over expression on cell size.

In order to determine whether hsps have any effect on cell size, expression vectors were transfected to over express hsps and monitor the effect on cell size. Primary cells were transfected with a cmv-β-gal expression vector and co-transfected with either: empty vector (control), pR19-27 to over express hsp27, pR19-56 to over express hsp56, pR19-70 to over express hsp70, or pR19-90 to over express hsp90. PR19 plasmids contain their respective heat shock protein cDNA with expression driven by the cmv promoter. As a positive control cells transfected with control plasmid were treated with CT-1. 48 hours later cells were fixed, X-gal stained and measured. Mean cell area and mean cell length was calculated and single factor ANOVA performed to test for significant differences between groups. The ANOVA showed significant differences in area ($p<0.0001$) and cell length ($p<0.0001$). Post-hoc Bonferroni tests were also performed to test for significant differences between transfection with control plasmid and each other plasmid. P values for Bonferroni tests are shown below as the effect of each hsp is discussed.

In cells transfected with control plasmid, treatment with CT-1 caused a 26% increase in mean cell area ($p=0.03$) (Figure 6.2.1).

Transfection with hsp27, hsp70 or hsp90 expression vectors caused no significant change in cell size. However, co-transfection with the hsp56 expression vector caused a 58% increase in mean cell area ($p<0.0001$) (Figure 6.2.1).

Cell length was also measured and a similar pattern was observed with a 33% increase in mean cell length in response to CT-1 ($p=0.01$) and a 55% increase with over expression of hsp56 ($p<0.0001$) (Figure 6.2.2).

This shows that over expression of hsp56 is sufficient to cause hypertrophy as defined by an increase in cell size in cardiac myocytes. Over expression of hsp27, hsp70 or hsp90 did not have any effect on cell size.

Figure 6.2.1 The effect of hsp over expression on cell area.

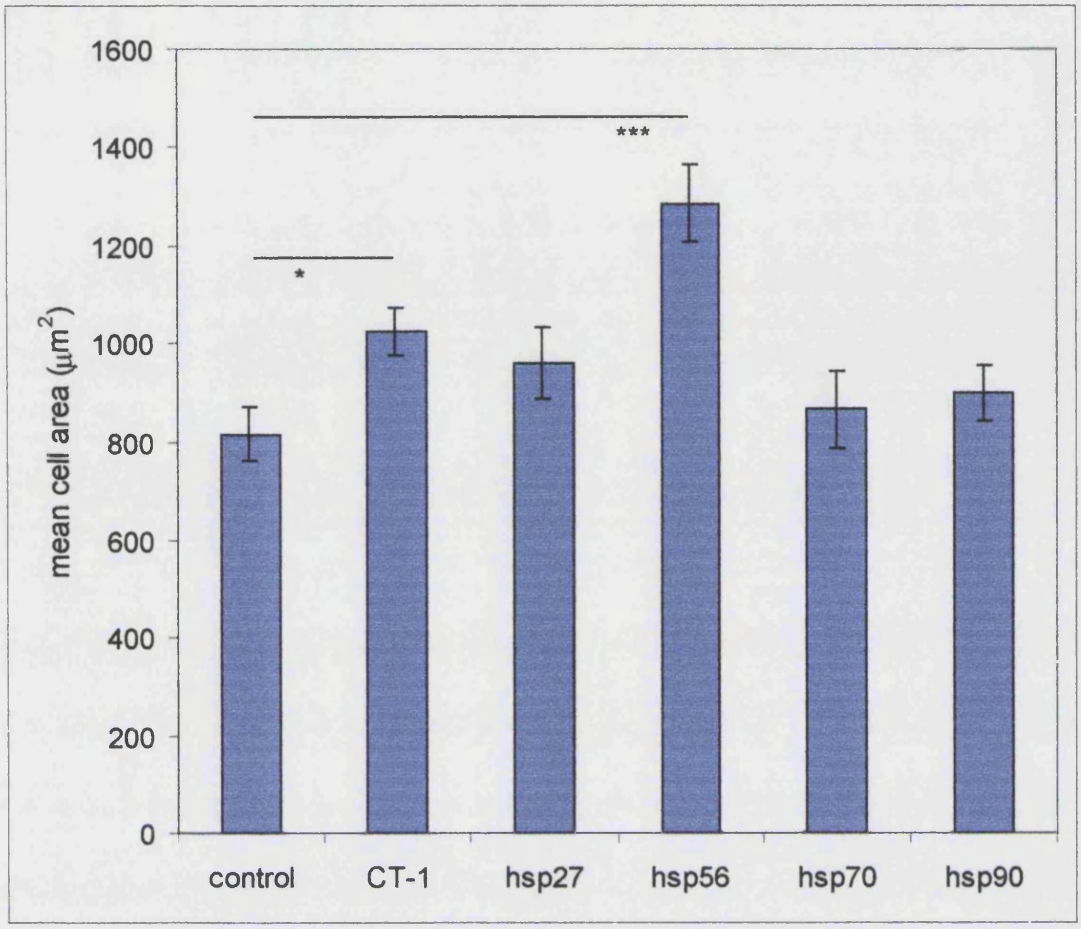


Figure 6.2.1 Primary cells were transfected with a cmv-β-gal expression vector and co-transfected with either: empty vector (control), pR19-27 (hsp27), pR19-56 (hsp56), pR19-70 (hsp70), or pR19-90 (hsp90). Cells transfected with control plasmid were left untreated or treated with CT-1 (10ng/ml). Cells transfected with hsp expression vectors were left untreated. 48 hours later cells were fixed, X-gal stained and measured. Mean cell area was calculated plotted on a graph +/- standard error. ANOVA showed significant differences in cell area ($p<0.0001$). Bonferroni tests were performed to test for significant differences between transfection with control plasmid and each other plasmid. Bonferroni tests are indicated by horizontal bars and p values are represented as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), the absence of a comparative bar indicates no significant difference.

Figure 6.2.2 The effect of hsp over expression on cell length

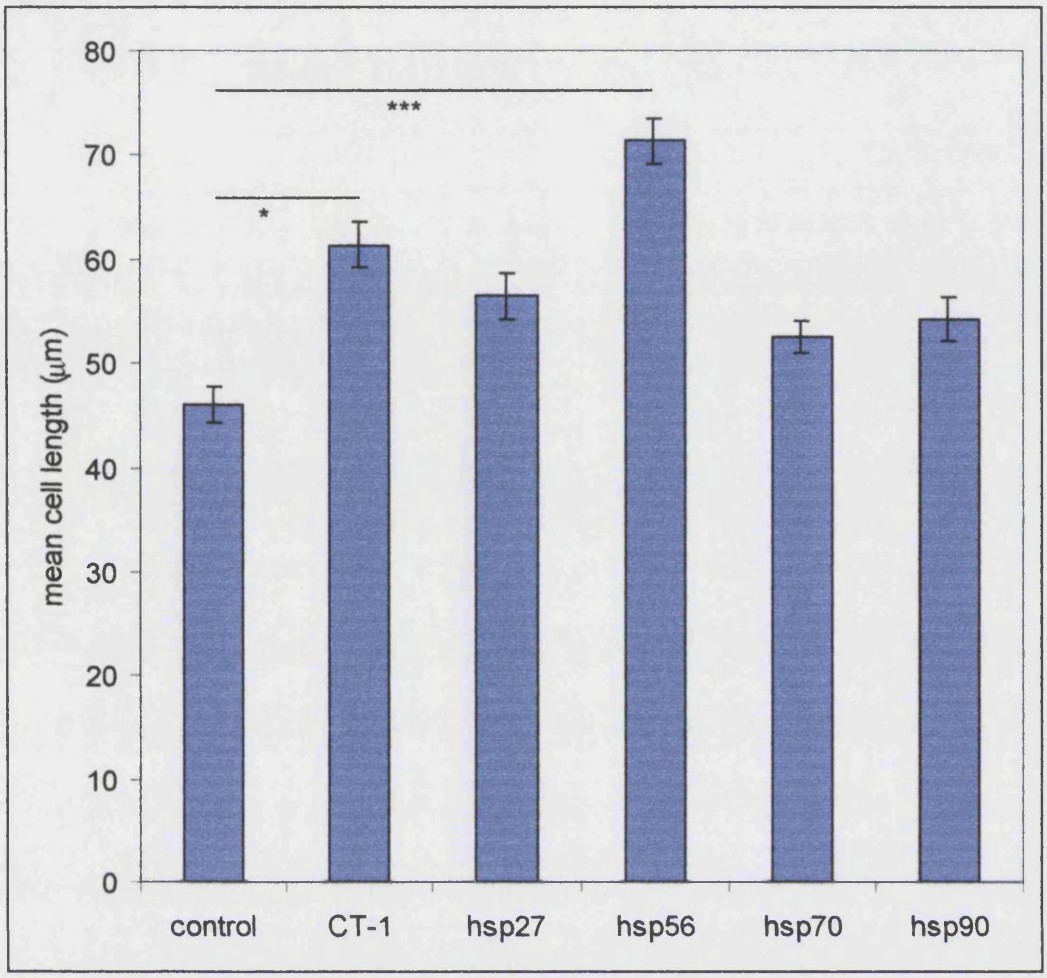


Figure 6.2.2 Primary cells were transfected with a cmv-β-gal expression vector and co-transfected with either: empty vector (control), pR19-27 (hsp27), pR19-56 (hsp56), pR19-70 (hsp70), or pR19-90 (hsp90). Cells transfected with control plasmid were left untreated or treated with CT-1 (10ng/ml). Cells transfected with hsp expression vectors were left untreated. 48 hours later cells were fixed, X-gal stained and measured. Mean cell length was calculated plotted on a graph +/- standard error. ANOVA showed significant differences in cell length ($p<0.0001$). Bonferroni tests were performed to test for significant differences between transfection with control plasmid and each other plasmid. Bonferroni tests are indicated by horizontal bars and p values are represented as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), the absence of a comparative bar indicates no significant difference.

6.3.0 Over expression of hsps using Herpes viral vectors.

To confirm the results presented in section 6.2, Herpes viral vectors were used to over express hsps with high efficiency in cardiac myocytes. Transfection of cardiac myocytes by the calcium phosphate method only successfully transfects 1-5% of cardiac myocytes, but these can be selectively measured by staining for a transfected β-galactosidase marker gene. Gene transfer with Herpes viral vectors, however, can achieve transfection of 80-100% of cells. Due to the high efficiency of gene transfer, cells infected with viral vectors can be measured without co-transfection of a marker gene.

6.3.1 Verification of hsp over expression by viral vectors.

In order to confirm that the viral vectors over express their relevant hsp, western blots were performed. Additionally, it was important to verify that hsps were not being induced by control viruses due to cells being stressed by viral infection.

Primary cells were infected with viral vectors at a multiplicity of infection (MOI) of 1, i.e. 1 plaque forming unit of virus per cell. 24 hours after plating cells were either left uninfected or infected with viral vector expressing; gfp (green fluorescent protein), β-gal (β-galactosidase), hsp56, or hsp70. Unfortunately, no hsp90 virus was available. Cells infected with gfp virus were observed under a fluorescent microscope to visualize green fluorescent cells and monitor infection efficiency. 48 hours after infection cells infected with gfp were observed under fluorescent microscopy and over 90% of cells fluoresced green. All cells were then harvested and western blots performed to quantify hsp levels. Membranes were probed with antibodies for hsp70, hsp56 and actin to equalize for protein loading. Due to its lack of effect on cell size, and shortage of antibody, hsp27 virus was not used in this experiment.

Hsp56 was strongly induced in cells infected with hsp56 virus and no hsp56 was detected in cells infected with the other viruses or in uninfected cells. Hsp70 was strongly induced in cells infected with hsp70 virus and was absent from cells infected with the other viruses. No hsp70 was detected in uninfected cells (Figure 6.3.1).

Induction of hsp56 was specific to cells infected with hsp56 virus and hsp70 virus caused a specific induction of hsp70. Therefore the viruses are successfully over

expressing their particular hsp specifically and to a high level. No endogenous hsp56 was detected because the antibody used was raised to rabbit hsp56 and did not recognise rat hsp56. This was the only antibody available at the time of these experiments. The hsp56 expressed by virus or plasmid in this study was derived from rabbit cDNA so was recognised by the antibody.

Hsp70 was not induced by infection with control virus. This shows that viral infection is not causing a stress response in the cells.

Figure 6.3.1 Over expression of hsps with Herpes viral vectors

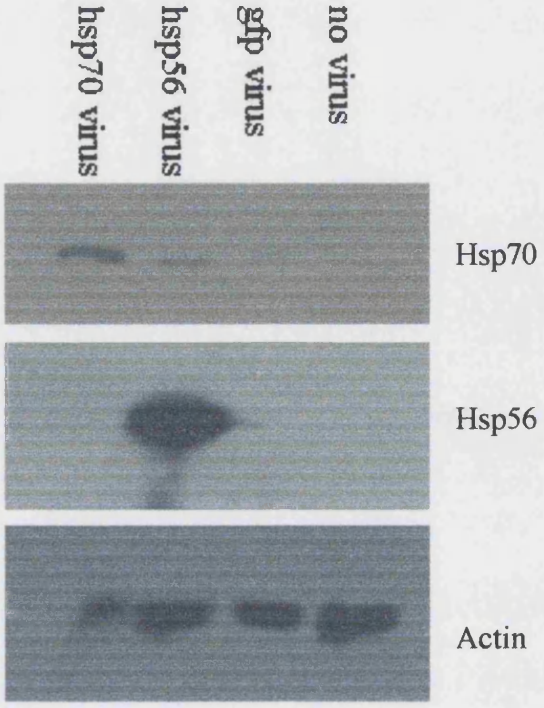


Figure 6.3.1 Primary cells were infected with gfp virus, hsp56 virus or hsp70 virus, and uninfected cells were used as a negative control. 48 hours after infection cells were harvested and western blots performed to quantify hsp levels. Membranes were probed with antibodies for hsp70, hsp56 and actin to equalize for protein loading.

6.3.2 The effect of viral hsp over expression on cell size

Primary cells were infected with viral vectors at a multiplicity of infection (MOI) of 1. 24 hours after plating cells were either left uninfected or infected with viral vector expressing; gfp, β-gal, hsp27, hsp56, or hsp70. 48 hours after infection cells were fixed. Cells infected with gfp virus were observed under a fluorescent microscope to visualize green fluorescent cells and monitor infection efficiency. Cells infected with β-gal virus were stained with X-gal to stain infected cells blue. All other cells were stained with crystal violet. At least 30 cells per condition were measured and mean cell length and mean cell area were calculated. Single factor ANOVA was carried out to test for significant differences between groups. The ANOVA showed significant differences in area ($p<0.0001$) and length ($p<0.0001$), and post-hoc Bonferroni tests were used to test for significant differences between specific sets of data. P values for Bonferroni tests are given below as infection with each virus is discussed.

Uninfected cells had a mean cell area of 951μm^2, this was increased by 33% to 1264μm^2 by infection with gfp virus ($p=0.01$) and by 28% to 1216μm^2 by infection with β-gal virus ($p=0.001$). This increase in cell size seen in response to viral infection is thought to be due to a reduction in cell plating density. Viral infection results in some cell death, so reduces the number of cells adhered to the plate. The remaining cells then expand to make contact with adjacent cells. This is the same effect as observed in section 4.2.1. Over 90% of cells were infected with β-gal virus or gfp virus as detected by X-gal staining or green fluorescence respectively. Cells infected with gfp or β-gal virus were used as controls for cells infected with viruses to over express hsps.

Viral over expression of hsp27 or hsp70 had no significant effect on cell size compared to cells infected with gfp virus or β-gal virus.

Infection with hsp56 virus caused an increase in mean cell area of 25% above cells infected with gfp virus ($p=0.1$) and a significant 30% increase above cells infected with β-gal virus ($p=0.01$) (Figure 6.3.2).

Cell length was also measured and showed a similar pattern. Infection with gfp virus caused a 27% increase in mean cell length compared to uninfected cells ($p=0.005$) and β-gal virus a 16% increase ($p=0.1$). Hsp27 and hsp70 viruses caused no significant

increase in cell size compared to uninfected cells. Hsp56 virus caused a 19% increase in mean cell length compared to cells infected with β-gal virus ($p=0.05$) and a non-significant 9% increase compared to cells infected with gfp virus (Figure 6.3.3).

This shows that hsp56 over expressed by a Herpes viral vector is causing hypertrophy in neonatal cardiac myocytes but hsp27 and hsp70 have no effect.

Figure 6.3.2 The effect of over expression of hsps with Herpes viral vectors on cell area.

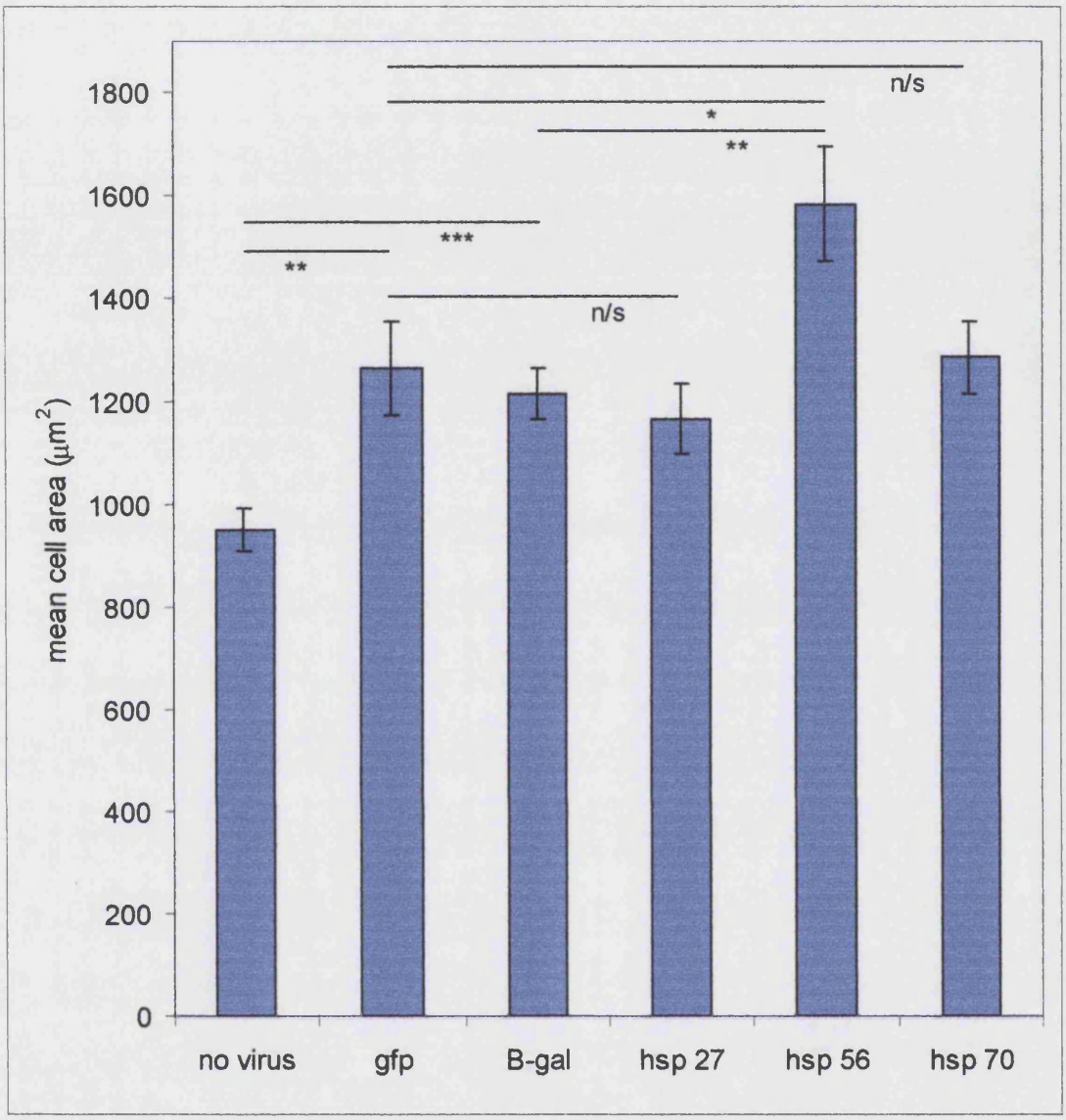


Figure 6.3.2. 24 hours after plating cells were either left uninfected or infected with viral vector expressing; gfp, β-gal, hsp27, hsp56, or hsp70. 48 hours after infection cells were fixed. Cells were measured and mean cell area was calculated and plotted on a graph +/- standard error. One way ANOVA showed significant differences in area ($p<0.0001$). Bonferroni tests were carried out to test for significant differences between specific sets of data, as depicted by horizontal bars. P values are represented as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or n/s (no significant difference).

Figure 6.3.3 The effect of over expression of hsps with Herpes viral vectors on cell length.

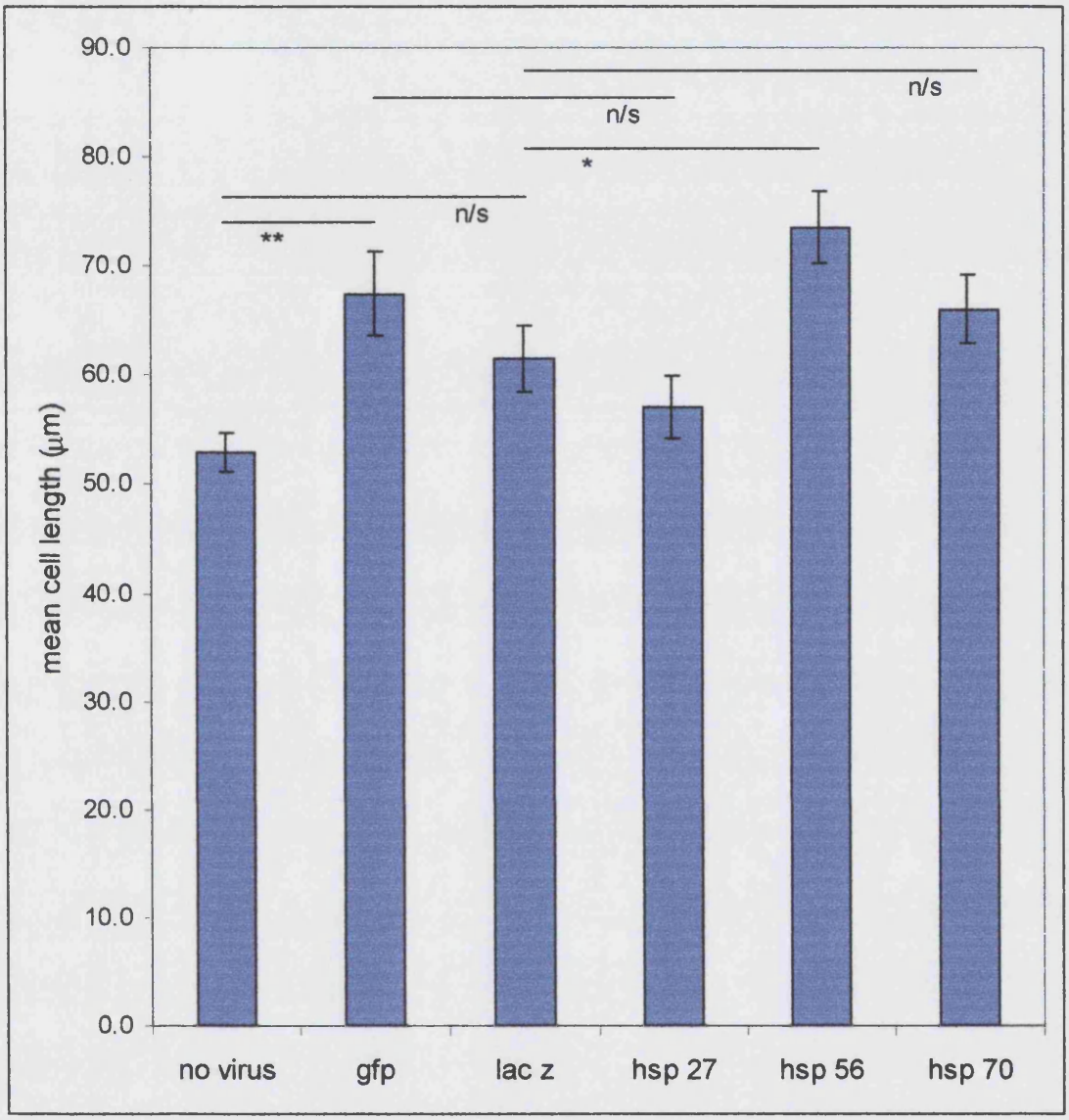


Figure 6.3.3. 24 hours after plating cells were either left uninfected or infected with viral vector expressing; gfp, β-gal, hsp27, hsp56, or hsp70. 48 hours after infection cells were fixed. Cells were measured and mean cell length was calculated and plotted on a graph +/- standard error. One way ANOVA showed significant differences in cell length ($p<0.0001$) Bonferroni tests were carried out to test for significant differences between specific sets of data, as depicted by horizontal bars. P values are represented as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or n/s (no significant difference).

6.4.0 Cloning of hsp56 antisense construct

It has been shown in this chapter that over expression of hsp56 by transient transfection or Herpes viral vectors is sufficient to cause hypertrophy in neonatal cardiac myocytes. Additionally, as shown in chapter 3, treatment with the hypertrophic agents CT-1 or urocortin increases hsp56 protein levels in cardiac myocytes. In order to further investigate the role of hsp56 in cardiac hypertrophy, an antisense construct was created to specifically inhibit hsp56 expression and to test the effect of depletion of hsp56 on the hypertrophic response to CT-1 and urocortin.

The pR19-56 expression vector (as shown in figure 6.4.1) was digested with BamHI and XhoI. This digestion produced the following fragments: Plamid backbone, large insert fragment (1.1kb) and small insert fragment (900kb) (Figure 6.4.2).

The plasmid backbone and large fragment were then purified from the gel using a 'gene clean' kit (see methods). The large insert fragment was ligated into the plasmid backbone. The sticky ends on the large fragment and plasmid backbone ensure that the insert should ligate into the plasmid in the antisense orientation. The ligation was then transformed into bacteria and cultured on agar plates containing 100μg/ml ampicillin to select for successfully transformed bacteria. 20 bacterial colonies were selected, cultured, and small scale DNA 'mini preps' performed to allow screening to identify clones containing the hsp56 insert in the antisense orientation. This DNA was screened by digesting with BamHI and XhoI to check for an insert and XhoI and StuI to check for orientation of the insert. There is a StuI site 200bp from the 3' end of the hsp56 cDNA. If the insert had ligated into the plasmid backbone in an antisense orientation, digestion with XhoI and StuI would result in a 900bp fragment while an insert in the sense orientation would yield a band of 200bp.

Of 20 colonies, 14 yielded DNA and were digested. Of these, one had the correct sized bands from both digests, so was identified as containing an hsp56 cDNA in the antisense orientation (Figure 6.4.3). Clone number 7 was named antisense hsp56, was cultured and a large scale DNA 'midi prep' was carried out to prepare DNA for further experiments.

A southern blot was then performed to verify that the hsp56 cDNA was present in the antisense construct. PR19-56 sense plasmid was left uncut or digested with BamHI and XhoI. Hsp56 antisense was also left uncut or digested with BamHI and XhoI. PR19-90 was run uncut as a negative control. The ethidium bromide gel was photographed on a transilluminator and transferred to membrane. The membrane was probed with a radioactively labelled cDNA probe which had been prepared by digesting pR19-56 with BamHI and 'gene cleaning' the insert. The southern blot confirmed the presence of the hsp56 cDNA in the insert of the antisense construct (Figure 6.4.4).

Figure 6.4.1 pR19-56 plasmid map.

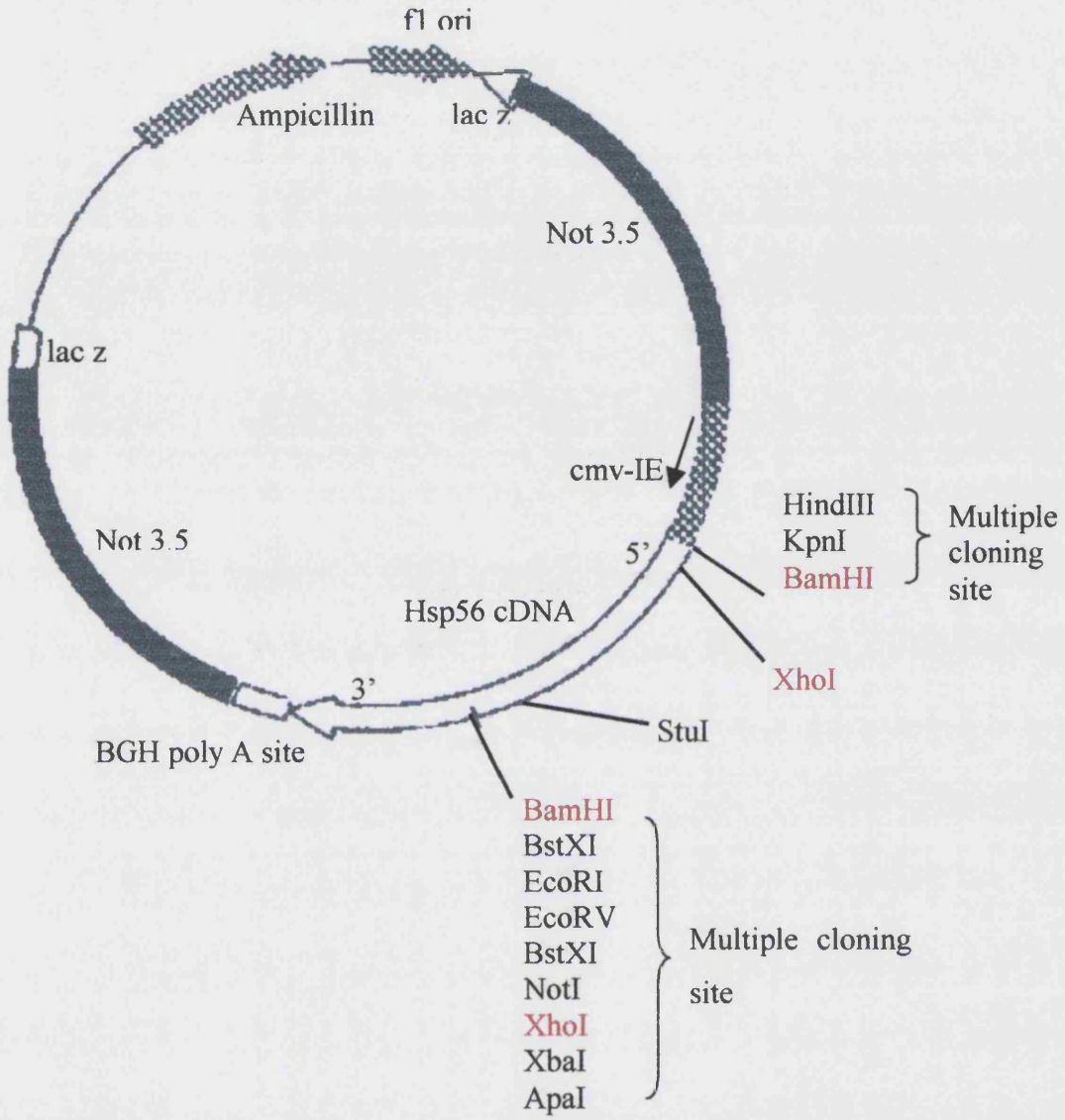


Figure 6.4.1 A schematic diagram of the pR19-56 plasmid. All pR19 plamids contain an ampicillin resistance gene. The Not 3.5 regions are viral flanking regions to allow incorporation into the Herpes viral genome. These are present as these plasmids were originally made in the preparation of Herpes viral vectors. The Not3.5 regions do not affect the ability of the plasmid to act as a mammalian expression vector. The hsp56 cDNA is inserted in the sense orientation at the BamHI site of the multiple cloning site.

Figure 6.4.2 Digestion of pR19-56 with BamHI and XhoI

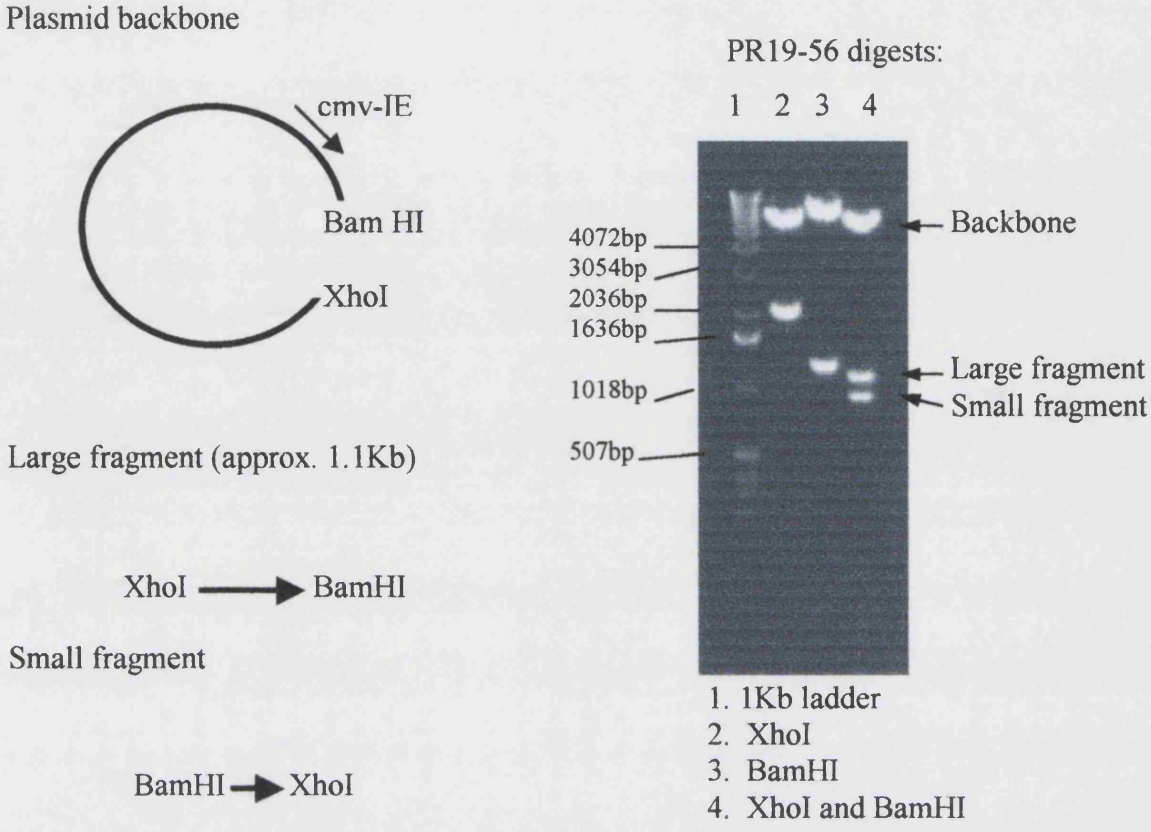


Figure 6.4.2 Digestion of pR19-56 with BamHI and XhoI yields the following fragments: Plasmid backbone with BamHI and XhoI 'sticky ends', large insert fragment (1.1Kb) with XhoI and BamHI sticky ends and small fragment with BamHI and XhoI sticky ends. Arrows indicate 5' to 3' direction. When large fragment is ligated into backbone it should be in antisense orientation due to position of 'sticky ends'.

Figure 6.4.3 Mini prep digests to screen for hsp56 cDNA in antisense orientation.

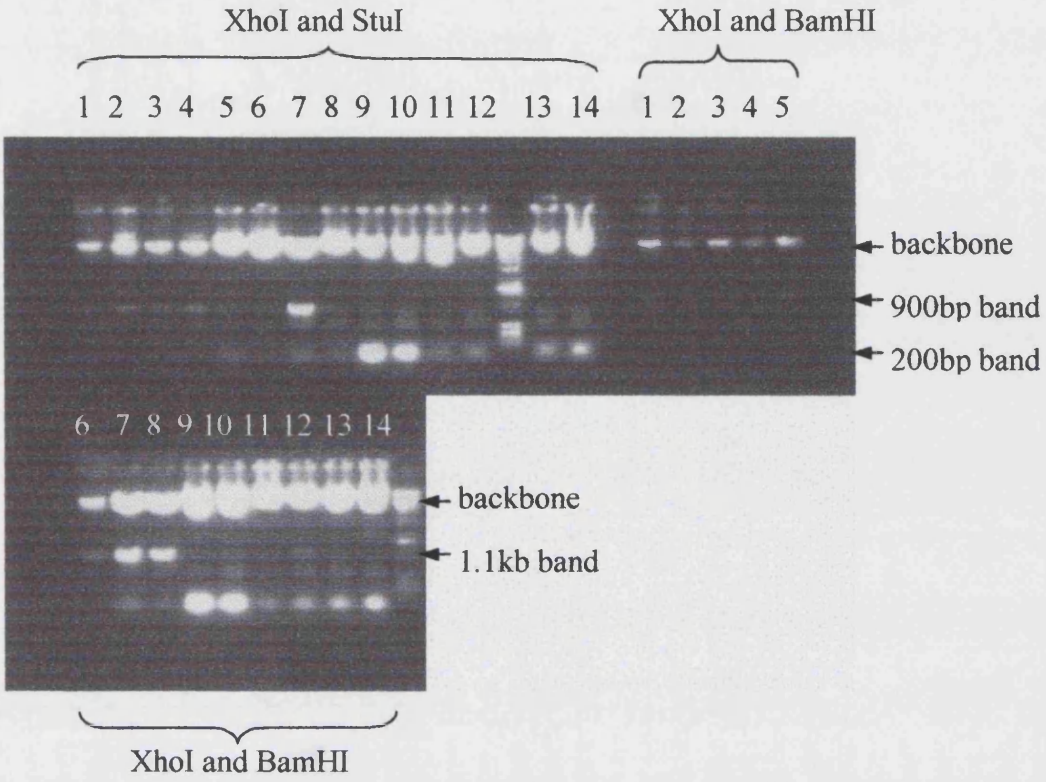
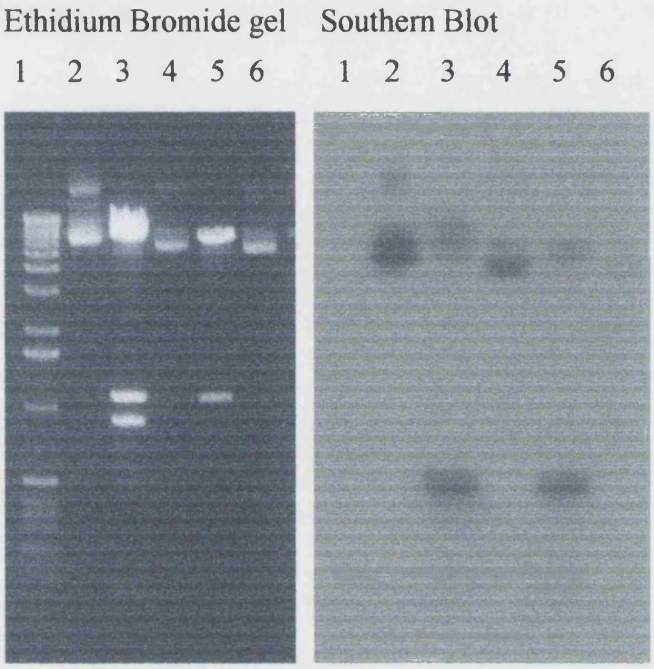


Figure 6.4.3 Small scale DNA 'mini preps' were carried out following transformation of plamids formed by the ligation of the hsp56 cDNA large fragment into the pR19-plamid backbone to make antisense. Digestion with XhoI and BamHI drops out the insert to give 1.1kb band as in lanes 7 and 8. Digestion with XhoI and StuI shows orientation of insert, antisense gives 900bp band as in lane 7. The clone from lane 7 was the most likely candidate for having hsp56 cDNA in the antisense orientation.

Figure 6.4.4 Southern Blot of hsp56 antisense



1. 1kb ladder
2. pR19-56 uncut
3. pR19-56 digested with BamHI and XhoI
4. hsp56 antisense uncut
5. hsp56 antisense digested with BamHI and XhoI

Figure 6.4.4 Ethidium bromide stained gel on left and southern blot on right. Southern blot was probed with hsp56 cDNA. This shows that the hsp56 cDNA is present in the antisense construct.

6.5.0 Hsp56 antisense inhibits hsp56 expression.

The restriction digests and southern blot in the previous section suggest that the construct made does contain hsp56 cDNA in the antisense orientation. It was therefore necessary to see whether the hsp56 antisense construct was capable of reducing levels of hsp56 protein when transfected into cells.

The only antibody to hsp56 available initially, recognised rabbit hsp56 but not rat hsp56. It was therefore impossible to look at endogenous hsp56 in rat cardiac cells, but rabbit hsp56 over expressed by transfection of the pR19-56 plamid or infection with the hsp56 virus could be easily detected. To monitor the effect of the hsp56 antisense construct on hsp56 levels, hsp56 was over expressed by transfection with pR19-56. This was co-transfected with either control plasmid (empty vector) or hsp56 antisense. 24 hours after transfection cells were harvested and western blotting was performed to visualize hsp56 levels. The neuroblastoma derived cell line ND7 was used for these experiments due to the high transfection efficiency achieved in these cells. Neonatal rat cardiac myocytes could not be used, as the transfection efficiency is too low to quantify transfected proteins in a total cell extract.

ND7 cells were transfected with combinations of control plasmid, pR19-56 and hsp56 antisense. Cells transfected with pR19-56 had elevated levels of hsp56. Hsp56 was not detected in cells where it was not over expressed because the antibody used does not recognise rat hsp56. Co-transfection with hsp56 antisense reduced the amount of hsp56 protein present compared to co-transfection with control plasmid or transfection with pR19-56 alone (Figure 6.5.1).

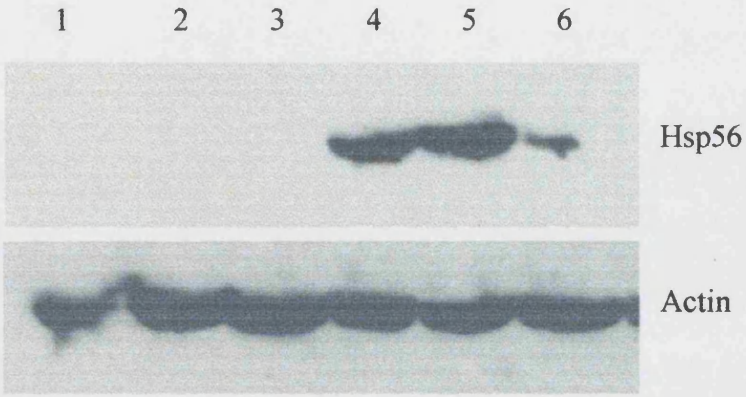
On subsequent receipt of an antibody that recognised rat hsp56 (UP30 serum) it was possible to monitor the effect of hsp56 antisense on endogenous hsp56 levels. Again, ND7 cells were used due to the high transfection efficiency possible in these cells. Cells were transfected with 10μg control plasmid (empty vector with cmv promoter), 5μg control plasmid + 5μg hsp56 antisense, or 10μg hsp56 antisense. 48 hours after transfection cells were harvested and extracts subjected to western blotting.

Hsp56 levels were reduced by transfection with antisense hsp56. A residual amount of hsp56 was still present in cells transfected with 10μg of antisense hsp56 (Figure 6.5.2).

This is because not all the cells were successfully transfected with antisense. In ND7 cells the transfection efficiency is approximately 50% using the calcium phosphate method.

Figures 6.5.1 and 6.5.2 show that transfection with hsp56 antisense significantly reduces hsp56 protein levels.

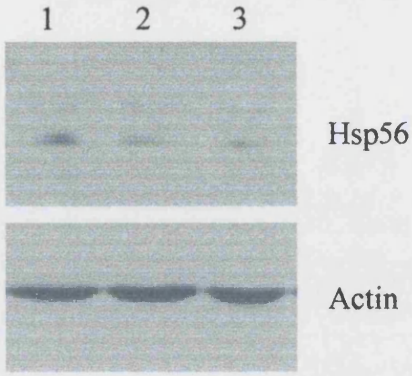
Figure 6.5.1 Reduction of hsp56 protein levels by transfection with hsp56 antisense construct.



1. 11μg control plasmid
2. 10μg hsp56 antisense + 1μg control plasmid
3. 10μg hsp56 antisense + 1μg pR19-56
4. 10μg control plasmid + 1μg pR19-56
5. 1μg pR19-56
6. 5μg hsp56 antisense + 5μg control plasmid + 1μg pR19-56

Figure 6.5.1 ND7 cells were transfected as numbered 1-6 above. 24 hours later cells were harvested and subjected to western blotting. Membranes were probed with antibody to hsp56 (Stressgen), then stripped and re-probed with antibody for actin. The hsp56 antibody used does not recognise endogenous hsp56, and only recognises rabbit hsp56 over expressed by the pR19-56 plasmid. 10μg of hsp56 antisense construct abolished hsp56 expression (lane 3) and 5μg hsp56 antisense construct strongly reduced hsp56 expression (lane 6).

Figure 6.5.2 Reduction of endogenous hsp56 levels by transfection with hsp56 antisense.



Lane 1. 10μg control Plasmid

Lane 2. 5μg hsp56 antisense + 5μg control plasmid

Lane 3. 10μg hsp56 antisense

Figure 6.5.2 ND7 cells were transfected as stated above. 48 hours after transfection cells were harvested and extracts subjected to western blotting. Membranes were probed with UP30 antibody to hsp56, then stripped and re-probed for actin.

6.6.1 The effect of antisense hsp56 on hypertrophy.

It was shown in sections 6.2 and 6.3 that over expression of hsp56 causes hypertrophy in cardiac myocytes. Following results showing that the hsp56 antisense construct reduces hsp56 levels, this construct was used to test the effect of depletion of hsp56 on hypertrophy in response to CT-1 or urocortin treatment.

Primary neonatal rat cardiac myocytes (primary cells) were transfected with a cmv-β-gal expression vector and co-transfected with the following constructs: control plasmid (empty vector), pR19-56 (hsp56 expression vector), or antisense hsp56. Transfections were performed in triplicate and 24 hours after transfection cells were either left untreated, or treated with CT-1 (10ng/ml), or urocortin ($1x10^{-7}$M). 48 hours later cells were fixed and X-gal stained. Blue (transfected) cells were measured and mean cell area and mean cell length were calculated. Single factor ANOVA was used to test for significant differences between groups. The ANOVA showed significant differences in cell area ($p<0.0001$) and cell length ($p<0.0001$). Post-hoc Bonferroni tests were performed to test for significant differences between particular sets of data.

As seen in chapter 5, CT-1 and urocortin both caused a highly significant increase in mean cell area in cells transfected with control plasmid. Untreated cells had a mean cell length of 653μm^2, which increased by 67% to 1093μm^2 with CT-1 treatment ($p<0.0001$), and by 38% to 898μm^2 with urocortin ($p<0.0001$). Over expression of hsp56 by transfection with pR19-56 also caused a highly significant 43% increase in mean cell area to 934μm^2 ($p<0.0001$). Hsp56 antisense caused no significant change in mean cell area (Figure 6.6.1).

In cells transfected with hsp56 antisense and then treated with CT-1, there was no significant increase in mean cell area compared to untreated cells transfected with hsp56 antisense. Conversely, urocortin treatment in cells transfected with hsp56 antisense caused a significant 36% increase in mean cell area from 667μm^2 to 907μm^2 ($p=0.001$) (Figure 6.6.1). This shows that antisense hsp56 is blocking the hypertrophic effect of CT-1, but not urocortin.

In cells transfected with pR19-56 to over express hsp56, treatment with CT-1 caused a significant increase in mean cell area to 1317μm^2 compared to cells transfected with

pR19-56 and left untreated (p=0.0001). Cells transfected with pR19-56 and treated with urocortin were not significantly different in size from cells transfected with pR19-56 and left untreated (p=0.08). This shows that the hypertrophic effect of CT-1 is enhanced by over expression of hsp56 but that of urocortin is not (Figure 6.6.1).

Cell length was also measured and showed the same pattern as shown for area but many of the changes in length were not significant (Figure 6.6.2). This loss of significance in measurements of cell length again shows that measurements of area are more reliable due to the increased variability seen in measurement of length.

These results show that hsp56 causes hypertrophy in cardiac myocytes. Antisense hsp56 blocks the hypertrophic effect of CT-1 but not urocortin. Likewise, over expression of hsp56 augments the hypertrophic effect of CT-1 but not urocortin. This shows that the hypertrophic response of CT-1 is dependent on induction of hsp56.

The hypertrophic effect of urocortin is not dependent on hsp56 levels. Urocortin causes an increase in hsp56 levels but its hypertrophic effect does not depend on this induction. Therefore, other factors must be important in mediating the hypertrophic effect of urocortin.

Figure 6.6.1 The effect of modulation of hsp56 levels on cell area.

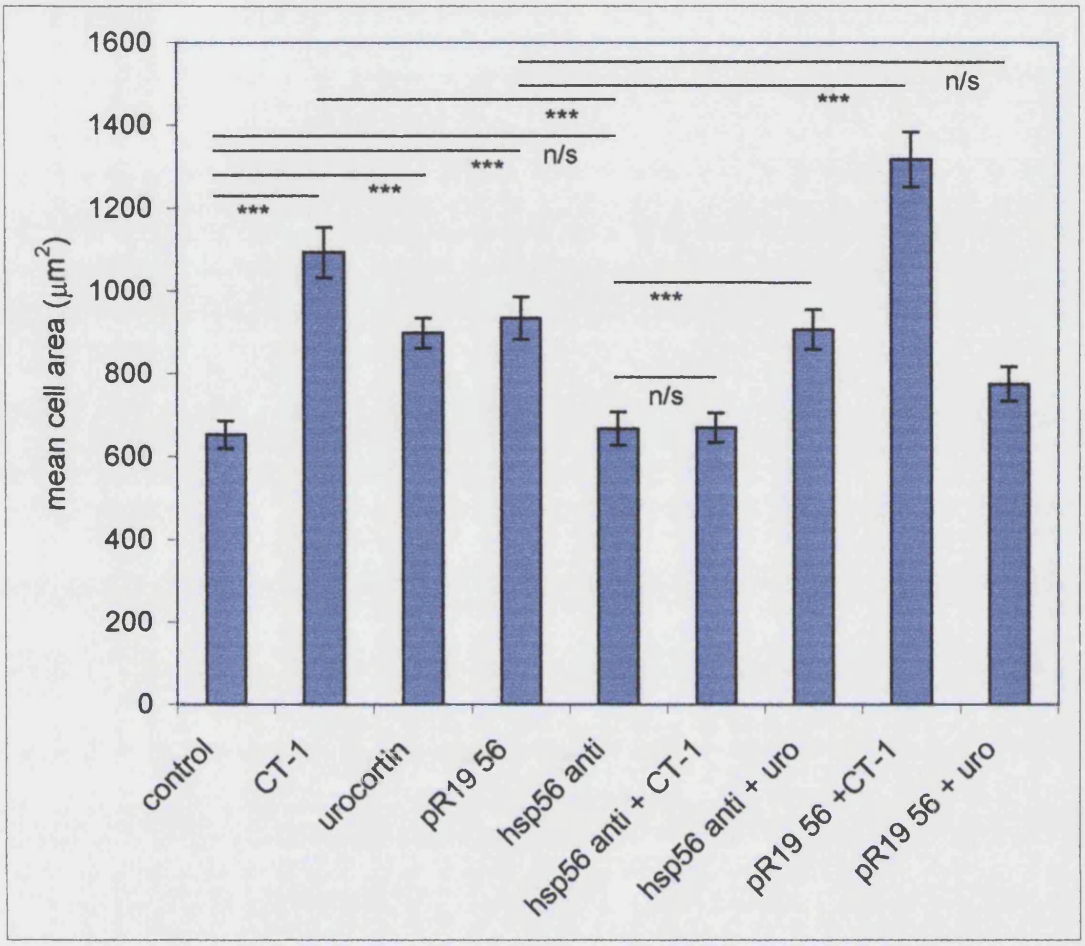


Figure 6.6.1 Primary cells were transfected with a cmv-β-gal expression vector and co-transfected with either control plasmid (empty vector), pR19-56 (hsp56 expression vector), or antisense hsp56. Transfections were performed in triplicate and 24 hours after transfection cells were either left untreated, treated with CT-1 (10ng/ml), or urocortin ($1x10^{-7}$M). 48 hours later cells were fixed and X-gal stained. Blue (transfected) cells were measured and mean cell area was plotted on a graph +/- standard error. Bonferroni tests were performed to test for significant differences between particular sets of data (horizontal bars). Significance levels are shown as follows: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), n/s (no significant difference).

Figure 6.6.2 The effect of modulation of hsp56 levels on cell length.

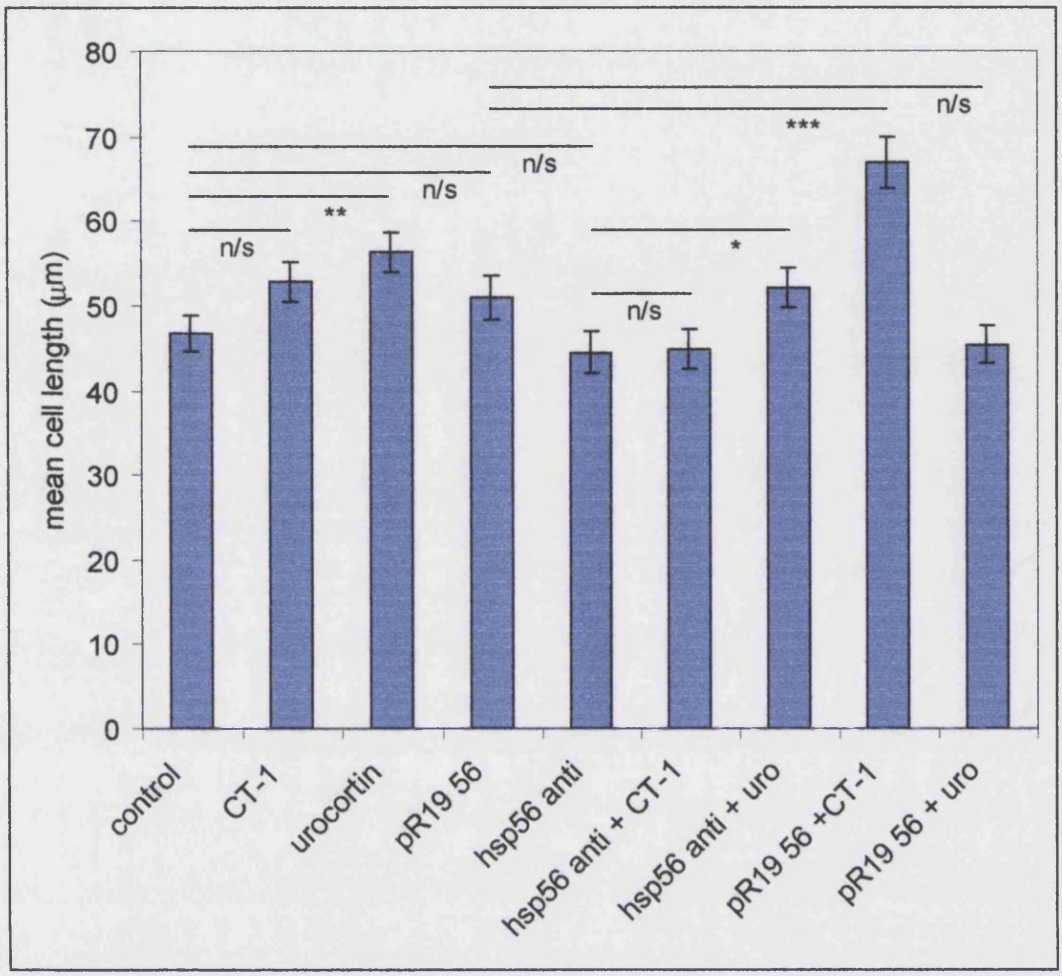


Figure 6.6.1 Primary cells were transfected with a cmv-β-gal expression vector and co-transfected with either control plasmid (empty vector), pR19-56 (hsp56 expression vector), or antisense hsp56. Transfections were performed in triplicate and 24 hours after transfection cells were either left untreated, treated with CT-1 (10ng/ml), or urocortin ($1x10^{-7}$M). 48 hours later cells were fixed and X-gal stained. Blue (transfected) cells were measured and mean cell length was plotted on a graph +/- standard error. Bonferroni tests were performed to test for significant differences between particular sets of data (horizontal bars). Significance levels are shown as follows: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), n/s (no significant difference).

6.7.0 Discussion

It is shown in this chapter that elevated levels of hsp56 are sufficient to cause hypertrophy in neonatal rat cardiac myocytes. None of the heat shock proteins have previously been investigated for hypertrophic effects and this is the first time that such an effect has been demonstrated.

Over expression of hsp56 by both transient transfection and viral infection caused a significant degree of hypertrophy. Hsp27, hsp70 and hsp90 did not have any effect on cell size. This indicates a specific role for hsp56 in the hypertrophic response. Herpes viral vectors are a useful tool to insert a gene of interest into a large percentage of cells. The hsp27 and hsp70 viral vectors used in this study have been shown to protect cardiac myocytes against lethal simulated ischemia and heat stress, while the hsp56 virus was not protective (Brar *et al.*, 1999b). Hsp90 was not tested due to the lack of an efficient viral vector. Infection with virus causes a small amount of cell death, which can affect the plating density of the cells. This was thought to be the reason for the increase in size seen after infection with control viruses. It is therefore important to use control virus infected cells and not uninfected cells as the control when measuring cell size.

CT-1 and urocortin both caused an increase in hsp56 levels in cardiac myocytes (as shown in chapter 3). CT-1 and urocortin also both increase hsp90 levels in heart cells (Stephanou *et al.*, 1998a; Brar *et al.*, unpublished observations). The induction of hsp56 by CT-1 was apparent after 6 hours treatment and maximal after 24 hours. This contrasts with the induction of hsp90, which commences after 10 minutes and is maximal after 3 hours (Figure 3.4.2). Additionally, increased hsp56 mRNA was detected after CT-1 treatment while hsp90 mRNA levels remained constant (Figure 3.8.1). This indicates that CT-1 is increasing levels of hsp56 and hsp90 via distinct transcriptional and post-transcriptional mechanisms. Further work will be necessary to elucidate the signalling pathways involved.

Urocortin caused a more rapid increase in hsp56 levels than CT-1 with maximal levels being reached within 2 hours (chapter 3). The hypertrophic effect of CT-1 could be blocked by antisense hsp56 while that of urocortin could not. This suggests that the more sustained induction of hsp56 by CT-1 may be involved in its hypertrophic

response. Conversely, the more transient induction of hsp56 by urocortin does not seem to be important in its hypertrophic response.

The mechanism by which urocortin causes hypertrophy remains elusive. The STAT3, STAT1 and p42/p44 MAPK pathways do not seem to be involved (chapter 5) and it is shown here that hsp56 is not required for the response.

The induction of hsp56 by urocortin was an unexpected finding and the function of hsp56 induced by urocortin remains unclear. In addition to this novel role in hypertrophy, hsp56 has many other roles in the cell. Hsp56 is involved in protein folding and is found in the steroid receptor complex. Urocortin also has functions other than its involvement in hypertrophy. For example, urocortin is important in the regulation of feeding behaviour (Heinrichs & Richard, 1999). It is possible that the induction of hsp56 by urocortin may be important in one of these other functions of urocortin.

The only previous work linking hsp56 to cardiac hypertrophy involves the immunosuppressive drug FK506. FK506 binding to hsp56 inhibits the cis-trans-peptidylprolyl isomerase activity of hsp56. Binding of FK506 to other FK506 binding proteins such as FKBP12 has been shown to form a complex, which can then interact with, and inhibit calcineurin (Liu *et al.*, 1991). Calcineurin inhibition is thought to be the mechanism by which FK506 prevents T-cell activation and causes immunosuppression. FKBP12 and FKBP13 share homology in the C-terminal FK506 binding domain with hsp56 (also known as FKBP56/59) (Yem *et al.*, 1992) and all these immunophillins bind to FK506. However, it has been shown that hsp56 complexed with FK506 is unable to inhibit calcineurin, compared to FKBP12 and FKBP13, which inhibit calcineurin when bound to FK506 (Lebeau *et al.*, 1994). This suggests that hsp56 may not be involved in the immunosuppressive role of FK506.

It has been shown that calcineurin activates NF-AT3 in cardiac hypertrophy and that activation of calcineurin is both necessary, and sufficient to induce cardiac hypertrophy (Molkentin *et al.*, 1998). In the same study, FK506 was shown to block the hypertrophic response to PE or Angiotensin II in cardiac myocytes. This work suggests that calcineurin is an important component of the hypertrophic response.

FK506 has also been shown to cause cardiac hypertrophy in the clinical setting, in particular in liver transplant patients treated with FK506 to prevent rejection. In one study, five paediatric patients were reported to have developed hypertrophic cardiomyopathy while on FK506, this hypertrophy regressed after reducing the dose or discontinuing FK506 (Atkison *et al.*, 1995). A subsequent study reported a further two cases of marked left ventricular hypertrophy in children on FK506 (Chang *et al.*, 1998).

It seems therefore, that FK506 can inhibit the development of certain forms of hypertrophy but that FK506 treatment may itself cause hypertrophy in some cases. It is shown here that FK506 treatment had no significant effect on cell size in neonatal rat cardiac myocytes. Treatment with FK506 reduced the hypertrophic effect of CT-1 and urocortin, but this effect was not statistically significant. This inhibition was not complete and cells treated with CT-1 or urocortin in the presence of FK506 were still significantly larger than control cells. The partial inhibition of the hypertrophic effect of CT-1 and urocortin by FK506 may indicate that calcineurin is a down stream effector of the CT-1 or urocortin mediated hypertrophic response, but that other factors not inhibited by FK506 are also involved. Additionally, FK506 may act on targets other than calcineurin to inhibit the hypertrophic response. Further work will be necessary to test whether CT-1 or urocortin activate calcineurin and whether such an activation is involved in their hypertrophic response.

There is some evidence linking hsp56 to control of the cell cycle and cell growth. The hsp56 related protein FKBP12 has been shown to interact with the type 1 TGF-β receptor, stimulation of which blocks cell cycle progression at the G1/S phase (Wang *et al.*, 1994). This interaction is competed by FK506, which shows that FKBP12 may be binding the TGF-β receptor via its FK506 binding domain. Since FKBP12 and hsp56 share homologous FK506 binding domains, it is likely that hsp56 may also interact with TGF-β receptors. An interaction between hsp56 and the TGF-β receptor could be involved in the hypertrophic response, such as by causing cell cycle arrest and a switch from mitotic to hypertrophic growth. Similarly, FKBP12 also interacts with RAFT1 (rapamycin and FKBP12 target 1), which is a mammalian homologue of the yeast TOR (target of rapamycin) gene product (Sabatini *et al.*, 1994). TOR proteins also cause cell

cycle arrest at the G1/S phase (Kunz *et al.*, 1993). If hsp56 is also able to interact with RAFT1, this could be another site for cell cycle regulation by hsp56.

Hsp56 mRNA levels are decreased in fibroblast cells subjected to serum withdrawal, but increased by growth factor stimulation (Doucet-Brutin *et al.*, 1995). Additionally, Hsp56 mRNA levels are increased in cells able to grow in low serum medium (Doucet-Brutin *et al.*, 1995). This study implies that hsp56 may be involved in the control of cell proliferation. Hsp56 mRNA is also expressed at high levels, in a stage specific manner during differentiation of male germ cells (Sananes *et al.*, 1998). This also points to a role for hsp56 in the control of cellular differentiation. Such cell cycle effects are very cell type specific and would depend on background levels of many other factors. In the heart, the effect of elevated hsp56 may be to stimulate hypertrophic growth while blocking cell division.

Hsp56 has been shown to mediate a neurotrophic effect in primary hippocampal neurons (Gold *et al.*, 1999). Like cardiac myocytes, hippocampal neurons are thought to be terminally differentiated. Hsp56, when released from the steroid receptor complex by hsp56 antibody or FK506 results in neurite outgrowth and nerve regeneration (Gold *et al.*, 1999). Gold *et al.* suggest that hsp56 mediates its effect by binding to microtubules and microfilaments. This is an example of hsp56 causing an increase in cell size in cultured terminally differentiated cells. A similar mechanism may be involved in the hypertrophic growth observed here, in cardiac myocytes in response to elevated hsp56.

A more direct link between hsp56 and the hypertrophic response is its effect on calcium signalling. Hsp56 binds calmodulin, which is an intracellular receptor for calcium (Massol *et al.*, 1992). Calmodulin regulates the activity of calmodulin binding proteins, including enzymes such as calcineurin and structural cytoskeletal proteins. The interaction between calmodulin and hsp56 is calcium dependent. Intracellular calcium levels are elevated in many forms of cardiac hypertrophy and this is often the common factor in the response to many different hypertrophic stimuli (Force *et al.*, 1999). One hypothesis for the hypertrophic effect of hsp56 is that hsp56 binds to and activates calmodulin in the presence of elevated intracellular calcium from hypertrophic stimuli. Calmodulin would then activate calcineurin (Klee *et al.*, 1998), which would cause

hypertrophy via activation of NF-AT3 transcription factors (Olson & Molkentin, 1999). This hypothesis would imply that the hypertrophic effect caused by hsp56 would be blocked by FK506 (through FK506-FKBP12 complex inhibition of calcineurin). In experiments presented here, the hypertrophic effect of CT-1 was blocked by antisense hsp56 but not significantly blocked by FK506. This suggests that calcineurin is not the sole factor involved in the hypertrophic response. It is possible that other calmodulin binding proteins may be involved.

In chapter 5, the hypertrophic effect of CT-1 was shown to be dependent on activation of STAT3. It is shown here that hsp56 induction is also required for CT-1 mediated cardiac hypertrophy. It is therefore likely that STAT3 and hsp56 are components in the same pathway leading to cardiac hypertrophy. It is possible that transcription of the hsp56 gene may be activated by STAT3 after CT-1 stimulation. Regulation of the hsp56 promoter is yet to be studied and it would be interesting to know if STAT3 is able to increase levels of hsp56.

CHAPTER 7

DISCUSSION

The work presented in this thesis is an investigation of the effects of cardiotrophin-1 (CT-1) on heat shock protein expression, protection and hypertrophy in the heart. CT-1 is known to be both protective and hypertrophic in the heart. The aim of this study was to look for divergence in the signalling pathways involved in these effects, with a view to blocking the detrimental hypertrophic effect while maintaining the beneficial protective effect. Urocortin was also studied, as it is also protective in heart cells and mediates its protective effect by the same pathway as CT-1.

The results presented in chapter 3 show that CT-1 increases levels of hsp90, which agrees with previous work in our laboratory (Stephanou *et al.*, 1998a). The increase in hsp90 levels was rapid (within 30 minutes) and sustained (lasted over 24 hours). The RNA synthesis inhibitor actinomycin D did not block the increase in hsp90 levels caused by CT-1, which indicated that a post-transcriptional mechanism may be involved. Despite clear elevation of hsp90 protein levels after CT-1 treatment, no increase in hsp90 mRNA level could be detected after CT-1 treatment.

No activation of the hsp90 promoter could be demonstrated in response to CT-1. Reporter constructs containing the largest available regions of the hsp90α and hsp90β promoters were tested and neither responded to CT-1 but both constructs were activated by heat shock. The same hsp90β reporter construct as used in this study shows clear activation in response to Interleukin-6 (IL-6) treatment (Stephanou *et al.*, 1997). This shows that although IL-6 and CT-1 share the common receptor component gp130, and both cause an increase in hsp90 protein levels, they do not activate the same region of the hsp90 promoter. This indicates that the two cytokines are acting via distinct mechanisms to increase hsp90 levels. IL-6 has been shown to activate the hsp90β promoter via the transcription factors NFIL-6 and STAT3 (Stephanou *et al.*, 1998b). CT-1 also activates STAT3, and NFIL-6 could be activated by p42/44 MAPK which is phosphorylated by CT-1 binding to its receptor (Robledo *et al.*, 1997b; Sheng *et al.*, 1997; Nakajima *et al.*, 1993). Thus, although the same pathways are activated by CT-1 and IL-6, a different mechanism is used to elevate hsp90 levels.

A clear elevation of hsp90 and hsp70 mRNA levels was observed in response to heat shock. Additionally, hsp90 and hsp70 production in response to heat shock was completely blocked by Actinomycin D. This shows that heat shock increases hsp90 and

hsp70 levels via transcriptional activation. This is a good positive control for experiments with CT-1, as heat shock is known to activate transcription of heat shock genes via the transcription factor HSF-1 (Sarge *et al.*, 1993).

The most likely mechanism for the increase in hsp90 caused by CT-1 would be at the translational or post-translational level. An increase in mRNA stability is unlikely as elevated hsp90 mRNA levels were not observed in response to CT-1 treatment. Regulation of mRNA splicing is also unlikely as no accumulation of a larger hsp90 mRNA was observed on Northern blots after CT-1 treatment. Having eliminated transcriptional regulation and regulation of mRNA splicing and stability, it is likely that CT-1 is increasing hsp90 protein levels by regulating translation, or post-translationally modifying the protein to enhance protein stability. It would be interesting to measure the half-life of the hsp90 protein with and without CT-1 treatment to determine whether CT-1 treatment increases protein stability.

CT-1 administered prior to heat shock caused a reduction in the amount of hsp90 and hsp70 produced compared to cells heat shocked without CT-1. This revealed a novel antagonistic effect of CT-1 on stress induced hsp production. This antagonistic effect was also observed at the mRNA and promoter levels indicating that it is occurring at the transcriptional level. The most likely mechanism for this effect is antagonism between STAT3 activated by CT-1, and HSF-1 activated by heat shock. STAT3 and HSF-1 have been shown to bind to overlapping regions in the hsp90β promoter, and when both are activated at the same time, they have an antagonistic effect on promoter activation (Stephanou *et al.*, 1998b). It is likely that HSF-1 and STAT3 compete for binding to the hsp90 promoter and as their binding sites overlap, and the two transcription factors do not interact with one another, they cannot bind to the promoter at the same time.

The results presented in chapter 3 therefore show a novel post-transcriptional mechanism for hsp90 induction by CT-1 and an antagonistic effect of CT-1 on stress induced hsp production. This demonstrates the complex multi-layered regulation of hsp levels in the heart. Hsp90 is an abundant protein constituting around 1% of total cell protein, it is therefore unsurprising that its regulation is controlled not only by HSF-1 but by interaction with other transcription factors and by post-transcriptional effects.

It was also shown that CT-1 causes a large increase in hsp56 mRNA and protein levels. This was unexpected, as regulation of hsp56 by IL-6 family cytokines has not previously been investigated. The increase in hsp56 mRNA level observed in response to CT-1 treatment implies that CT-1 is causing transcriptional activation of hsp56 or is increasing the mRNA stability. This is distinct to the post-transcriptional increase in hsp90 in response to CT-1. It would be interesting to study regulation of the hsp56 promoter, as this has not previously been investigated. Hsp56 levels are increased by heat so it is likely that the promoter contains heat shock elements (Sanchez, 1990). It is likely that the promoter may also contain STAT3 binding sites as these are found in other hsp promoters overlapping the heat shock elements (Stephanou *et al.*, 1998b). The mechanism for the induction of hsp56 by CT-1 may therefore be transcriptional activation by STAT3.

A method to measure the hypertrophic effect of CT-1 while allowing the manipulation of specific signalling pathways was developed in the work presented in chapter 4. It was shown that cell size is dependent on plating density and that thinly plated cells become larger in culture. This was thought to be because cells need to be in contact with neighbouring cells. In-vivo, heart cells are tightly joined to one another with each cell being surrounded by other cells. Cultured primary neonatal cardiac myocytes beat if cultured in a confluent monolayer but do not beat if cultured at low density. This is an example of the requirement for cell-to-cell contact for normal cellular function. In the light of these results, experiments were carried out on cells forming a confluent monolayer in culture.

When cells were in a confluent monolayer it was difficult to identify the boundary of each cell. To allow easy visualisation of individual cells, transfection with a β-galactosidase (β-gal) reporter gene was carried out. Cells transfected with β-gal could be stained blue, and easily measured. This allowed the random selection and visualisation of a subset of cells to be measured from a confluent monolayer. The selective measurement of transfected cells allowed other DNA constructs to be co-transfected with β-gal, so that the effect of these constructs on cell size could be determined. It is assumed that when a DNA precipitate containing more than one plamid is made for transfection, the precipitate will contain DNA from both plasmids, and therefore successfully transfected cells will be transfected with both plasmids.

It was shown in chapter 5, that urocortin causes hypertrophy as defined by an increase in cardiac myocyte planimetric area in both neonatal and adult rat cardiac myocytes. This supports results from a previous study where urocortin was shown to stimulate atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) release from neonatal rat cardiac myocytes (Ikeda *et al.*, 1998), but is the first time that a direct hypertrophic effect on cell size has been demonstrated for a CRH family member. The induction of ANP and BNP demonstrated by Ikeda *et al.* was at least partly dependent on protein kinase A (PKA) phosphorylation. In the same study, urocortin treatment was also shown to increase protein synthesis in neonatal cardiac myocytes and to increase cAMP production (Ikeda *et al.*, 1998). Urocortin binds to the CRF-R2β receptor in the heart, which is Gs-protein coupled, so causes cAMP release (Vaughan *et al.*, 1995). cAMP binds to and activates PKA (Brown & Birnbaumer, 1990) (also known as cAMP dependent protein kinase) which phosphorylates components of calcium channels causing calcium to enter the cell (Curtis & Catterall, 1985). Activated PKA also acts directly on transcription factors such as CREB (cAMP response element binding protein) and many regulatory proteins (Karin & Smeal, 1992).

Elevated intracellular calcium as a result of urocortin induced PKA activation could be the mechanism by which urocortin causes hypertrophy. Elevated intracellular calcium precedes most forms of hypertrophy and results in the activation of calcium dependent signalling pathways. One such pathway known to cause hypertrophy would be activation of calcineurin by calmodulin. However, calcineurin is inhibited by FK506 and FK506 only partially blocked the hypertrophic effect of urocortin. It would therefore seem that calcineurin may be involved in the hypertrophic response to urocortin but is not the sole mediator of the response.

The hypertrophic effect of urocortin was independent from STAT3 and p42/p44 MAPK signalling, and did not require the expression of hsp56. Following the elimination of these pathways, it would be interesting to investigate whether the hypertrophic effect of urocortin is dependent on PKA activation, and to study the role of CREB in the hypertrophic response.

CT-1 also caused hypertrophy in neonatal and adult rat cardiac myocytes. This supports previous reports from neonatal cells (Pennica *et al.* 1995a; Wollert *et al.*, 1996), but is the first time that a hypertrophic effect for CT-1 has been demonstrated in isolated adult cardiac myocytes.

The hypertrophic effect of CT-1 was blocked by inhibition of the STAT3 pathway but was not affected by inhibition of the STAT1, p42/p44 MAPK, or PI3 kinase pathways. Activated STAT3 has been demonstrated in the hypertrophic response to various stimuli (Pan *et al.*, 1999; Pan *et al.*, 1998). Transgenic mice with heart specific over expression of STAT3 have cardiac hypertrophy at 12 weeks of age (Kunisada *et al.*, 2000). Additionally, over expression of wild type STAT3 augments the hypertrophic effect of the CT-1 related factor LIF, while over expression of mutant STAT3 attenuated its hypertrophic effect (Kunisada *et al.*, 1998). Activated STAT3 translocates to the nucleus and acts as a transcription factor. Transcription of the angiotensinogen gene is activated by STAT3 (Mascareno *et al.*, 1998). Angiotensinogen is the precursor for angiotensin II, which has well documented hypertrophic effects. It has also been shown that CT-1 increases levels of angiotensinogen mRNA via activation of STAT3, and that an angiotensin II receptor antagonist partly inhibits the hypertrophic effect of CT-1 (Fukuzawa *et al.*, 2000). It seems, therefore that the hypertrophic effect of CT-1 is at least partly mediated by activation of angiotensinogen gene expression by STAT3.

In addition to its role in hypertrophy, STAT3 has also been implicated in the protection of cardiac myocytes against cell death. STAT3 activation was necessary for *bcl-2* gene expression and anti-apoptotic effects in pro-B cell lines expressing chimeric gp130 receptors (Fukada *et al.*, 1996). Additionally, STAT3 transgenic mice were protected against administration of the cardiotoxic drug doxorubicin (Kunisada *et al.*, 2000). The aim of this study was to see if it is possible to block the hypertrophic effect of CT-1 while maintaining its protective effects. It was therefore important to discover whether inhibition of STAT3 signalling caused cell death or had any effect on the protective effect of CT-1.

Inhibition of the STAT3 pathway had no effect on the protective effects of CT-1 or urocortin. Importantly, STAT3 inhibition did not cause any significant increase cell death in control cells or in cells subjected to hypoxic stress. This shows that although

STAT3 is protective in some circumstances, it is not essential for the protective effect of CT-1. The protective effects of both CT-1 and urocortin were dependent on the p42/p44 MAPK pathway as shown previously (Sheng *et al.*, 1997)(Brar *et al.* unpublished observations). It was therefore established that the hypertrophic effect of CT-1 could be blocked by inhibition of the STAT3 pathway while maintaining its protective effects.

It was shown in chapter 3 that hsp56 mRNA and protein levels were significantly increased in response to CT-1 treatment, and urocortin also caused an increase in hsp56 protein levels. Hsp56 over expression does not protect cardiac myocytes against stress induced cell death (Cumming *et al.*, 1996b), but a potential role for hsp56 in hypertrophy had not previously been investigated. It was shown in chapter 6 that over expression of hsp56 by plasmid or Herpes viral vectors caused hypertrophy in neonatal rat cardiac myocytes. Furthermore, over expression of hsp56 significantly enhanced the hypertrophic effect of CT-1 but not urocortin. Transfection with antisense hsp56 blocked the hypertrophic effect of CT-1 but had no effect on the hypertrophic effect of urocortin. These results demonstrated that hsp56 is able to cause hypertrophy and that hsp56 expression is required for the hypertrophic effect of CT-1 but not urocortin.

Work presented in chapters 5 and 6 has revealed two points at which the hypertrophic effect of CT-1 can be blocked. It seems likely that STAT3 and hsp56 must be components of the same pathway, which mediates the hypertrophic effect of CT-1.

One possible connection between STAT3 and hsp56 is TGFβ (Transforming growth factor β). The hypertrophic effect of CT-1 is dependent on STAT3 mediated angiotensin II induction (Mascareno *et al.*, 1998). Angiotensin II causes TGFβ release from fibroblasts which mediates its hypertrophic effect on cardiac myocytes (Gray *et al.*, 1998; Azuma *et al.*, 2000). TGFβ receptors are known to interact with FKBP12, a close relative of hsp56, and this interaction can be competed by high doses of FK506 (Wang *et al.*, 1994). FKBP12 and hsp56 share homologous FK506 binding sites (Yem *et al.*, 1992), so it is likely that hsp56 is also able to interact with the TGFβ receptor. If such an interaction could be demonstrated between hsp56 and the TGFβ receptor it may prove to be involved in the hypertrophic response to CT-1.

In addition to the possible interaction of hsp56 with the TGFβ receptor, it is also possible that STAT3 may directly activate transcription of hsp56 after CT-1 treatment. Hsp56 may then cause hypertrophy through interactions with cytoskeletal proteins (Ruff *et al.*, 1992), or other unknown effects. One of the best-characterised roles of hsp56 is its participation in the steroid receptor complex and binding to hsp90 (Sanchez, 1990). It is however; unlikely that the steroid receptor complex is involved in the hypertrophic response as if this was the case other components of the complex should also cause hypertrophy. Over expression of hsp90 did not cause hypertrophy so it seems unlikely that the complex is directly involved.

To summarize, CT-1 increases levels of hsp90 by a post-transcriptional mechanism and hsp56 by an alternative transcriptional mechanism or by increasing mRNA stability. The hypertrophic effect of CT-1 is dependent on both STAT3 signalling and the induction of hsp56. The protective effect of CT-1 is independent from STAT3 inhibition. Urocortin was shown to have a hypertrophic effect, which unlike CT-1, does not depend on STAT3 signalling or hsp56 expression.

The work presented in this thesis opens up several new and interesting avenues for research. In particular an investigation of the regulation of hsp56 gene expression and effects of over expressed hsp56 should prove interesting. Hsp56 has been studied very little to date and the novel hypertrophic effect presented here highlights a potentially important role for this protein in cardiac physiology.

Reference List

Adams, J.M. & Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science* **281**, 1322-1326.

Agnello, D., Bertini, R., Sacco, S., Meazza, C., Villa, P. & Ghezzi, P. (1998). Corticosteroid-independent inhibition of tumor necrosis factor production by the neuropeptide urocortin. *Am J Physiol* **275**, E757-E762

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. & Kishimoto, T. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J* **9**, 1897-1906.

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. & Pestell, R.G. (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* **270**, 23589-23597.

Ali, A., Bharadwaj, S., O'Carroll, R. & Ovsenek, N. (1998). HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Mol Cell Biol* **18**, 4949-4960.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. & Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171

Ando, T., Rivier, J., Yanaihara, H. & Arimura, A. (1998). Peripheral corticotropin-releasing factor mediates the elevation of plasma IL-6 by immobilization stress in rats. *Am J Physiol* **275**, R1461-R1467

Andreeva, L., Heads, R. & Green, C.J. (1999). Cyclophilins and their possible role in the stress response. *Int J Exp Pathol* **80**, 305-315.

Anfinsen, C.B. (1973). Principles that govern the folding of protein chains. *Science* **181**, 223-230.

Anversa, P. & Kajstura, J. (1998). Ventricular myocytes are not terminally differentiated in the adult mammalian heart. *Circ Res* **83**, 1-14.

Aoki, H., Richmond, M., Izumo, S. & Sadoshima, J. (2000). Specific role of the extracellular signal-regulated kinase pathway in angiotensin II-induced cardiac hypertrophy in vitro. *Biochem J* **347**, 275-284.

Arce, V., Garces, A., deBovis, B., Filippi, P., Henderson, C., Pettmann, B. & Br. (1999). Cardiotrophin-1 requires LIFR beta to promote survival of mouse motoneurons purified by a novel technique. *Journal Of Neuroscience Research* **55**, 119-126.

Arrigo, A.P. & Preville, X. (1999). Role of hsp27 and related proteins. In *Stress Proteins* (Latchman, D.S., ed.), Springer-Verlag, Berlin, pp. 101-132.

Asakawa, A., Inui, A., Ueno, N., Makino, S., Fujino, M.A. & Kasuga, M. (1999). Urocortin reduces food intake and gastric emptying in lean and ob/ob obese mice. *Gastroenterology* **116**, 1287-1292.

Ashkenazi, A. & Dixit, V.M. (1998). Death receptors: signaling and modulation. *Science* **281**, 1305-1308.

Atkinson, E.A. & Bleackley, R.C. (1995)a. Mechanisms of lysis by cytotoxic T cells. *Crit Rev Immunol* **15**, 359-384.

Atkison, P., Joubert, G., Barron, A., Grant, D., Paradis, K., Seidman, E., Wall, W., Rosenberg, H., Howard, J. & Williams, S. (1995)b. Hypertrophic cardiomyopathy associated with tacrolimus in paediatric transplant patients. *Lancet* **345**, 894-896.

Azuma, M., Takahashi, K., Fukuda, T., Ohyabu, Y., Yamamoto, I., Kim, S., Iwao, H., Schaffer, S.W. & Azuma, J. (2000). Taurine attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac myocytes. *Eur J Pharmacol* **403**, 181-188.

Bardwell, J.C. & Craig, E.A. (1988). Ancient heat shock gene is dispensable [published erratum appears in J Bacteriol 1988 Oct;170(10):4999. *J Bacteriol* **170**, 2977-2983.

Baulieu, E.E. (1991). The connection between steroid receptors and stress protein (hsp90). *Methods Achiev Exp Pathol* **15**, 104-125.

Becker, J. & Craig, E.A. (1994). Heat-shock proteins as molecular chaperones. *Eur J Biochem* **219**, 11-23.

Bogoyevitch, M.A., Gillespie-Brown, J., Ketterman, A.J., Fuller, S.J., Ben-Levy, R., Ashworth, A., Marshall, C.J. & Sugden, P.H. (1996). Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ Res* **79**, 162-173.

Bogoyevitch, M.A., Parker, P.J. & Sugden, P.H. (1993). Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. *Circ Res* **72**, 757-767.

Bokemeyer, D., Sorokin, A. & Dunn, M.J. (1996). Multiple intracellular MAP kinase signaling cascades. *Kidney Int* **49**, 1187-1198.

Booz, G.W. & Baker, K.M. (1996). Role of type 1 and type 2 angiotensin receptors in angiotensin II-induced cardiomyocyte hypertrophy. *Hypertension* **28**, 635-640.

Bordet, T., Schmalbruch, H., Pettmann, B., Hagege, A., Castelnau-Ptakhine, L., Kahn, A. & Haase, G. (1999). Adenoviral cardiotrophin-1 gene transfer protects pmn mice from progressive motor neuronopathy. *J Clin Invest* **104**, 1077-1085.

Braell, W., Schlossman, D., Schmid, S. & Rothmann, J. (1984). Dissociation of clathrin coats coupled to the hydrolysis of ATP: role of an uncoating ATPase. *J Cell Biol* **99**, 734-741.

Brar, B.K., Jonassen, A.K., Stephanou, A., Santilli, G., Railson, J., Knight, R.A., Yellon, D.M. & Latchman, D.S. (2000). Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway. *J Biol Chem* **275**, 8508-8514.

Brar, B.K., Stephanou, A., Okosi, A., Lawrence, K.M., Knight, R.A., Marber, M.S. & Latchman, D.S. (1999)a. CRH-like peptides protect cardiac myocytes from lethal ischaemic injury. *Mol Cell Endocrinol* **158**, 55-63.

Brar, B.K., Stephanou, A., Wagstaff, M.J., Coffin, R.S., Marber, M.S., Engelmann, G. & Latchman, D.S. (1999)b. Heat shock proteins delivered with a virus vector can protect cardiac cells against apoptosis as well as against thermal or hypoxic stress. *J Mol Cell Cardiol.* **31**, 135-146.

Brown, A.M. & Birnbaumer, L. (1990). Ionic channels and their regulation by G protein subunits. *Annu Rev Physiol* **52**, 197-213.

Buchner, J. (1996). Supervising the fold: functional principles of molecular chaperones. *FASEB J* **10**, 10-19.

Caldenhoven, E., van-Dijk, T.B., Solari, R., Armstrong, J., Raaijmakers, J.A.M., Lammers, J.W.J., Koenderman, L. & de-Groot, R.P. (1996). STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. *J Biol Chem.* **271**, 13221-13227.

Cano, E. & Mahadevan, L.C. (1995). Parallel signal processing among mammalian MAPKs. *Trends Biochem Sci* **20**, 117-122.

Chambraud, B., Rouviere-Fourmy, N., Radanyi, C., Hsiao, K., Peattie, E.A., Livingston, D.J. & Baulieu, E.E. (1993). Overexpression of p59-HBI (FKBP59), full length and domains, and characterization of PPIase activity. *Biochem Biophys Res Commun* **196**, 160-166.

Chang, R.K., Alzona, M., Alejos, J., Jue, K. & McDiarmid, S.V. (1998). Marked left ventricular hypertrophy in children on tacrolimus (FK506) after orthotopic liver transplantation. *Am.J Cardiol.* **81**, 1277-1280.

Chen, L., Xin, X., Eckhart, A.D., Yang, N. & Faber, J.E. (1995). Regulation of vascular smooth muscle growth by alpha 1-adrenoreceptor subtypes in vitro and in situ. *J Biol Chem* **270**, 30980-30988.

Chen, R., Lewis, K.A., Perrin, M.H. & Vale, W.W. (1993). Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci U S A* **90**, 8967-8971.

Chen, R.H., Abate, C. & Blenis, J. (1993). Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc Natl Acad Sci U S A* **90**, 10952-10956.

Chien, K.R., Knowlton, K.U., Zhu, H. & Chien, S. (1991). Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. *FASEB J* **5**, 3037-3046.

Chu, B., Soncin, F., Price, B.D., Stevenson, M.A. & Calderwood, S.K. (1996). Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem* **271**, 30847-30857.

Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P. & Shuai, K. (1997). Specific inhibition of Stat3 signal transduction by PIAS3. *Science* **278**, 1803-1805.

Chung, J., Uchida, E., Grammer, T.C. & Blenis, J. (1997). STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* **17**, 6508-6516.

Coffer, P., Lutticken, C., van Puijenbroek, A., Klop-de, J.M., Horn, F. & Kruijer, W. (1995). Transcriptional regulation of the junB promoter: analysis of STAT-mediated signal transduction. *Oncogene* **10**, 985-994.

Cohen, P. (1996). Dissection of protein kinase cascades that mediate cellular response to cytokines and cellular stress. *Adv.Pharmacol.* **36**, 15-27.

Collins, J.A., Schandi, C.A., Young, K.K., Vesely, J. & Willingham, M.C. (1997). Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem* **45**, 923-934.

Conde, A.G., Lau, S.S., Dillmann, W.H. & Mestril, R. (1997). Induction of heat shock proteins by tyrosine kinase inhibitors in rat cardiomyocytes and myogenic cells confers protection against simulated ischemia. *J Mol Cell Cardiol.* **29**, 1927-1938.

Cooper, G., Kent, R.L., Uboh, C.E., Thompson, E.W. & Marino, T.A. (1985). Hemodynamic versus adrenergic control of cat right ventricular hypertrophy. *J Clin Invest* **75**, 1403-1414.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z. & Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol Ther* **79**, 129-168.

Cumming, D.V., Heads, R.J., Coffin, R.S., Yellon, D.M. & Latchman, D.S. (1996)a. Pharmacological preconditioning of primary rat cardiac myocytes by FK506. *Basic.Res Cardiol.* **91**, 367-373.

Cumming, D.V., Heads, R.J., Watson, A., Latchman, D.S. & Yellon, D.M. (1996)b. Differential protection of primary rat cardiocytes by transfection of specific heat stress proteins. *J Mol Cell Cardiol.* **28**, 2343-2349.

Curtis, B.M. & Catterall, W.A. (1985). Phosphorylation of the calcium antagonist receptor of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **82**, 2528-2532.

Czar, M.J., Owens-Grillo, J.K., Dittmar, K.D., Hutchison, K.A., Zacharek, A.M., Leach, K.L., Deibel, M.R.J. & Pratt, W.B. (1994). Characterization of the protein-protein interactions determining the heat shock protein (hsp90.hsp70.hsp56) heterocomplex. *J Biol Chem* **269**, 11155-11161.

Dana, A. & Walker, M. (1999). Acute myocardial infarction. Extended review. *J R Coll Physicians.Lond.* **33**, 131-140.

Darnell-JE, J. (1997). STATs and gene regulation. *Science* **277**, 1630-1635.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T. & Traganos, F. (1997). Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* **27**, 1-20.

De Windt, L.J., Lim, H.W., Haq, S., Force, T. & Molkentin, J.D. (2000)a. Calcineurin promotes protein kinase C and c-Jun NH2-terminal kinase activation in the heart. Cross-talk between cardiac hypertrophic signaling pathways. *J Biol Chem* **275**, 13571-13579.

De Windt, L.J., Lim, H.W., Taigen, T., Wencker, D., Condorelli, G., Dorn, G.W., Kitsis, R.N. & Molkentin, J.D. (2000)b. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis In vitro and In vivo : An apoptosis-independent model of dilated heart failure. *Circ Res* **86**, 255-263.

Delcayre, C., Samuel, J.L., Marotte, F., Best-Belpomme, M., Mercadier, J.J. & Rappaport, L. (1988). Synthesis of stress proteins in rat cardiac myocytes 2-4 days after imposition of hemodynamic overload. *J Clin Invest* **82**, 460-468.

Dhillon, V.B., Mccallum, S., Norton, P., Twomey, B.M., Erkelleryuksel, F., Br, Isenberg, D.A. & Latchman, D.S. (1993). Differential heat-shock protein overexpression and its clinical Relevance in systemic lupus-erythematosus. *Annals Of The Rheumatic Diseases* **52**, 436-442.

DiDomenico, B.J., Bugaisky, G.E. & Lindquist, S. (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. *Cell* **31**, 593-603.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. & Healy, J.I. (1997). Differential activation of transcription factors induced by Ca2+ response amplitude and duration [published erratum appears in Nature 1997 Jul 17;388(6639):308. *Nature* **386**, 855-858.

Donaldson, C.J., Sutton, S.W., Perrin, M.H., Corrigan, A.Z., Lewis, K.A., Rivier, J.E., Vaughan, J.M. & Vale, W.W. (1996). Cloning and characterization of human urocortin [published erratum appears in Endocrinoogy 1996 Sep;137(9):3896. *Endocrinology* **137**, 2167-2170.

Dostal, D.E. & Baker, K.M. (1998). Angiotensin and endothelin: messengers that couple ventricular stretch to the Na+/H+ exchanger and cardiac hypertrophy. *Circ Res* **83**, 870-873.

Doucet-Brutin, S., Renoir, M., Le Gallic, L., Vincent, S., Marty, L. & Fort, P. (1995). Growth-regulated expression of FKBP-59 immunophilin in normal and transformed fibroblastic cells. *Exp Cell Res* **220**, 152-160.

Duhe, R.J. & Farrar, W.L. (1998). Structural and mechanistic aspects of Janus kinases: how the two-faced god wields a double-edged sword. *J Interferon Cytokine Res* **18**, 1-15.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. & Reed, S.I. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* **76**, 1013-1023.

Edington, B.V. & Hightower, L.E. (1990). Induction of a chicken small heat shock (stress) protein: evidence of multilevel posttranscriptional regulation. *Mol Cell Biol* **10**, 4886-4898.

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. & 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.

Engelmann, G.L., Birchenall-Roberts, M., Ruscetti, F.W. & Samarel, A.M. (1993). Formation of fetal cardiac cell clones by retroviral transformation: retention of select myocyte characteristics. *J Mol Cell Cardiol.* **25**, 197-213.

Erdmann, J., Hassfeld, S., Kallisch, H., Fleck, E. & Regitz, Z., V. (1998). Cloning and characterization of the 5'-flanking region of the human cardiotrophin-1 gene. *Biochem Biophys Res Commun.* **244**, 494-497.

Feinberg, A.P. & Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal.Biochem.* **132**, 6.

Force, T. & Bonventre, J.V. (1998). Growth factors and mitogen-activated protein kinases. *Hypertension* **31**, 152-161.

Force, T., Hajjar, R., Del Monte, F., Rosenzweig, A. & Choukroun, G. (1999). Signaling pathways mediating the response to hypertrophic stress in the heart. *Gene Expr.* **7**, 337-348.

Force, T., Pombo, C.M., Avruch, J.A., Bonventre, J.V. & Kyriakis, J.M. (1996). Stress-activated protein kinases in cardiovascular disease. *Circ Res* **78**, 947-953.

Fortin, A., Raybaud-Diogene, H., Tetu, B., Huot, J., Landry, J. & Deschenes, R. (2000). Overexpression of the 27 KDa heat shock protein is associated with thermoresistance and chemoresistance but not with radioresistance. *Int J Radiat Oncol Biol Phys* **46**, 1259-1266.

Freeman, B.C. & Morimoto, R.I. (1996). The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J* **15**, 2969-2979.

Fujio, Y., Kunisada, K., Hirota, H., YamauchiTakihara, K. & Kishimoto, T. (1997). Signals through gp130 upregulate bcl-x gene expression via STAT1- binding cis-element in cardiac myocytes. *Journal Of Clinical Investigation* **99**, 2898-2905.

Fukada, T., Hibi, M., Yamanaka, Y., Takahashi, T.M., Fujitani, Y., Yamaguchi, T., Nakajima, K. & Hirano, T. (1996). Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity.* **5**, 449-460.

Fukuzawa, J., Booz, G.W., Hunt, R.A., Shimizu, N., Karoor, V., Baker, K.M. & Dostal, D.E. (2000). Cardiotrophin-1 increases angiotensinogen mRNA in rat cardiac myocytes through STAT3 : An autocrine loop for hypertrophy. *Hypertension* **35** , 1191-1196.

Fuller, S.J., Gillespie, B.J. & Sugden, P.H. (1998). Oncogenic src, raf, and ras stimulate a hypertrophic pattern of gene expression and increase cell size in neonatal rat ventricular myocytes. *J Biol Chem.* **273**, 18146-18152.

Funamoto, M., Hishinuma, S., Fujio, Y., Matsuda, Y., Kunisada, K., Oh, H., Negoro, S., Tone, E., Kishimoto, T. & Yamauchi-Takihara, K. (2000). Isolation and characterization of the murine cardiotrophin-1 gene: expression and norepinephrine-induced transcriptional activation. *J Mol Cell Cardiol* **32**, 1275-1284.

Gabai, V.L., Meriin, A.B., Yaglom, J.A., Volloch, V.Z. & Sherman, M.Y. (1998). Role of Hsp70 in regulation of stress-kinase JNK: implications in apoptosis and aging. *FEBS Lett* **438**, 1-4.

Galron, R., Kloog, Y., Bdolah, A. & Sokolovsky, M. (1989). Functional endothelin/sarafotoxin receptors in rat heart myocytes: structure-activity relationships and receptor subtypes. *Biochem Biophys Res Commun* **163**, 936-943.

Gans, J.H. & Cater, M.R. (1970). Norepinephrine induced cardiac hypertrophy in dogs. *Life Sci [I]* **9**, 731-740.

Gavrieli, Y., Sherman, Y. & Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119**, 493-501.

Geng, Y. & Lotz, M. (1995). Increased intracellular Ca2+ selectively suppresses IL-1-induced NO production by reducing iNOS mRNA stability. *J Cell Biol* **129**, 1651-1657.

Gerhartz, C., Heesel, B., Sasse, J., Hemmann, U., Landgraf, C., Schneider-Mergener, J., Horn, F., Heinrich, P.C. & Graeve, L. (1996). Differential activation of acute phase response factor/STAT3 and STAT1 via the cytoplasmic domain of the interleukin 6 signal transducer

gp130. I. Definition of a novel phosphotyrosine motif mediating STAT1 activation. *J Biol Chem* **271**, 12991-12998.

Gerner, E. & Scheider, M.J. (1975). Induced thermal resistance in HeLa cells. *Nature* **256**, 500-502.

Gibbons, I.R. (1988). Dynein ATPases as microtubule motors. *Journal Of Biological Chemistry* **263** , 15837-15840.

Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M.H. & Shaw, P.E. (1995). ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J* **14**, 951-962.

Gillespie-Brown, J., Fuller, S.J., Bogoyevitch, M.A., Cowley, S. & Sugden, P.H. (1995). The mitogen-activated protein kinase kinase MEK1 stimulates a pattern of gene expression typical of the hypertrophic phenotype in rat ventricular cardiomyocytes. *J Biol Chem* **270**, 28092-28096.

Glennon, P.E., Kaddoura, S., Sale, E.M., Sale, G.J., Fuller, S.J. & Sugden, P.H. (1996). Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. *Circ.Res.* **78**, 954-961.

Gold, B.G., Densmore, V., Shou, W., Matzuk, M.M. & Gordon, H.S. (1999). Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *J Pharmacol Exp Ther* **289**, 1202-1210.

Gonzalez, F.A., Raden, D.L. & Davis, R.J. (1991). Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J Biol Chem* **266**, 22159-22163.

Gottowik, J., Goetschy, V., Henriot, S., Kitas, E., Fluhman, B., Clerc, R.G., Moreau, J.L., Monsma, F.J. & Kilpatrick, G.J. (1997). Labelling of CRF1 and CRF2 receptors using the novel radioligand, [3H]-urocortin. *Neuropharmacology* **36**, 1439-1446.

Gottshall, K.R., Hunter, J.J., Tanaka, N., Dalton, N., Becker, K.D., Ross, J.J. & Chien, K.R. (1997). Ras-dependent pathways induce obstructive hypertrophy in echo-selected transgenic mice. *Proc Natl Acad Sci U S A* **94**, 4710-4715.

Gray, M.O., Long, C.S., Kalinyak, J.E., Li, H.T. & Karliner, J.S. (1998). Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts. *Cardiovasc Res* **40**, 352-363.

Green, D.R. & Reed, J.C. (1998). Mitochondria and apoptosis. *Science* **281**, 1309-1312.

Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI). (1986). Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. *Lancet* **1**, 397-402.

Gupta, S., Campbell, D., Derijard, B. & Davis, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**, 389-393.

Haas, I.G. & Wabl, M. (1983). Immunoglobulin heavy chain binding protein. *Nature* **306**, 387-389.

Hadley, M.E. (1996). *Endocrinology*. Prentice-Hall Inc. New Jersey,

Hamanaka, I., Saito, Y., Nishikimi, T., Magaribuchi, T., Kamitani, S., Kuwahara, K., Ishikawa, M., Miyamoto, Y., Harada, M., Ogawa, E., Kajiyama, N., Takahashi, N., Izumi, T., Shirakami, G., Mori, K., Inobe, Y., Kishimoto, I., Masuda, I., Fukuda, K. & Nakao, K. (2000). Effects of cardiotrophin-1 on hemodynamics and endocrine function of the heart. *Am J Physiol Heart Circ Physiol* **279**, H388-H396

Hanford, D.S., Thuerauf, D.J., Murray, S.F. & Glembotski, C.C. (1994). Brain natriuretic peptide is induced by alpha 1-adrenergic agonists as a primary response gene in cultured rat cardiac myocytes. *J Biol Chem* **269**, 26227-26233.

Harada, M., Saito, Y., Kuwahara, K., Ogawa, E., Ishikawa, M., Nakagawa, O., Miyamoto, Y., Kamitani, S., Hamanaka, I., Kajiyama, N., Takahashi, N., Masuda, I., Itoh, H. & Nakao, K. (1998). Interaction of myocytes and nonmyocytes is necessary for mechanical stretch to induce ANP/BNP production in cardiocyte culture. *J Cardiovasc Pharmacol* **31 Suppl 1**, S357-S359

Harroch, S., Revel, M. & Chebath, J. (1994). Induction by interleukin-6 of interferon regulatory factor 1 (IRF-1) gene expression through the palindromic interferon response element pIRE and cell type-dependent control of IRF-1 binding to DNA. *EMBO J* **13**, 1942-1949.

He, B., Meng, Y. & Mivechi, N.F. (1998). Glycogen synthase kinase 3β and extracellular signal-regulated kinase inactivate heat shock transcription factor 1 by facilitating the disappearance of transcriptionally active granules after heat shock. *Mol Cell Biol* **18**, 6624-6633.

Heads, R.J., Latchman, D.S. & Yellon, D.M. (1994). Stable high level expression of a transfected human HSP70 gene protects a heart-derived muscle cell line against thermal stress. *J Mol Cell Cardiol* **26**, 695-699.

Heinrich, P.C., Behrmann, I., MullerNewen, G., Schaper, F. & Graeve, L. (1998). Interleukin-6 type cytokine signalling through the gp130/Jak/STAT pathway. *Biochemical Journal* **334**, 297-314.

Heinrichs, S.C. & Richard, D. (1999). The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. *Neuropeptides* **33**, 350-359.

Hemmann, U., Gerhartz, C., Heesel, B., Sasse, J., Kurapkat, G., Grotzinger, J., Wollmer, A., Zhong, Z., Darnell, J.E.J., Graeve, L., Heinrich, P.C. & Horn, F. (1996). Differential activation of acute phase response factor/Stat3 and Stat1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. II. Src homology SH2 domains define the specificity of stat factor activation. *J Biol Chem* **271**, 12999-13007.

Hickey, E., Brandon, S.E., Smale, G., Lloyd, D. & Weber, L.A. (1989). Sequence and regulation of a gene encoding a human 89-kilodalton heat shock protein. *Mol Cell Biol* **9**, 2615-2626.

Hill, C.S. & Treisman, R. (1995). Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J* **14**, 5037-5047.

Hirata, Y. (1989). Endothelin-1 receptors in cultured vascular smooth muscle cells and cardiocytes of rats. *J Cardiovasc Pharmacol* **13 Suppl 5**, S157-S158

Hishinuma, S., Funamoto, M., Fujio, Y., Kunisada, K. & Yamauchi-Takihara, K. (1999). Hypoxic stress induces cardiotrophin-1 expression in cardiac myocytes. *Biochem Biophys Res Commun* **264**, 436-440.

Hoey, T., Sun, Y.L., Williamson, K. & Xu, X. (1995). Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity* **2**, 461-472.

Horvai, A.E., Xu, L., Korzus, E., Brard, G., Kalafus, D., Mullen, T.M., Rose, D.W., Rosenfeld, M.G. & Glass, C.K. (1997). Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. *Proc Natl Acad Sci U S A* **94**, 1074-1079.

Horvath, C.M., Stark, G.R., Kerr, I.M. & Darnell, J.E.J. (1996). Interactions between STAT and non-STAT proteins in the interferon-stimulated gene factor 3 transcription complex. *Mol Cell Biol* **16**, 6957-6964.

Huang, S.N., Yang, H., Zhang, C.H., Wen, J.K., Tang, C.S., Tang, J. & Zhou, A.R. (1994). Transcription of HSP 70 gene induced by pressure overload in left ventricule of rats. *Sheng Li Hsueh Pao* **46**, 333-337.

Hunter, J.J., Tanaka, N., Rockman, H.A., Ross, J.J. & Chien, K.R. (1995). Ventricular expression of a MLC-2v-ras fusion gene induces cardiac hypertrophy and selective diastolic dysfunction in transgenic mice. *J Biol Chem* **270**, 23173-23178.

Hutter, M.M., Sievers, R.E., Barbosa, V. & Wolfe, C.L. (1994). Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. *Circulation* **89**, 355-360.

Iannotti, A.M., Rabideau, D.A. & Dougherty, J.J. (1988). Characterization of purified avian 90,000-Da heat shock protein. *Arch Biochem Biophys* **264**, 54-60.

Ikeda, K., Tojo, K., Sato, S., Ebisawa, T., Tokudome, G., Hosoya, T., Harada, M., Nakagawa, O. & Nakao, K. (1998). Urocortin, a newly identified corticotropin-releasing factor-related mammalian peptide, stimulates atrial natriuretic peptide and brain natriuretic peptide secretions from neonatal rat cardiomyocytes. *Biochem.Biophys.Res.Commun.* **250**, 298-304.

Ishikawa, M., Saito, Y., Miyamoto, Y., Kuwahara, K., Ogawa, E., Nakagawa, O., Harada, M., Masuda, I. & Nakao, K. (1996). cDNA cloning of rat cardiotrophin-1 (CT-1): augmented expression of CT-1 gene in ventricle of genetically hypertensive rats. *Biochem Biophys Res Commun.* **219**, 377-381.

ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. (1988). Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. *Lancet* **2**, 349-360.

Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Taniguchi, K. & Marumo, F. (1991). Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ Res* **69**, 209-215.

Iwai, N., Shimoike, H. & Kinoshita, M. (1995). Cardiac renin-angiotensin system in the hypertrophied heart. *Circulation* **92**, 2690-2696.

Izumo, S., Lompre, A.M., Matsuoka, R., Koren, G., Schwartz, K., Nadal-Ginard, B. & Mahdavi, V. (1987). Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. Interaction between hemodynamic and thyroid hormone-induced signals. *J Clin Invest* **79**, 970-977.

Izumo, S., Nadal-Ginard, B. & Mahdavi, V. (1988). Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci U S A* **85**, 339-343.

Jin, H., Yang, R., Keller, G.A., Ryan, A., Ko, A., Finkle, D., Swanson, T.A., Li, W., Pennica, D., Wood, W.I. & Paoni, N.F. (1996). In vivo effects of cardiotrophin-1. *Cytokine* **8**, 920-926.

Jin, H.K., Yang, R.H., Ko, A., Pennica, D., Wood, W.I. & Paoni, N.F. (1998). Effects of cardiotrophin-1 on haemodynamics and cardiac function in conscious rats. *Cytokine* **10**, 19-25.

Johnson, D.B. & Dell'Italia, L.J. (1996). Cardiac hypertrophy and failure in hypertension. *Curr.Opin.Nephrol.Hypertens.* **5**, 186-191.

Jolly, C., Morimoto, R.I., RobertNicoud, M. & Vourch, C. (1997). HSF-1 transcription factor concentrates in nuclear foci during heat shock: relationship with transcription sites. *Journal Of Cell Science* **110**, 2935-2941.

Jolly, C., Usson, Y. & Morimoto, R.I. (1999). Rapid and reversible relocalization of heat shock factor 1 within seconds to nuclear stress granules. *Proc Natl Acad Sci U S A* **96**, 6769-6774.

Jougasaki, M., Tachibana, I., Luchner, A., Leskinen, H., Redfield, M.M. & Burnett, J.C.J. (2000). Augmented Cardiac Cardiotrophin-1 in Experimental Congestive Heart Failure. *Circulation* **101**, 14-17.

Kadonaga, J.T. (1998). Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell* **92**, 307-313.

Kajstura, J., Cheng, W., Reiss, K., Clark, W.A., Sonnenblick, E.H., Krajewski, S., Reed, J.C., Olivetti, G. & Anversa, P. (1996). Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* **74**, 86-107.

Kanie-ishii, C., Tanikawa, J., Nakai, A., Morimoto, R.I. & Ishii, S. (1997). Activation of HSF-3 by c-Myb in the absence of cellular stress. *Science* **277**, 246-248.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270**, 16483-16486.

Karin, M. & Smeal, T. (1992). Control of transcription factors by signal transduction pathways: the beginning of the end. *Trends Biochem Sci* **17**, 418-422.

Kariya, K., Karns, L.R. & Simpson, P.C. (1991). Expression of a constitutively activated mutant of the beta-isozyme of protein kinase C in cardiac myocytes stimulates the promoter of the beta-myosin heavy chain isogene. *J Biol Chem* **266**, 10023-10026.

King, K.L., Winer, J., Phillips, D.M., Quach, J., Williams, P.M. & Mather, J.P. (1998). Phenylephrine, endothelin, prostaglandin F2alpha' and leukemia inhibitory factor induce different cardiac hypertrophy phenotypes in vitro. *Endocrine* **9**, 45-55.

Kira, Y., Kochel, P.J., Gordon, E.E. & Morgan, H.E. (1984). Aortic perfusion pressure as a determinant of cardiac protein synthesis. *Am J Physiol* **246**, C247-C258

Klee, C.B., Draetta, G.F. & Hubbard, M.J. (1988). Calcineurin. *Adv Enzymol Relat Areas Mol Biol* **61**, 149-200.

Klee, C.B., Ren, H. & Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J Biol Chem* **273**, 13367-13370.

Knowlton, K.U., Baracchini, E., Ross, R.S., Harris, A.N., Henderson, S.A., Evans, S.M., Glembotski, C.C. & Chien, K.R. (1991). Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during alpha-adrenergic stimulation of neonatal rat ventricular cells. Identification of cis sequences within an embryonic and a constitutive contractile protein gene which mediate inducible expression. *J Biol Chem.* **266**, 7759-7768.

Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F. & Yazaki, Y. (1990). Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* **265**, 3595-3598.

Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F. & 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.

Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. & Yahara, I. (1986). Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins. *Proc Natl Acad Sci U S A* **83**, 8054-8058.

Kunisada, K., Hirota, H., Fujio, Y., Matsui, H., Tani, Y., Yamauchi, T.K. & Kishimoto, T. (1996). Activation of JAK-STAT and MAP kinases by leukemia inhibitory factor through gp130 in cardiac myocyte [published erratum appears in Circulation 1997 Apr 1;95(7):1975]. *Circulation* **94**, 2626-2632.

Kunisada, K., Negoro, S., Tone, E., Funamoto, M., Osugi, T., Yamada, S., Okabe, M., Kishimoto, T. & Yamauchi-Takihara, K. (2000). Signal transducer and activator of transcription 3 in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy. *Proc Natl Acad Sci U S A* **97**, 315-319.

Kunisada, K., Tone, E., Fujio, Y., Matsui, H., Yamauchi-Takihara, K. & Kishimoto, T. (1998). Activation of gp130 transduces hypertrophic signals via STAT3 in cardiac myocytes. *Circulation* **98**, 346-352.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R. & Hall, M.N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* **73**, 585-596.

Kuwahara, K., Saito, Y., Harada, M., Ishikawa, M., Ogawa, E., Miyamoto, Y., Hamanaka, I., Kamitani, S., Kajiyama, N., Takahashi, N., Nakagawa, O., Masuda, I. & Nakao, K. (1999). Involvement of Cardiotrophin-1 in Cardiac Myocyte-Nonmyocyte Interactions During Hypertrophy of Rat Cardiac Myocytes In Vitro. *Circulation* **100**, 1116-1124.

Kuwahara, K., Saito, Y., Kishimoto, I., Miyamoto, Y., Harada, M., Ogawa, E., Hamanaka, I., Kajiyama, N., Takahashi, N., Izumi, T., Kawakami, R. & Nakao, K. (2000). Cardiotrophin-1 Phosphorylates Akt and BAD, and Prolongs Cell Survival via a PI3K-dependent Pathway in Cardiac Myocytes. *J Mol Cell Cardiol* **32**, 1385-1394.

Kuwahara, K., Saito, Y., Ogawa, Y., Tamura, N., Ishikawa, M., Harada, M., Ogawa, E., Miyamoto, Y., Hamanaka, I., Kamitani, S., Kajiyama, N., Takahashi, N., Nakagawa, O., Masuda, I. & Nakao, K. (1998). Endothelin-1 and cardiotrophin-1 induce brain natriuretic peptide gene expression by distinct transcriptional mechanisms. *J Cardiovasc Pharmacol* **31 Suppl 1**, S354-S356

Laks, M.M. & Morady, F. (1976). Norepinephrine--the myocardial hypertrophy hormone? *Am Heart J* **91**, 674-675.

Lane, D.P. (1993). Cancer. A death in the life of p53. *Nature* **362**, 786-787.

Larson, J.S., Schuetz, T.J. & Kingston, R.E. (1988). Activation in vitro of sequence-specific DNA binding by a human regulatory factor. *Nature* **335**, 372-375.

Latchman, D.S. (1999). Cardiotrophin-1 (CT-1): a novel hypertrophic and cardioprotective agent. *Int J Exp Pathol* **80**, 189-196.

Lavoie, J.N., L'Allemain, G., Brunet, A., Muller, R. & Pouyssegur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem.* **271**, 20608-20616.

Layton, M.J., Lock, P., Metcalf, D. & Nicola, N.A. (1994). Cross-species receptor binding characteristics of human and mouse leukemia inhibitory factor suggest a complex binding interaction. *J Biol Chem* **269**, 17048-17055.

Lebeau, M.C., Myagkikh, I., Rouviere-Fourmy, N., Baulieu, E.E. & Klee, C.B. (1994). Rabbit FKBP-59/HBI does not inhibit calcineurin activity in vitro. *Biochem Biophys Res Commun* **203**, 750-755.

Lee, H.R., Henderson, S.A., Reynolds, R., Dunnmon, P., Yuan, D. & Chien, K.R. (1988). Alpha 1-adrenergic stimulation of cardiac gene transcription in neonatal rat myocardial cells. Effects on myosin light chain-2 gene expression. *J Biol Chem* **263**, 7352-7358.

Lee, R.T., Bloch, K.D., Pfeffer, J.M., Pfeffer, M.A., Neer, E.J. & Seidman, C.E. (1988). Atrial natriuretic factor gene expression in ventricles of rats with spontaneous biventricular hypertrophy. *J Clin Invest* **81**, 431-434.

Lee, W.C., Lin, K.Y., Chiu, Y.T., Lin, J.H., Cheng, H.C., Huang, H.C., Yang, P.C., Liu, S.K. & Mao, S.J. (1996). Substantial decrease of heat shock protein 90 in ventricular tissues of two sudden death pigs with hypertrophic cardiomyopathy. *FASEB J* **10**, 1198-1204.

Leitch, I.M., Boura, A.L., Botti, C., Read, M.A., Walters, W.A. & Smith, R. (1998). Vasodilator actions of urocortin and related peptides in the human perfused placenta in vitro. *J Clin Endocrinol Metab* **83**, 4510-4513.

Leite, M., Quinta-Costa, M., Leite, P.S. & Guimaraes, J.E. (1999). Critical evaluation of techniques to detect and measure cell death--study in a model of UV radiation of the leukaemic cell line HL60. *Anal Cell Pathol* **19**, 139-151.

Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A. & Pouyssegur, J. (1993). Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J Cell Biol* **122**, 1079-1088.

Levine, B., Kalman, J., Mayer, L., Fillit, H.M. & Packer, M. (1990). Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* **323**, 236-241.

Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B. & Castelli, W.P. (1990). Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* **322**, 1561-1566.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. & Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489.

Lindquist, S. (1986). The heat-shock response. *Annu.Rev.Biochem* **55**, 1151-1191.

Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D. & Shuai, K. (1998). Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* **95**, 10626-10631.

Liu, J., Farmer, J.D.J., Lane, W.S., Friedman, J., Weissman, I. & Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815.

Liu, Y.Z., Chrivia, J.C. & Latchman, D.S. (1998). Nerve growth factor up-regulates the transcriptional activity CBP through activation of the p42/p44(MAPK) cascade. *Journal Of Biological Chemistry* **273**, 32400-32407.

Locke, M., Tanguay, R.M., Klabunde, R.E. & Ianuzzo, C.D. (1995). Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *Am J Physiol.* **269**, H320-H325

Loh, C., Shaw, K.T., Carew, J., Viola, J.P., Luo, C., Perrino, B.A. & Rao, A. (1996). Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. *J Biol Chem* **271**, 10884-10891.

Lorell, B.H. (1995). Cardiac renin-angiotensin system: role in development of pressure-overload hypertrophy. *Can J Cardiol* **11 Suppl F**, 7F-12F.

Lovenberg, T.W., Chalmers, D.T., Liu, C. & De Souza, E.B. (1995). CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. *Endocrinology* **136**, 4139-4142.

Lowe, H., Schulz, U. & Blasig, I.E. (1988). The effect of amrinone on LDH release and perfusion pressure in isolated ischemic rabbit hearts. *Pharmazie* **43**, 501-502.

Lundgren, E., Borg, T. & Mardh, S. (1984). Isolation, characterization and adhesion of calcium-tolerant myocytes from the adult rat heart. *J Mol Cell Cardiol* **16**, 355-362.

MacLellan, W.R. & Schneider, M.D. (1997). Death by design. Programmed cell death in cardiovascular biology and disease. *Circ Res* **81**, 137-144.

Mann, D.L., Kent, R.L. & Cooper, G. (1989). Load regulation of the properties of adult feline cardiocytes: growth induction by cellular deformation. *Circ Res* **64**, 1079-1090.

Marber, M.S., Mestril, R., Chi, S.H., Sayen, M.R., Yellon, D.M. & Dillmann, W.H. (1995). Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest.* **95**, 1446-1456.

Margulies, K.B., Hildebrand, F.L.J., Lerman, A., Perrella, M.A. & Burnett, J.C.J. (1990). Increased endothelin in experimental heart failure. *Circulation* **82**, 2226-2230.

Mascareno, E., Dhar, M. & Siddiqui, M.A. (1998). Signal transduction and activator of transcription (STAT) protein- dependent activation of angiotensinogen promoter: a cellular signal for hypertrophy in cardiac muscle. *Proc Natl Acad Sci U S A* **95**, 5590-5594.

Mascotti, K., McCullough, J. & Burger, S.R. (2000). HPC viability measurement: trypan blue versus acridine orange and propidium iodide. *Transfusion* **40**, 693-696.

Massol, N., Lebeau, M.C., Renoir, J.M., Faber, L.E. & Baulieu, E.E. (1992). Rabbit FKBP59-heat shock protein binding immunophillin (HBI) is a calmodulin binding protein. *Biochem Biophys Res Commun* **187**, 1330-1335.

Matsuda, S., Gotoh, Y. & Nishida, E. (1993). Phosphorylation of Xenopus mitogen-activated protein (MAP) kinase kinase by MAP kinase kinase kinase and MAP kinase. *J Biol Chem* **268** , 3277-3281.

Matsumura, K., Jeremy, R.W., Schaper, J. & Becker, L.C. (1998). Progression of myocardial necrosis during reperfusion of ischemic myocardium. *Circulation* **97**, 795-804.

McMillan, D.R., Xiao, X., Shao, L., Graves, K. & Benjamin, I.J. (1998). Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol.Chem.* **273**, 7523-7528.

Mehlen, P., Preville, X., Chareyron, P., Briolay, J., Klemenz, R. & Arrigo, A.P. (1995). Constitutive expression of human hsp27, Drosophila hsp27, or human alpha B-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. *J Immunol* **154**, 363-374.

Mehlen, P., Schulze-Osthoff, K. & Arrigo, A.P. (1996). Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. *J Biol Chem* **271**, 16510-16514.

Mercadier, J.J., Lompre, A.M., Duc, P., Boheler, K.R., Fraysse, J.B., Wisnewsky, C., Allen, P.D., Komajda, M. & Schwartz, K. (1990). Altered sarcoplasmic reticulum Ca2(+)-ATPase gene expression in the human ventricle during end-stage heart failure. *J Clin Invest* **85**, 305-309.

Mesner-PW, J. & Kaufmann, S.H. (1997). Methods utilized in the study of apoptosis. *Adv.Pharmacol.* **41**, 57-87.

Mestril, R., Giordano, F.J., Conde, A.G. & Dillmann, W.H. (1996). Adenovirus-mediated gene transfer of a heat shock protein 70 (hsp 70i) protects against simulated ischemia. *J Mol Cell Cardiol.* **28**, 2351-2358.

Mezger, V., Rallu, M., Morimoto, R.I., Morange, M. & Renard, J.P. (1994). Heat shock factor 2-like activity in mouse blastocysts. *Dev Biol* **166**, 819-822.

Minami, Y., Kimura, Y., Kawasaki, H., Suzuki, K. & Yahara, I. (1994). The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function in vivo. *Mol Cell Biol* **14**, 1459-1464.

Minshull, J., Sun, H., Tonks, N.K. & Murray, A.W. (1994). A MAP kinase-dependent spindle assembly checkpoint in Xenopus egg extracts. *Cell* **79**, 475-486.

Mivechi, N.F. & Giaccia, A.J. (1995). Mitogen-activated protein kinase acts as a negative regulator of the heat shock response in NIH3T3 cells. *Cancer Res* **55**, 5512-5519.

Miyashita, T., Harigai, M., Hanada, M. & Reed, J.C. (1994). Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* **54**, 3131-3135.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R. & Olson, E.N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93**, 215-228.

Monopoli, A. & Ongini, E. (1994). Effects of antihypertensive drugs on cardiac hypertrophy. *Pharmacol.Res.* **29**, 197-215.

Morimoto, R.I., Sarge, K.D. & Abravaya, K. (1992). Transcriptional regulation of heat shock genes. A paradigm for inducible genomic responses. *J Biol Chem.* **267**, 21987-21990.

Morisco, C., Zebrowski, D., Condorelli, G., Tsichlis, P., Vatner, S.F. & Sadoshima, J. (2000). The Akt-glycogen synthase kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by beta-adrenergic receptor stimulation in cardiac myocytes. *J Biol Chem* **275**, 14466-14475.

Morris, S.D., Cumming, D.V., Latchman, D.S. & Yellon, D.M. (1996). Specific induction of the 70-kD heat stress proteins by the tyrosine kinase inhibitor herbimycin-A protects rat neonatal cardiomyocytes. A new pharmacological route to stress protein expression? *J Clin Invest.* **97**, 706-712.

Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. & Kishimoto, T. (1991). Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc Natl Acad Sci U S A* **88**, 11349-11353.

Murphy, S.P., Gorzowski, J.J., Sarge, K.D. & Phillips, B. (1994). Characterisation of constitutive HSF2 DNA binding activity in mouse embryonal carcinoma cells. *Mol Cell Biol* **14** , 5309-5317.

Nadeau, K., Das, A. & Walsh, C.T. (1993). Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J Biol Chem* **268**, 1479-1487.

Nagai, R., Zarain-Herzberg, A., Brandl, C.J., Fujii, J., Tada, M., MacLennan, D.H., Alpert, N.R. & Periasamy, M. (1989). Regulation of myocardial Ca2+-ATPase and phospholamban mRNA expression in response to pressure overload and thyroid hormone. *Proc Natl Acad Sci U S A* **86**, 2966-2970.

Nakai, A., Tanabe, M., Kawazoe, Y., Inazawa, J., Morimoto, R.I. & Nagata, K. (1997). HSF4, a new member of the human heat shock factor family which lacks properties of a transcriptional activator. *Mol Cell Biol* **17**, 469-481.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. & Akira, S. (1993). Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc.Natl.Acad.Sci U.S.A.* **90**, 2207-2211.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. & Akira, S. (1993). Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc.Natl.Acad.Sci U.S.A.* **90**, 2207-2211.

Narula, J., Pandey, P., Arbustini, E., Haider, N., Narula, N., Kolodgie, F.D., Dal-Bello, B., Semigran, M.J., Bielsa, M.A., Dec, G.W., Israels, S., Ballester, M., Virmani, R., Saxena, S. & Kharbanda, S. (1999). Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. *Proc Natl Acad Sci U S A* **96**, 8144-8149.

Neckers, L., Mimnaugh, E. & Schulte, T.W. (1999). The Hsp90 Chaperone Family. In *Stress Protiens* (Latchman, D.S., ed.), Springer-Verlag, pp. 9-42.

Nemoto, S., Sheng, Z. & Lin, A. (1998). Opposing effects of Jun kinase and p38 mitogen-activated protein kinases on cardiomyocyte hypertrophy. *Mol Cell Biol* **18**, 3518-3526.

Neyses, L. & Pelzer, T. (1995). The biological cascade leading to cardiac hypertrophy. *Eur.Heart J* **16 Suppl N**, 8-11.

Nicholson, D.W. & Thornberry, N.A. (1997). Caspases: killer proteases. *Trends Biochem Sci* **22**, 299-306.

Nishida, K., Yoshida, Y., Itoh, M., Fukada, T., Ohtani, T., Shirogane, T., Atsumi, T., Takahashi-Tezuka, M., Ishihara, K., Hibi, M. & Hirano, T. (1999). Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood* **93**, 1809-1816.

O'Brien, C.A. & Manolagas, S.C. (1997). Isolation and characterization of the human gp130 promoter. Regulation by STATS. *J Biol Chem.* **272**, 15003-15010.

Ohan, N.W. & Heikkila, J.J. (1995). Involvement of differential gene expression and mRNA stability in the developmental regulation of the hsp 30 gene family in heat-shocked Xenopus laevis embryos. *Dev.Genet.* **17**, 176-184.

Oie, E., Bjornerheim, R., Clausen, O.P. & Attramadal, H. (2000). Cyclosporin A inhibits cardiac hypertrophy and enhances cardiac dysfunction during postinfarction failure in rats. *Am J Physiol Heart Circ Physiol* **278**, H2115-H2123

Okosi, A., Brar, B.K., Chan, M., DSouza, L., Smith, E., Stephanou, A., Latchman, D.S., Chowdrey, H.S. & Knight, R.A. (1998). Expression and protective effects of urocortin in cardiac myocytes. *Neuropeptides* **32**, 167-171.

Olivetti, G., Abbi, R., Quaini, F., Kajstura, J., Cheng, W., Nitahara, J.A., Quaini, E., Di-Loreto, C., Beltrami, C.A., Krajewski, S., Reed, J.C. & Anversa, P. (1997). Apoptosis in the failing human heart. *N.Engl.J Med* **336**, 1131-1141.

Olson, E.N. & Molkentin, J.D. (1999). Prevention of cardiac hypertrophy by calcineurin inhibition: hope or hype?. *Circ.Res.* **84**, 623-632.

Ostle, B. (1963). *Statistics in Research.* Iowa State University Press pp. 466-469.

Owens-Grillo, J.K., Hoffmann, K., Hutchison, K.A., Yem, A.W., Deibel, M.R.J., Handschumacher, R.E. & Pratt, W.B. (1995). The cyclosporin A-binding immunophilin CyP-40 and the FK506-binding immunophilin hsp56 bind to a common site on hsp90 and exist in independent cytosolic heterocomplexes with the untransformed glucocorticoid receptor. *J Biol.Chem.* **270**, 20479-20484.

Pan, J., Fukuda, K., Kodama, H., Makino, S., Takahashi, T., Sano, M., Hori, S. & Ogawa, S. (1997). Role of angiotensin II in activation of the JAK/STAT pathway induced by acute pressure overload in the rat heart. *Circ.Res* **81**, 611-617.

Pan, J., Fukuda, K., Kodama, H., Sano, M., Takahashi, T., Makino, S., Kato, T., Manabe, T., Hori, S. & Ogawa, S. (1998). Involvement of gp130-mediated signaling in pressure overload-induced activation of the JAK/STAT pathway in rodent heart. *Heart Vessels* **13**, 199-208.

Pan, J., Fukuda, K., Saito, M., Matsuzaki, J., Kodama, H., Sano, M., Takahashi, T., Kato, T. & Ogawa, S. (1999). Mechanical stretch activates the JAK/STAT pathway in rat cardiomyocytes. *Circ Res* **84**, 1127-1136.

Panniers, R. (1994). Translational control during heat shock. *Biochimie* **76**, 737-747.

Parker, T.G., Packer, S.E. & Schneider, M.D. (1990). Peptide growth factors can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. *J Clin Invest* **85**, 507-514.

Parkes, D.G., Vaughan, J., Rivier, J., Vale, W. & May, C.N. (1997). Cardiac inotropic actions of urocortin in conscious sheep. *Am J Physiol* **272**, H2115-H2122

Parsell, D.A. & Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu Rev Genet* **27**, 437-496.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J. & Sturgill, T.W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J* **10**, 885-892.

Pennica, D., Arce, V., Swanson, T.A., Vejsada, R., Pollock, R.A., Armanini, M., Dudley, K., Phillips, H.S., Rosenthal, A., Kato, A.C. & Henderson, C.E. (1996)a. Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons. *Neuron* **17**, 63-74.

Pennica, D., King, K.L., Shaw, K.J., Luis, E., Rullamas, J., Luoh, S.M., Darbonne, W.C., Knutzon, D.S., Yen, R., Chien, K.R. & et, a. (1995)a. Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc.Natl.Acad.Sci U.S.A.* **92**, 1142-1146.

Pennica, D., Shaw, K.J., Swanson, T.A., Moore, M.W., Shelton, D.L., Zioncheck, K.A., Rosenthal, A., Taga, T., Paoni, N.F. & Wood, W.I. (1995)b. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem.* **270**, 10915-10922.

Pennica, D., Swanson, T.A., Shaw, K.J., Kuang, W.J., Gray, C.L., Beatty, B.G. & Wood, W.I. (1996)b. Human cardiotrophin-1: protein and gene structure, biological and binding activities, and chromosomal localization. *Cytokine* **8**, 183-189.

Peters, M., Roeb, E., Pennica, D., Meyer-zum, B.K. & Rose, J.S. (1995). A new hepatocyte stimulating factor: cardiotrophin-1 (CT-1). *FEBS Lett.* **372**, 177-180.

Picard, D., Khursheed, B., Garabedian, M.J., Fortin, M.G., Lindquist, S. & Yamamoto, K.R. (1990). Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* **348**, 166-168.

Pickart, C.M. (1999). Ubiquitin and the stress response. In *Stress Proteins* (Latchman, D.S., ed.), Springer-Verlag, Berlin, pp. 133-152.

Piper, H.M., Probst, I., Schwartz, P., Hutter, F.J. & Spieckermann, P.G. (1982). Culturing of calcium stable adult cardiac myocytes. *J Mol Cell Cardiol* **14**, 397-412.

Plumier, J.C., Ross, B.M., Currie, R.W., Angelidis, C.E., Kazlaris, H., Kollias, G. & Pagoulatos, G.N. (1995). Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. *J Clin Invest.* **95**, 1854-1860.

Poliak, S., Mor, F., Conlon, P., Wong, T., Ling, N., Rivier, J., Vale, W. & Steinman, L. (1997). Stress and autoimmunity: the neuropeptides corticotropin-releasing factor and urocortin suppress encephalomyelitis via effects on both the hypothalamic-pituitary-adrenal axis and the immune system. *J Immunol* **158**, 5751-5756.

Pratt, W.B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J Biol Chem* **268**, 21455-21458.

Pratt, W.B. (1997). The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. *Annu.Rev.Pharmacol.Toxicol.* **37**, 297-326.

Pulido, E.J., Shames, B.D., Pennica, D., O'leary, R.M., Bensard, D.D., Cain, B.S. & McIntyre, R.C.J. (1999). Cardiotrophin-1 attenuates endotoxin-induced acute lung injury. *J Surg Res* **84**, 240-246.

Qureshi, S.A., Salditt-Georgieff, M. & Darnell, J.E.J. (1995). Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3. *Proc Natl Acad Sci U S A* **92**, 3829-3833.

Rabindran, S.K., Haroun, R.I., Clos, J., Wisniewski, J. & Wu, C. (1993). Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. *Science* **259**, 230-234.

Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. & Davis, R.J. (1996). MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* **16**, 1247-1255.

Rebbe, N.F., Hickman, W.S., Ley, T.J., Stafford, D.W. & Hickman, S. (1989). Nucleotide sequence and regulation of a human 90-kDa heat shock protein gene. *J Biol Chem* **264**, 15006-15011.

Reed, J.C. & Paternostro, G. (1999). Postmitochondrial regulation of apoptosis during heart failure. *Proc Natl Acad Sci U S A* **96**, 7614-7616.

Richard, S., Leclercq, F., Lemaire, S., Piot, C. & Nargeot, J. (1998). Ca2+ currents in compensated hypertrophy and heart failure. *Cardiovasc Res* **37**, 300-311.

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in Drosophila. *Experientia* **18**, 571-573.

Robledo, O., Chevalier, S., Froger, J., Barthelaix, P.A., Pennica, D. & Gascan, H. (1997)a. Regulation of interleukin 6 expression by cardiotrophin 1. *Cytokine* **9**, 666-671.

Robledo, O., Fourcin, M., Chevalier, S., Guillet, C., Auguste, P., Pouplard, B.A., Pennica, D. & Gascan, H. (1997)b. Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component. *J Biol Chem.* **272**, 4855-4863.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. & Nebreda, A.R. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037.

Ruff, V.A., Yem, A.W., Munns, P.L., Adams, L.D., Reardon, I.M., Deibel, M.R.J. & Leach, K.L. (1992). Tissue distribution and cellular localization of hsp56, an FK506- binding protein. Characterization using a highly specific polyclonal antibody. *J Biol.Chem.* **267**, 21285-21288.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**, 35-43.

Sadoshima, J. & Izumo, S. (1993). Signal transduction pathways of angiotensin II--induced c-fos gene expression in cardiac myocytes in vitro. Roles of phospholipid-derived second messengers. *Circ Res* **73**, 424-438.

Sadoshima, J., Xu, Y., Slayter, H.S. & Izumo, S. (1993). Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* **75**, 977-984.

Saito, S., Aikawa, R., Shiojima, I., Nagai, R., Yazaki, Y. & Komuro, I. (1999). Endothelin-1 induces expression of fetal genes through the interleukin- 6 family of cytokines in cardiac myocytes. *FEBS Lett.* **456**, 103-107.

Samali, A. & Cotter, T.G. (1996). Heat shock proteins increase resistance to apoptosis. *Exp Cell Res* **223**, 163-170.

Sananes, N., Baulieu, E.E. & Le Goascogne, C. (1998). Stage-specific expression of the immunophilin FKBP59 messenger ribonucleic acid and protein during differentiation of male germ cells in rabbits and rats. *Biol Reprod* **58**, 353-360.

Sanchez, E.R. (1990). Hsp56: A novel heat shock protein associated with untransformed steroid receptor complexes. *J Biol Chem* **265**, 22067-22070.

Sarge, K.D., Murphy, S.P. & Morimoto, R.I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA binding activity, and nuclear localization and can occur in the absence of stress. *Mol Cell Biol* **13**, 1392-1407.

Sarge, K.D., Park-Sarge, O.K., Kirby, J.D., Mayo, K.E. & Morimoto, R.I. (1994). Expression of Heat shock factor 2 in mouse testis: potential role as a regulator of heat-shock protein gene expression during spermatogenesis. *Biol Reprod* **50**, 1334-1343.

Schaefer, T.S., Sanders, L.K. & Nathans, D. (1995). Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. *Proc Natl Acad Sci U S A* **92**, 9097-9101.

Schaefer, T.S., Sanders, L.K., Park, O.K. & Nathans, D. (1997). Functional differences between Stat3alpha and Stat3beta. *Mol Cell Biol* **17**, 5307-5316.

Schaeper, U., Gehring, N.H., Fuchs, K.P., Sachs, M., Kempkes, B. & Birchmeier, W. (2000). Coupling of gab1 to c-met, grb2, and shp2 mediates biological responses. *J Cell Biol* **149**, 1419-1432.

Schaper, F., Gendo, C., Eck, M., Schmitz, J., Grimm, C., Anhuf, D., Kerr, I.M. & Heinrich, P.C. (1998). Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression. *Biochem J* **335 (Pt 3)**, 557-565.

Schett, G., Redlich, K., Xu, Q., Bizan, P., Groger, M., Tohidast-Akrad, M., Kiener, H., Smolen, J. & Steiner, G. (1998). Enhanced expression of heat shock protein 70 (hsp70) and heat shock factor 1 (HSF1) activation in rheumatoid arthritis synovial tissue. Differential regulation of hsp70 expression and hsf1 activation in synovial fibroblasts by proinflammatory cytokines, shear stress, and antiinflammatory drugs. *J Clin Invest* **102**, 302-311.

Schilling, L., Kanzler, C., Schmiedek, P. & Ehrenreich, H. (1998). Characterization of the relaxant action of urocortin, a new peptide related to corticotropin-releasing factor in the rat isolated basilar artery. *Br J Pharmacol* **125**, 1164-1171.

Schindler, C. & Darnell-JE, J. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu.Rev.Biochem* **64**, 621-651.

Schlesinger, M.J. (1990). Heat shock proteins. *J Biol Chem.* **265**, 12111-12114.

Schreiber, S.L. & Crabtree, G.R. (1992). The mechanism of action of cyclosporin A and FK506. *Immunol Today* **13**, 136-142.

Schwartz, K., de la Bastie, D., Bouveret, P., Oliviero, P., Alonso, S. & Buckingham, M. (1986). Alpha-skeletal muscle actin mRNA's accumulate in hypertrophied adult rat hearts. *Circ Res* **59**, 551-555.

Seger, R., Ahn, N.G., Posada, J., Munar, E.S., Jensen, A.M., Cooper, J.A., Cobb, M.H. & Krebs, E.G. (1992). Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. *J Biol Chem* **267**, 14373-14381.

Seidel, H.M., Milocco, L.H., Lamb, P., Darnell, J.E.J., Stein, R.B. & Rosen, J. (1995). Spacing of palindromic half sites as a determinant of selective STAT (signal transducers and activators of transcription) DNA binding and transcriptional activity. *Proc Natl Acad Sci U S A* **92**, 3041-3045.

Seth, A., Gonzalez, F.A., Gupta, S., Raden, D.L. & Davis, R.J. (1992). Signal transduction within the nucleus by mitogen-activated protein kinase. *J Biol Chem* **267**, 24796-24804.

Shaknovich, R., Shue, G. & Kohtz, D.S. (1992). Conformational activation of a basic helix-loop-helix protein (MyoD1) by the C-terminal region of murine HSP90 (HSP84). *Mol Cell Biol* **12**, 5059-5068.

Shen, Y., Liu, J., Wang, X., Cheng, X., Wang, Y. & Wu, N. (1997). Essential role of the first intron in the transcription of hsp90beta gene. *FEBS Lett.* **413**, 92-98.

Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J.H. & Chien, K.R. (1997). Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. *J Biol Chem.* **272**, 5783-5791.

Sheng, Z., Pennica, D., Wood, W.I. & Chien, K.R. (1996). Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. *Development* **122**, 419-428.

Shenolikar, S. (1994). Protein serine/threonine phosphatases--new avenues for cell regulation. *Annu Rev Cell Biol* **10**, 55-86.

Shi, Y., Mosser, D.D. & Morimoto, R.I. (1998). Molecular Chaperones as HSF1-specific transcriptional repressors. *Genes & Development* **12**, 654-666.

Shima, D.T., Deutsch, U. & D'Amore, P.A. (1995). Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. *FEBS Lett.* **370**, 203-208.

Shuai, K., Horvath, C.M., Huang, L.H., Qureshi, S.A., Cowburn, D. & Darnell, J.E.J. (1994). Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* **76**, 821-828.

Shubeita, H.E., McDonough, P.M., Harris, A.N., Knowlton, K.U., Glembotski, C.C., Brown, J.H. & Chien, K.R. (1990). Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem* **265**, 20555-20562.

Simpson, P. (1983). Norepinephrine-stimulated hypertrophy of cultured rat myocardial cells is an alpha 1 adrenergic response. *J Clin Invest* **72**, 732-738.

Simpson, P., McGrath, A. & Savion, S. (1982)a. Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ.Res* **51**, 787-801.

Simpson, P. & Savion, S. (1982)b. Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Cross-striations, ultrastructure, and chronotropic response to isoproterenol. *Circ Res* **50**, 101-116.

Sistonen, L., Sarge, K.D., Phillips, B., Abravaya, K. & Morimoto, R.I. (1992). Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. *Mol Cell Biol* **12**, 4104-4111.

Song, H.Y., Dunbar, J.D., Zhang, Y.X., Guo, D. & Donner, D.B. (1995). Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor. *J Biol Chem* **270**, 3574-3581.

Sorger, P.K. (1991). Heat Shock Factor and The Heat Shock Response. *Cell* **65**, 363-366.

Stahl, N., Farruggella, T.J., Boulton, T.G., Zhong, Z., Darnell-JE, J. & Yancopoulos, G.D. (1995)a. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 β receptor components. *Science* **263**, 92-95.

Stahl, N., Farruggella, T.J., Boulton, T.G., Zhong, Z., Darnell, J.E.J. & Yancopoulos, G.D. (1995)b. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* **267**, 1349-1353.

Stenzel, P., Kesterson, R., Yeung, W., Cone, R.D., Rittenberg, M.B. & Stenzel-Poore, M.P. (1995). Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart. *Mol Endocrinol* **9**, 637-645.

Stephanou, A., Amin, V., Isenberg, D.A., Akira, S., Kishimoto, T. & Latchman, D.S. (1997). Interleukin 6 activates heat-shock protein 90 beta gene expression. *Biochem J* **321**, 103-106.

Stephanou, A., Brar, B., Heads, R., Knight, R.D., Marber, M.S., Pennica, D. & Latchman, D.S. (1998)a. Cardiotrophin-1 induces heat shock protein accumulation in cultured cardiac cells and protects them from stressful stimuli. *J Mol Cell Cardiol.* **30**, 849-855.

Stephanou, A., Brar, B.K., Scarabelli, T.M., Jonassen, A.K., Yellon, D.M., Marber, M.S., Knight, R.A. & Latchman, D.S. (2000). Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. *J Biol Chem* **275**, 10002-10008.

Stephanou, A., Isenberg, D.A., Akira, S., Kishimoto, T. & Latchman, D.S. (1998)b. The nuclear factor interleukin-6 (NF-IL6) and signal transducer and activator of transcription-3 (STAT-3) signalling pathways co-operate to mediate the activation of the hsp90 beta gene by interleukin-6 but have opposite effects an its inducibility by heat shock. *Biochemical Journal* **330**, 189-195.

Stephanou, A., Isenberg, D.A., Nakajima, K. & Latchman, D.S. (1999). STAT-1 and HSF-1 interact and activate the transcription of the Hsp-70 and Hsp-90 gene promoters. *Journal Of Biological Chemistry* **274**, 1723-1728.

Stephanou, A. & Latchman, D.S. (1999). Transcriptional regulation of the heat shock protein genes by STAT family transcription factors. *Gene Expr.* **7**, 311-319.

Stephanou, A., Latchman, D.S. & Isenberg, D.A. (1998)c. The regulation of heat shock proteins and their role in systemic lupus erythematosus. *Semin Arthritis Rheum* **28**, 155-162.

Stokoe, D., Campbell, D.G., Nakielny, S., Hidaka, H., Leevers, S.J., Marshall, C. & Cohen, P. (1992). MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J* **11**, 3985-3994.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. & Hancock, J.F. (1994). Activation of Raf as a result of recruitment to the plasma membrane [published erratum appears in Science 1994 Dec 16;266(5192):1792-3]. *Science* **264**, 1463-1467.

Su, C.Y., Chong, K.Y., Chen, J., Ryter, S., Khardori, R. & Lai, C.C. (1999). A physiologically relevant hyperthermia selectively activates constitutive hsp70 in H9c2 cardiac myoblasts and confers oxidative protection. *J Mol Cell Cardiol.* **31**, 845-855.

Sugden, P.H. (1999). Signaling in myocardial hypertrophy: life after calcineurin? *Circ Res* **84**, 633-646.

Sussman, M.A., Lim, H.W., Gude, N., Taigen, T., Olson, E.N., Robbins, J., Colbert, M.C., Gualberto, A., Wieczorek, D.F. & Molkentin, J.D. (1998). Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science* **281**, 1690-1693.

Suzuki, K., Sawa, Y., Kaneda, Y., Ichihara, H., Shirakura, R. & Matsuda, H. (1998). In vivo gene transfection with heat shock protein 70 enhances myocardial tolerance to ischemia-reperfusion injury in rat. *J Cardiol.* **31**, 187-188.

Taga, T. & Kishimoto, T. (1997). Gp130 and the interleukin-6 family of cytokines. *Annu.Rev.Immunol.* **15**, 797-819.

Tai, P.K., Maeda, Y., Nakao, K., Wakim, N.G., Duhring, J.L. & Faber, L.E. (1986). A 59-kilodalton protein associated with progestin, estrogen, androgen and glucocorticoid receptors. *Biochemistry* **25**, 5269-5275.

Taigen, T., De Windt, L.J., Lim, H.W. & Molkentin, J.D. (2000). Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A* **97**, 1196-1201.

Takahashi-Tezuka, M., Yoshida, Y., Fukada, T., Ohtani, T., Yamanaka, Y., Nishida, K., Nakajima, K., Hibi, M. & Hirano, T. (1998). Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Mol Cell Biol* **18**, 4109-4117.

Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. & Masaki, T. (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J Biol Chem* **264**, 7856-7861.

Talwar, S., Downie, P.F., Squire, I.B., Barnett, D.B., Davies, J.D. & Ng, L.L. (1999). An immunoluminometric assay for cardiotrophin-1: a newly identified cytokine is present in normal human plasma and is increased in heart failure. *Biochem.Biophys.Res.Commun.* **261**, 567-571.

Talwar, S., Squire, I.B., Downie, P.F., O'brien, R.J., Davies, J.E. & Ng, L.L. (2000). Elevated circulating cardiotrophin-1 in heart failure: relationship with parameters of left ventricular systolic dysfunction. *Clin Sci (Colch)* **99**, 83-88.

Tanabe, M., Nakai, A., Kawazoe, Y. & Nagata, K. (1997). Different thresholds in the responses of two heat shock transcription factors, HSF1 and HSF3. *J Biol Chem* **272**, 15389-15395.

Tatsuguchi, M., Hiratsuka, E., Nishikawa, T., Imamura, S. & Matsuoka, R. (1998). Expression of an embryonic skeletal myosin heavy chain and cardiotrophin-1 in the cardiomyopathic Syrian hamster heart. *Journal Of Muscle Research And Cell Motility* **19**, 455

Tetu, B., Brisson, J., Landry, J. & Huot, J. (1995). Prognostic significance of heat-shock protein-27 in node-positive breast carcinoma: an immunohistochemical study. *Breast Cancer Res Treat* **36**, 93-97.

Tetu, B., Lacasse, B., Bouchard, H.L., Lagace, R., Huot, J. & Landry, J. (1992). Prognostic influence of HSP-27 expression in malignant fibrous histiocytoma: a clinicopathological and immunohistochemical study. *Cancer Res* **52**, 2325-2328.

Theodorakis, N.G. & Morimoto, R.I. (1987). Posttranscriptional regulation of hsp70 expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. *Mol Cell Biol* **7**, 4357-4368.

Thorburn, J., Frost, J.A. & Thorburn, A. (1994). Mitogen-activated protein kinases mediate changes in gene expression, but not cytoskeletal organization associated with cardiac muscle cell hypertrophy. *J Cell Biol* **126**, 1565-1572.

Tissieres, A., Mitchell, H.K. & Tracy, U.M. (1974). Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol Biol* **84**, 389-398.

Twomey, B.M., Amin, V., Isenberg, D.A. & Latchman, D.S. (1993). Elevated levels of the 70 kD heat shock protein in patients with systemic lupus erythematosus are not dependent on enhanced transcription of the hsp70 gene. *Lupus* **2**, 297-301.

Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213**, 1394-1397.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D. & Rivier, C. (1995). Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* **378**, 287-292.

Vaux, D.L., Weissman, I.L. & Kim, S.K. (1992). Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. *Science* **258**, 1955-1957.

Vernallis, A.B., Hudson, K.R. & Heath, J.K. (1997). An antagonist for the leukemia inhibitory factor receptor inhibits leukemia inhibitory factor, cardiotrophin-1, ciliary neurotrophic factor, and oncostatin M. *J Biol Chem.* **272**, 26947-26952.

Vojtek, A.B. & Der, C.J. (1998). Increasing complexity of the Ras signaling pathway. *J Biol Chem* **273**, 19925-19928.

Voss, A.K., Thomas, T. & Gruss, P. (2000). Mice lacking HSP90beta fail to develop a placental labyrinth. *Development* **127**, 1-11.

Wagstaff, M. J. The Neuroprotective Effect of The Heat Shock Proteins. 1997. University of London Thesis.

Wagstaff, M.J.D., Smith, J., CollacoMoraes, Y., deBelleroche, J.S., Voellmy, R., Coffin, R.S. & Latchman, D.S. (1998). Delivery of a constitutively active form of the heat shock factor using a virus vector protects neuronal cells from thermal or ischaemic stress but not from apoptosis. *European Journal Of Neuroscience* **10**, 3343-3350.

Wang, G., Klostergaard, J., Khodadadian, M., Wu, J., Wu, T.-W., Fung, K.P., Carper, S.W. & Tomasovic, S.P. (1996). Murine cells transfected with human Hsp27 cDNA resist TNF-induced cytotoxicity. *J Immunother Emphasis Tumor Immunol* **19**, 9-20.

Wang, T., Donahoe, P.K. & Zervos, A.S. (1994). Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. *Science* **265**, 674-676.

Wang, Y., Su, B., Sah, V.P., Brown, J.H., Han, J. & Chien, K.R. (1998). Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. *J Biol Chem.* **273**, 5423-5426.

Watanabe, T.X., Kumagaye, S., Nishio, H., Nakajima, K., Kimura, T. & Sakakibara, S. (1989). Effects of endothelin-1 and endothelin-3 on blood pressure in conscious hypertensive rats. *J Cardiovasc Pharmacol* **13 Suppl 5**, S207-S208

Waters, S.B., Holt, K.H., Ross, S.E., Syu, L.J., Guan, K.L., Saltiel, A.R., Koretzky, G.A. & Pessin, J.E. (1995). Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. *J Biol Chem* **270**, 20883-20886.

Westwood, J.T., Clos, J. & Wu, C. (1991). Stress induced oligomerisation and chromosomal relocalization of heat-shock factor. *Nature* **353**, 822-827.

Williams, N.E. & Nelsen, E.M. (1997). HSP70 and HSP90 homologs are associated with tubulin in hetero-oligomeric complexes, cilia and the cortex of Tetrahymena. *J Cell Sci* **110 (Pt 14)**, 1665-1672.

Willingham, M.C. (1999). Cytochemical methods for the detection of apoptosis. *J Histochem.Cytochem.* **47**, 1101-1110.

Wollert, K.C. & Chien, K.R. (1997). Cardiotrophin-1 and the role of gp130-dependent signaling pathways in cardiac growth and development. *J Mol Med* **75**, 492-501.

Wollert, K.C., Taga, T., Saito, M., Narazaki, M., Kishimoto, T., Glembotski, C.C., Vernallis, A.B., Heath, J.K., Pennica, D., Wood, W.I. & Chien, K.R. (1996). Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways. *J Biol Chem.* **271**, 9535-9545.

Wood, J.N., Beval, S.J., Coete, P., Darn, P.M., Hogan, P., Latchman, D.S., Morrison, C., Rougon, G., Theveniau, M. & Wheatley, S.C. (1990). Novel cell lines display the properties of nociceptive sensory neurons. *Proc.R.Soc.Lond.[Biol.]* **241**, 187-194.

Yamada, M., Ohnishi, H., Sano, S., Araki, T., Nakatani, A., Ikeuchi, T. & Hatanaka, H. (1999). Brain-derived neurotrophic factor stimulates interactions of Shp2 with phosphatidylinositol 3-kinase and Grb2 in cultured cerebral cortical neurons. *J Neurochem* **73**, 41-49.

Yamazaki, T., Komuro, I. & Yazaki, Y. (1995). Molecular mechanism of cardiac cellular hypertrophy by mechanical stress. *J Mol Cell Cardiol.* **27**, 133-140.

Yan, M. & Templeton, D.J. (1994). Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. *J Biol Chem* **269**, 19067-19073.

Yem, A.W., Tomasselli, A.G., Heinrikson, R.L., Zurcher-Neely, H., Ruff, V.A., Johnson, R.A. & Deibel, M.R.J. (1992). The Hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP-13. *J Biol Chem* **267**, 2868-2871.

Yin, T., Sandhu, G., Wolfgang, C.D., Burrier, A., Webb, R.L., Rigel, D.F., Hai, T. & Whelan, J. (1997). Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. *J Biol Chem* **272**, 19943-19950.

Yoshida, K., Taga, T., Saito, M., Suematsu, S., Kumanogoh, A., Tanaka, T., Fujiwara, H., Hirata, M., Yamagami, T., Nakahata, T., Hirabayashi, T., Yoneda, Y., Tanaka, K., Wang, W.Z., Mori, C., Shiota, K., Yoshida, N. & Kishimoto, T. (1996). Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proc.Natl.Acad.Sci U.S.A.* **93**, 407-411.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. & Horvitz, H.R. (1993). The C.elegans Cell Death Gene ced-3 Encodes a Protein Similar to Mammalian Interlekin-1β-converting Enzyme. *Cell* **75**, 641-652.

Zhang, D., Sun, M., Samols, D. & Kushner, I. (1996). STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. *J Biol Chem* **271**, 9503-9509.

Zhang, J.J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C.M. & Darnell, J.E.J. (1996). Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. *Proc Natl Acad Sci USA* **93**, 15092-15096.

Zhong, Z., Wen, Z. & Darnell-JE, J. (1994). Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* **264**, 95-98.

Zhu, X., Wen, Z., Xu, L.Z. & Darnell-JE, J. (1997). Stat1 serine phosphorylation occurs independently of tyrosine phosphorylation and requires an activated Jak2 kinase. *Mol Cell Biol* **17**, 6618-6623.

Zhu, Z. & Mivechi, N.F. (1999). Regulatory domain of human heat shock transcription factor-2 is not regulated by hemin or heat shock. *J Cell Biochem* **73**, 56-69.

Zou, J., Guo, Y., Guettouche, T., Smith, D.F. & Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**, 471-480.