Purinergic Signalling in

Chronic Venous Insufficiency and Penile Erection

A thesis submitted to the
University of London for the degree of
Doctor of Medicine (M.D.)

by

Matthew James Metcalfe, MBChB, MRCS
Department of Surgery
Royal Free and University College Medical School, London

November 2006

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Chronic venous insufficiency (CVI) describes diseases of the lower limb veins in which venous return is impaired and varicose veins and skin ulceration may develop. The roles of purinergic signalling in regulation of vascular tone in the long saphenous vein (LSV) and in the trophic changes occurring in LSV muscle cells and epidermal keratinocytes in lower leg skin were studied. The purinergic role in penile blood flow was also studied, where regulation of penile blood flow affects tumescence.

Purinergic signalling was studied using immunohistochemistry, organ bath pharmacology and electron microscopy. $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor immunoreactivity was present on LSV smooth muscle. Purine-mediated muscle contractions were weaker in varicose veins. Electron microscopy and immunohistochemistry findings support the view that smooth muscle cells change from the contractile to the synthetic phenotype in varicose veins, associated with an upregulation of $P2Y_1$ and $P2Y_2$ receptors and a down regulation of $P2X_1$ receptors. CVI skin showed a decrease in $P2X_7$ receptor expression and an increase in $P2Y_1$, $P2Y_2$ and $P2X_5$ receptor expression in different epidermal layers. Mean skin epidermal area in CVI was reduced. Immunohistochemistry and RT-PCR techniques were used to study the presence of $P2Y_6$ receptors in corpus cavernosal tissue. Using organ bath pharmacology, $P2Y_6$ receptors on cavernosal tissue mediated relaxation which was diminished by a $P2Y_6$ antagonist.

In conclusion it is suggested that the LSV muscle cell phenotype change may be a causal factor in the development of varicose veins. The thinner epidermis found in CVI might be the result of the changes in expression of P2Y and P2X receptors on keratinocytes. Increased keratinocyte $P2X_5$ receptor activity may, in part, be accountable for epidermal thinning in CVI. Purinergic modulation of human cavernosal smooth muscle cells via the $P2Y_6$ receptor subtype might play a physiological role in penile erection.

Acknowledgements

The studies described and presented in this thesis are the original work of the author. All studies were performed by the author with the following exceptions:

- Electron microscopy was performed in conjunction with Mr M Turmaine, Department of Anatomy, University College London.
- RT-PCR of cavernosal smooth muscle was performed in conjunction with Mr D Lau, Royal Free Hospital, who also performed the functional pharmacology of cavernosal smooth muscle

Specimens were obtained by surgeons at The Heart Hospital, The Royal Free Hospital and Charing Cross Hospital. The studies in this thesis were performed in accordance with protocols approved by the Ethical Committee and with patients' informed consent.

I would like to thank the vascular surgeons (in particular Mr J Menon, Mr R Pratak, Mr B Krijsman and Mr N Garg) at the Royal Free Hospital, Pond St, London and the cardiothoracic surgeons at the Heart Hospital, Westmoreland St, London for their careful dissection and handling of the LSV tissue and excision of skin biopsies. I am grateful to Mr. J. Belringer and Mr. P. Thomas for providing penile tissues from patients undergoing gender reassignment surgery.

I would like to thank Dr GE Knight for her teaching and guidance of the pharmacology experiments, subsequent interpretation of the results and assistance in the preparation of the manuscripts.

This thesis would not have been undertaken without the continued support of my supervisors Professor G Burnstock and Mr D Baker who have directed and encouraged me from the very beginning. Professor Burnstock has helped guide me through this challenging and complex world of purines.

Abbreviations

ACh...acetylcholine

ADP... adenosine diphosphate

ANS...autonomic nervous system

ATP... adenosine 5'-triphosphate

BDNF...brain-derived neurotrophic factor

bFGF...basic fibroblast growth factor

BM...basement membrane

BOO...bladder outlet obstruction

BPH...benign prostatic hypertrophy

CABG...coronary artery bypass graft

CEAP... Clinical, a(E)tiology, Anatomical and Pathophysiological classification

cGMP...cyclic GMP

CNS...central nervous system

CGRP...calcitonin gene-related peptide

CSM...cavernosal smooth muscle

CT...connective tissue

CVH...chronic venous hypertension

CVI.....chronic venous insufficiency

DVT...deep venous thrombosis

EC...endothelial cell

ECM...extracellular matrix

ED...erectile dysfunction

EDHF...endothelium-dependent hyperpolarizing factor

EDRF...endothelium-dependent relaxing factor

EJP...excitatory junction potential

H&E...Haematoxylin and Eosin

ICAM-1...intercellular adhesion molecule-1

IJP...inhibitory junction potential

IL...interleukin

LSV...long saphenous vein

LUTS...lower urinary tract symptoms

MAPK...mitogen-activated protein kinase

α,β-meATP ...α,β-meylene ATP

2-MeSADP...2-Methylthio ADP

MMP...matrix metalloproteinases

NA.....noradrenaline

NANC... non-adrenergic, non-cholinergic

NHS...normal horse serum

NO.....nitric oxide

NOS.....nitric oxide synthase

NPY....neuropeptide Y

PBS...phosphate buffer solution

PDE5...phosphodiesterase type 5

PDGF...platelet derived growth factor

PGE_1...prostaglandin

PPADS... pyridoxal-phosphate-6-azophenyl-2', 4'-disulphonic acid

RBC...red blood cells

RT-PCR...Reverse Transcription-Polymerase Chain Reaction

SCG...superior cervical ganglion

SMC...smooth muscle cells

SP...substance P

TGF-β1...transforming growth factor-β1

TNF-α ...tumour necrosis factor-α

t-PA...tissue-type plasminogen activator

TTX...tetrodotoxin

UDP... uridine diphosphate

UTP... uridine 5'-triphosphate

VEGF...vascular endothelial growth factor

VIP...vasoactive intestinal polypeptide

WBC...white blood cells

List of contents

Preface

Receptors for purines and pyrimidines are known to mediate regulation of blood vessel tone and long term trophic pathways. This study was initially intended to identify purine receptor variation between control and varicose long saphenous vein (LSV). Measuring smooth muscle contractions would provide vessel tone related information whilst structural changes would identify trophic changes occurring. The first hypothesis was 'Purinoceptor changes occur between control and varicose LSV, and these changes are related to functional properties of the smooth muscle, either causal or consequential to varicose veins'.

Reflux within the superficial veins is thought to be an initial factor in the chain of events during the development of chronic venous insufficiency (CVI). The condition can result in ulcer formation which may take years to heal, can be debilitating for patients and may require surgery for debridement or amputation. Stripping of refluxing superficial veins is a common surgical procedure in both prevention of CVI skin changes and treatment for non-healing ulcers. The end organ of CVI, the skin, was investigated. Purine-mediated trophic changes to keratinocytes have been previously identified and so our second hypothesis was 'Purine-mediated effects on keratinocytes change in CVI skin'.

Having learnt new experimental techniques, I repeated them on penile tissue obtained from gender reassignment surgery. We focused on the $P2Y_6$ receptor as it had earlier shown contractile effects in the LSV, is unreported in cavernosal smooth muscle (CSM), and P2Y receptor activation is thought to modulate CSM function as reported in previous studies. Our hypothesis was 'The $P2Y_6$ receptor plays a short term role in dilatations underlying penile erection'. Smooth muscle function is a possible target in the treatment of erectile dysfunction and thus we might identify new targets for its treatment.

Chapter 1

Introduction

A) Chronic Venous Insufficiency

The venous system

The venous system holds approximately 2/3^{rds} of the human body's blood volume[1]. Apart from the pulmonary veins and portal vein, it returns deoxygenated blood back to the heart. Their muscular walls allow them to contract and relax so as to act as a major reservoir of blood with little change in the venous pressure. Blood flows along a pressure gradient from approximately 15-20mmHg in the venules to 0-6mmHg in the right atrium[2].

The blood flow through a vessel can be calculated using Ohm's law:

Blood flow = Pressure difference between the ends of the vessel ÷ resistance

Venous pressure within the abdominal cavity is about 2mmHg. This can rise to as high as 20mmHg in pregnancy, with large abdominal tumours and with ascites. This impedes the blood flow from the legs back to the heart. Venous pressure within the legs must rise above this pressure in order for the abdominal veins to open and allow the flow of blood back to the heart.

Hydrostatic pressure is caused by the weight of a body of water. In an adult man standing still, the column of blood from his right atrium to his foot creates a hydrostatic pressure of about 90mmHg at the foot. However valves are located within most veins and break up this column. Muscle contraction, during movement, compresses the veins and squeezes blood out. These valves, when healthy, allow blood to flow past them in one direction only, towards the heart. Contraction of the calf muscles compresses the deep veins, forcing blood in a cephalic direction, the venous muscle pump. During relaxation of these muscles, the deep venous system fills with blood from the superficial veins along a pressure gradient, via perforating and distal veins. Normal valve function prevents the reflux of blood[2]. In a walking man, the average venous pressure in the foot is 20mmHg. If he was to stand still without contracting his muscles, the venous leg pressure would rise to 90mmHg in about 30secs. A rise in venous pressure increases the pressure within the capillaries causing fluid to leak out of the circulatory system into the surrounding tissue.

Venous flow out of the lower limb can be reduced in three physiological settings:

- Obstruction of the blood flow from either a thrombus within the blood vessel higher up or from external compression on the vein from an abdominal tumour.
- Refluxing valves, causing blood to flow back down the leg and accumulate
- A failure of the muscles to adequately pump blood along the veins, seen in patients with a reduced muscle bulk. This could result from insufficient exercise or from muscle wasting disorders[3].

When a reduction in the flow of venous blood out of the leg is long term, blood accumulates in the leg, increasing in volume and thus pressure. This is known as chronic venous hypertension (CVH) or chronic venous insufficiency (CVI).

CVI can also be divided into primary and secondary

- Primary CVI results from abnormalities of the valves and the venous wall, which leads to reflux. Inflammatory reactions may play a role.
- Secondary CVI is the result of deep vein thrombosis (DVT) or superficial vein thrombophlebitis. Recanalisation of the vein following these insults may result in obstruction and incompetent valves.

Clinical presentations of CVI

CVI leads to several different clinical features:

- Increased capillary pressure in the circulatory system forces out fluid at the capillaries, resulting in swelling.
- The increase in pressure within the veins leads to dilated tortuous superficial veins known as varicose veins.

- The skin can undergo several different changes including varicose eczema, pigmentation, fibrosis and ulceration.
- The leg becomes heavy and painful, especially after standing.
- Venous claudication, where exercise increases the arterial flow, but due to the reduced venous outflow the leg becomes distended and gives a painful bursting sensation. The pain settles after 15 minutes of rest.

Aetiology of CVI

The prevalence of CVI in the adult population is between 5 and 10% and costs the National Health Service £230-600 million per year. Venous ulceration is more prevalent in elderly females, and as the female population is greater and has a higher life expectancy; its prevalence will continue to increase. Changes in the macrocirculation and microcirculation occur in CVI.

- Macrocirculation

Competent veins are one of the three mechanisms ensuring adequate venous outflow from the lower limb. Reflux in the iliofemoral, long saphenous and popliteal veins has the greatest effect on venous flow[4]. Perforating veins are also believed to be to contributors to CVI, as duplex scanning has shown the number of incompetent calf-perforating veins increase along with the severity of CVI[5].

Arterial influx into the extremity is thought to be significantly increased in CVI. This suggests the presence of arteriovenous fistulae and shunting[6]. A combination of venous reflux and venous hypertension is thought to initiate a cascade of inflammatory events.

- Microcirculation

CVI affects the overlying skin and may result in varicose eczema, lipodermatosclerosis and ultimately venous ulceration. Erythrocytes extravasate

into the skin, resulting in the deposition of haemosiderin within the macrophages. This stimulates melanin production, pigmenting the skin brown. The development of ulceration from lipodermatosclerosis is thought to be due to enhanced activity of matrix metalloproteinases (MMP). MMPs are naturally occurring enzymes that are able to degrade the extracellular matrix and participate in remodeling of tissues[7]. An underlying chronic inflammatory process is present. There are several theories to the pathophysiological pathway:

a) The 'white cell' trapping theory

Increased venous pressure reduces the perfusion pressure (pressure gradient) across the capillary bed causing white blood cells (WBC) to plug the capillaries and red blood cells (RBC) to accumulate proximally. At the post capillary venule the WBCs are forced to marginate by the RBC. The reduced pressure gradient allows the WBC to roll for longer. Endothelial cells (EC) upregulate their surface adhesion molecules in venous hypertension. There is corresponding upregulation on the WBC of integrin CD11b, which increases the binding affinity to the ligands on the endothelial surface. Thus a reduction in physical forces and an increase in the expression of adhesion molecules increase the chance of adherence.

The trapped WBCs become activated, releasing enzymes and oxygen-free radicals, damaging EC and surrounding tissue. This stimulates the release of Vascular Endothelial Growth Factor (VEGF), increasing the microvascular permeability and producing excess nitric oxide (NO), causing further tissue damage. The trapping of WBCs reduces perfusion and generates local ischaemia which be a may trigger for white cell activation. This theory was first proposed by Coleridge Smith et al[8].

Studies of CVH skin show both T-cell and macrophage deposits, suggesting a chronic inflammatory process and neutrophil activation.

Claudy et al[9] proposed that leukocyte activation also released proteolytic enzymes and elastase activity increased. These factors led to endothelial

damage, increased vessel permeability and deposition of pericapillary fibrin from fibrinogen. Elastase, a lysosomal constituent released from polymorphonuclear cells, affects the role of elastin degradation in tissue remodeling and wound healing. Leukocytes were also noted to release tumour necrosis factor α (TNF-α) which decreases fibrinolytic activity resulting in the formation of a pericapillary fibrin cuff.

b) The fibrin cuff theory

The pressure rise in CVI is thought to cause capillary elongation[10] and widening of the pores between EC[11]. This increases the surface area for exchange allowing larger molecules such as fibrinogen to leave the intravascular compartment. Fibrinogen is converted to fibrin, which accumulates and, along with fibronectin and denatured collagen macromolecules, is thought to act as a barrier to oxygen and other nutrients, resulting in ischaemia and cell death[12,13]. However Falanga et al showed that the fibrin cuffs were not a barrier to diffusion, as they observed the cuffs were discontinuous around the capillaries, and that venous ulcers healed despite the cuff presence on the ulcer border[14].

c) The 'Trap' hypothesis due to macromolecule leakage

Capillary distension or injury to endothelial cells due to CVH leads to extravasation of fibrinogen and α_2–macroglobulins from veins to dermis. These macromolecules can cause a functional inhibition of endogenous growth factors, eg transforming growth factor β (TGF-β), preventing them maintaining tissue integrity and aiding wound repair[14]. The abundance of growth factors in venous ulcers supports this hypothesis.

The CEAP classification

CVI is internationally classified into the Clinical, a(E)tiology, Anatomical and Pathophysiological classification. This was developed in 1994 and has been expanded in 2003[15,16].

- Clinical – seven classes of clinical signs are described:

  Class 0 = no visible or palpable signs of venous disease
  Class 1 = telangiectasia and reticular veins, malleolar flare
  Class 2 = varicose veins
  Class 3 = oedema without skin changes
  Class 4a = skin eczema (erythema, scaling, weeping and itching)
  Class 4b = lipodermatosclerosis, where the dermis and subcutaneous tissue become indurated and fibrosed without the presence of pitting oedema. The skin can become atrophic, losing sweat glands and hair follicles. A rigid 'woody' hardness of the skin may develop and is thought to resemble an inverted champagne bottle.
  Class 5 = previous ulceration now healed
  Class 6 = active ulceration

- A(E)tiology – Congenital (G), Primary (P, unknown) and Secondary (S, cause known e.g. post-thrombosis, post-traumatic)

- Anatomical – Superficial (S), Deep (D), and Perforating (P)

- Pathophysiological – Reflux (R) and Obstruction (O)

Investigations used to detect venous reflux

Hand held doppler enables the clinician to detect reflux in a superficial vein. In a standing patient the probe is placed over the vein, the calf squeezed manually, and an audible reflux is listened for on release of calf pressure indicating reflux along the vein. This is then repeated in the presence of a tourniquet, which should

reduce the reflux in the superficial vein when tightened. This technique, though operator dependent, is often satisfactory to detect reflux in the LSV. In patients in whom further clarification of the site of reflux is required, duplex scanning is usually sufficient, where doppler ultrasonography enables the flow along individual vessels to be studied.

Venography is an invasive technique where contrast is injected directly into varicose veins or superficial foot veins. This can be useful in the obese patient where duplex findings are limited

Calf Muscle Pump

This greatly affects the venous system of the lower leg. An efficient muscle pump induces venous hypotension on exercise, with blood pumped back towards the heart. A lack of this results in venous hypertension and skin changes. Functional calf volume measurements include foot volumetry, ambulatory venous pressure measurements and plethysmography.

Treatment of CVI

This can be divided into conservative and surgical management.

Conservative management:

- Leg elevation – legs elevated to above the level of the heart will aid venous drainage of the legs and reduce venous hypertension. This will help reduce leg oedema and improve the rate of ulcer healing.
- Prevention of risk factors – avoiding immobility (so as to encourage muscle pump activity), reducing obesity and avoiding jobs that require long periods of standing still.
- Graduated compression stockings – the application of a graduated compression stocking to the lower leg, with greatest pressure at the ankle, encourages the flow of venous blood up and out of the leg. There are four classes of compression stockings available:

Class I – ankle pressure < 25mmHg

Class II – ankle pressure 25-35mmHg

Class III – ankle pressure 35-45mmHg

Class IV – ankle pressure 45-60mmHg

The greater the degree of CVI, the higher the class of the compression stocking that should be worn.

- Dressings – a wide variety exist to aid the healing of ulcers.
- Treating infections - venous ulcers often become infected and require antibiotic therapy. The commonest organisms are Staphylococcus aureus, Pseudomonas aeruginosa and β-haemolytic streptococci.
- The drug 'Pentoxifylline' alone and in combination with compression stockings may contribute to ulcer healing. Its actions are thought to be related to inhibition of synthesis of proinflammatory cytokines, inhibition of leukocyte activation by reducing their adhesion and inhibition of platelet aggregation[17]. Another drug 'Flavanoid' may have clinical benefit. Flavanoids are natural compounds that protect cells from the effects of hypoxia, decrease the fragility of the vein valves and increase venous tone. Flavanoids also affect leukocyte adhesion and free radical formation. Neither of these drugs are widely used in present clinical practice[18].

Surgical management:

- Superficial venous surgery is indicated if significant reflux is present in the superficial component. Reflux in the long saphenous vein (LSV) requires high long saphenous vein ligation at the saphenofemoral junction, stripping of the LSV and multiple avulsions. Reflux of the saphenopopliteal junction requires saphenopopliteal disconnection and multiple avulsions. These two operations disconnect the long saphenous vein and the short saphenous vein respectively from the deep leg veins, and thus prevent the blood flowing

back towards the foot through the incompetent valves. This prevents venous blood recycling and prevents a build up in venous pressure.

- Subfascial Endoscopic Perforating Vein Surgery – this relatively new technique ligates the perforating veins connecting superficial and deep veins, preventing blood recycling itself in the leg via incompetent valves[19]. This surgery avoids disrupting the long and short saphenous veins when they are competent and functioning normally.

- Venous valve reconstruction – this is rarely performed as the majority of patients obtain satisfactory benefit form superficial venous surgery and conservative treatments. This surgery is complex. Various methods have been described including valvuloplasty (where valves are sutured together to render them competent once more) and valve transplantation (where segments of brachial or axillary veins containing competent valves are transposed into the leg vein)[20].

- Venous outflow obstruction can result following a DVT. Recanalisation of the thrombosis commonly occurs, following anticoagulation treatment, and collateral veins develop to bypass the venous occlusion. In patients where the venous outflow obstruction persists who develop symptomatic swollen legs and skin changes, bypass surgery using the LSV as a conduit is feasible, for example a femoro-femoral cross-over vein graft in patients with an iliac vein occlusion[21].

- Skin grafting – this is suitable for large ulcers, but the ulcer bed must be free from infection and slough. Grafting aims to increase the rate of ulcer healing, however the cause of the venous ulcer will still need to be addressed.

i) Varicose Veins

Varicose veins are dilated tortuous thickened superficial veins. They are most commonly found in the distribution of the long and short saphenous veins in the lower leg. Female patients often relate their vv to pregnancy and childbirth (male: female ratio 1.5-3.5 : 1)[22]. The prevalence of vv increases with age and a hereditary element is thought to exist.

They can be divided into primary and secondary veins.

- Primary varicose veins (95%) are due to damaged valves leading to reflux of blood from deep to the superficial veins, increasing the superficial venous pressure.
- Secondary varicose veins are due to changes in blood flow that lead to back pressure and therefore an increase in venous pressure (eg an arteriovenous malformation or obstruction due to a pelvic thrombosis).

Several theories exist as to the aetiology of varicose veins. Incompetent venous valves certainly occur. An inherent weakness of the muscle wall due to defective smooth muscle and CT metabolism leading to vessel dilatation is also thought to be a contributing factor. Dilatation of the vessel increases the cross sectional area. The valves do not change in size resulting in separation of valve leaflets and a failure of the valves to close completely, allowing blood to reflux through the gaps[3,23]. This causes an even greater hydrostatic pressure on the vein below. Similar structural, biochemical and functional changes in varicose tributaries and in non varicosed veins from the same patient[24] supports the hypothesis that abnormalities within the vein wall exist before the varicosities develop.

The LSV

The LSV is an important structure within the human body. It commonly becomes varicosed and due to chronic venous hypertension leads to venous ulceration[3]. It is the most widely used autogenous venous graft due to its thick walls, free availability and being the longest vein in the human body.

The LSV is a three-layered structure:

- Intima: this inner layer consists of flattened endothelial cells resting on a subendothelial connective tissue. This connective tissue consists of collagen, elastin and longitudinal smooth muscle fibres. This muscle layer thickens at the site of valves[25].
- Media: The media contains circular smooth muscle fibres, interspersed by fibroblasts and collagen.
- Adventitia: This outer layer makes up the bulk of the vein wall. It contains collagen fibres, fibroblasts and the vasa vasorum. The vasa vasorum is a network of blood vessels supplying nutrients to the vein wall. Thick bundles of longitudinal smooth muscle fibres are found in the adventitia[25-28].

Structure of varicose veins

Their wall structure varies from hypertrophic to atrophic regions, and there is loss of individual layers.

- Atrophic regions

In atrophic regions the medial SMC and extracellular matrix are diminished. The vein wall consists of a thin media lying on the adventitial fibrous tissue.[26] Vein wall thickness varies to half that of controls and may represent aneurysmal segments.[26]

- Hypertrophic regions

In hypertrophic regions the organisation is greatly disturbed. Smooth muscle bundles lose their longitudinal and circular orientation, and are broken up by an accumulation of fibrous tissue. There is an increase in the quantity of extracellular matrix and in the number of vasa vasorum within the media. The intima is diffusely thickened with both hyperplasia and hypertrophy of the intimal SMC and increased and disorganized collagen bundles[26,29].

In the hypertrophic media there is reduced staining from outer to inner for SM-α-actin and desmin. Thickened intimal SMC stain strongly for SM-α-actin and vimentin[26]. Badier-Commander et al suggested that variations in protein staining seen in SMC represent different SMC populations. They also stated that the alterations of SMC, CT metabolism and BM suggest modulation of the SMC from a contractile to a synthetic phenotype, explaining the altered functional properties.[26] Electron microscopy of SMC have shown them to contain collagen fibres, suggesting they have taken up a phagocytic role.[26]

In the vv wall, increases of TGFβ1 and bFGF have been observed[26]. TGFβ1 is known to stimulate the synthesis of ECM components especially collagens and elastins. bFGF is known to be chemotactic and mitogenic for SMC. The increase in these growth factors would explain the increase in ECM and SMC proliferation. Furthermore is would support the concept of a proliferative phenotype.

The number of vasa vasorum increase in hypertrophic areas. Proliferation factor Ki67 has been identified on endothelial cells within the vasa vasorum, suggesting angiogenesis occurs in the LSV wall[26]. Mast cells accumulating around the vasa vasorum may contribute to angiogenesis.

LSV bypass grafts

As the LSV is the longest vein in the human body, is relatively easily surgically accessible and is often expendable, it is commonly used as the bypass allograft in surgery eg CABG and revascularization in peripheral vascular disease. It is of great value to a surgeon and thus its preservation is important. Preventing varicose changes in the LSV would reduce the incidence of surgical stripping of the LSV, maintaining its availability for a bypass graft in later years.

When the LSV is exposed to arterial pressures, histological changes occur due to the hypertension (arterial blood in a venous vessel). These pressure changes could be similar in nature to the changes seen in venous hypertension. It must be stressed that these two environments are not identical, as arterial flow is pulsatile, causing both longitudinal and circular strains. Arterial flow is also of a greater pressure and arterial blood differs from venous in its composition.

Vein grafts have a limited life span eg 82% at 5yrs post CABG[30]. Factors that affect this include the diameter of the distal vascular bed and LSV manipulation during harvesting. Atherosclerosis is a process where endothelial damage leads to platelet aggregation, lipid deposition, smooth muscle formation and plaque formation. Atherosclerosis is known to occur in arteries, and risk factors include smoking, hypertension and hypercholesterolaemia. Atherosclerotic lesions have been identified in vein grafts too[31]. SMC migration and proliferation are involved in intimal hyperplasia of vein grafts. Prevention of LSV atherosclerosis would further the use of the LSV, reducing the need for revision surgery and for prosthetic grafts. Varicose LSV, assuming it has not been stripped, may deter the surgeon from using it as a graft.

ii) Skin in Chronic Venous Insufficiency

Skin is an organ that consists of two parts, an outer epidermis and a deeper underlying dermis.

Epidermis

The epidermis is a multilayered organ. It consists of keratinised, stratified squamous epithelia that divide and flatten as they move outwards away from their basal layer. In areas vulnerable to wear and tear, such as the soles of the feet and palms of the hands, the epidermis is extremely thick. In contrast it is very thin on the anterior surface of the forearm.

The deepest layer is the proliferative cell layer. It comprises of a single row of stem cells resting on a basal lamina, which is adherent to the underlying dermis. Stem cells divide by mitosis producing the majority of the cells in the epidermis (keratinocytes), which are destined to mature into the uppermost layer of keratin.

The stratum spinosum, the second deepest layer, is approximately 5 keratinocyte cells thick. A strong supporting framework lies between and within the cells. Lamellar granules are seen in the cells representing initial development of lipid rich substances, which continues in the more superficial cells.

The third layer, the stratum granulosum, is characterised by accumulation of numerous dense cytoplasmic keratohyalin granules containing proteins that promote the aggregation of the tonofilaments to form increasing quantities of keratin. At the same time the nucleus and organelles break down and their destruction results in cells filled with keratin only. These cells are programmed to destroy their nuclei and organelles, yet at the same time synthesize keratin and lamellar bodies. The contents of the lamellar granules in the granular cells are discharged into the extracellular space and provide a lipid layer, establishing a permeability barrier for the skin.

The stratum lucidum is seen in thick skin. It consists of flattened, dead cells with abundant keratin proteins.

The most superficial layer is the stratum corneum, consisting of dead, anucleate squamous cells containing keratin. It ranges from 0.1mm to >1mm in thickness. The cells are constantly shed from the surface, and replaced from cells arising from the deeper layers. Transit time from a stem cell to desquamation is about 1 month[32]. The stratum corneum plays a crucial role as the water-impermeable barrier, protecting the underlying water-rich internal organs from environmental dryness.

Dermis

This consists of connective tissue, blood vessels, lymphatic vessels and nerves. In general it is thinner on anterior surfaces and thinner in women. The dermis is connected to underlying fascia and bones by the superficial fascia.

Hair grows from follicles which are invaginations of the epidermis into the dermis. The hair bulb is the expanded end that lies deep within the dermis. The blood supply to the hair enters via a concavity within the base of the hair bulb, deep within the dermis. Sebaceous glands release secretions, known as sebum, onto the shafts of the hairs within the dermis. Sebum is an oily fluid that helps maintain the flexibility of the hair.

Sweat glands are the most deeply penetrating structures in skin. They lie beneath the dermis in the superficial fascia. The sweat duct passes from the gland to a pore on the epidermal surface. Sweat is secreted as a mechanism of heat control via sympathetic cholinergic nerves although adrenaline and noradrenaline also stimulate sweat production. In severe conditions, up to 2L/hr of total body sweat can be produced in man.

Ulcers

An ulcer is a defect in an epithelial surface. They can be classified into 4 types:

- Arterial ulcers

Tissue hypoxia and ischaemia occur as a result of a reduced blood flow and result in ulceration. The most common cause of reduced blood flow is atherosclerosis which affects medium and large sized arteries, however other causes include diabetes, vasculitis, thalassaemia and sickle cell disease. Thrombotic and embolic events may accelerate their development. Peripheral vascular disease describes a condition where there is reduced blood flow to the limbs. It commonly affect the lower limbs and produces symptoms ranging from intermittent claudication (calf pain brought on with exercise that is eased with rest) to rest pain (constant foot pain that disturbs sleep and is relieved by hanging the foot over the edge of the bed). In severe cases, regular analgesia may be required and gangrene develops. Infected gangrene, known as wet gangrene, requires intervention, either surgically or radiologically. If it progresses it will lead to systemic sepsis and death, unless amputation is performed.

Arterial ulcers commonly occur at limb extremities such as toes, heels and over bony prominences. The ulcers are punched out with well demarcated edges. The ulcer base is commonly pale and non-granulating. Skin surrounding the ulcer is often cold, dusky in colour, hairless, thin and shiny. The peripheral pulses are often absent.

- Venous

As mentioned earlier, venous ulceration is a result of CVI. 50% of venous ulcers are due to superficial venous insufficiency and/or perforating vein incompetence with a normal deep venous system. Venous ulcers are commonly persistent and painful, occurring in the gaiter area. The ulcer bed is often covered with a fibrinous layer mixed with granulation tissue, surrounded by an irregular,

gently sloping edge. The surrounding skin is often fibrotic, oedematous and pigmented[2].

- Diabetic

Due to peripheral neuropathy in diabetic patients, these ulcers are commonly painless and may go unnoticed by the person. These can be divided into neuropathic and neuroischaemic.

Neuropathic ulcers develop in warm feet with an adequate blood supply. Repetitive forces, often from walking, are the commonest cause. Peripheral nerves are damaged due to the diabetes and result in reduced peripheral sensation eg of the foot. A callus forms and if allowed to thicken, it will compress underlying soft tissue and lead to ulceration.

Neuroischaemic ulcers develop in cold feet with an insufficient blood supply. Peripheral blood vessels are often absent. Microvessels become occluded due to endothelial cell and basal lamina damage from the diabetes. They commonly develop on the margins of the foot, especially on the medial aspect of the first metatarsophalangeal joint. High friction forces eg from ill-fitting shoes, lead to blister formation. This then develops into a shallow ulcer.

- Others

Ulcers can occur due to dual pathology for example an ischaemic ulcer in a diabetic patient who therefore has both microvascular and macrovascular disease. Premature atherosclerosis occurs in diabetics, hence these patients may have stenosed large blood vessels along with small blood vessel occlusion. Another example would include a patient who has an ischaemic ulcer and CVI, here compression therapy would aid the CVI but worsen the ischaemia. This can complicate ulcer treatments as more than one pathology needs to be addressed at the same time in order to aid ulcer healing. These are sometimes referred to as mixed ulcers. Others conditions where ulcers develop include:

  - Tropical infections (eg yaws and leishmaniasis)
  - Malignancy (eg basal cell carcinoma and squamous cell carcinoma)

- Drugs (eg hydroxycarbamide)
- Coagulation disorders (abnormalities of coagulation factors can lead to skin ischaemia and ulceration eg protein C deficiency, protein S deficiency, antithrombin 3 deficiency, homocystinaemia and factor V Leiden mutation)
- Calciphylaxis (intramural hyperplasia, intravascular calcification and thrombosis occur)
- Many vasculitis conditions can result in ulceration (eg Wegener's granulomatosis and polyarteritis nodosa)
- Inflammatory disorders can lead to ulceration (eg pyeoderma gangrenosum, Behcet's syndrome, rheumatoid arthritis, systemic lupus erythematosus and Sjogren's syndrome)
- Infections (Leishmania, syphilis, tuberculosis, herpes simplex, cytomegalovirus, β-haemolytic Streptococcus pyogenes and Staphylococcus aureus)

B) The Penis

Structure and mechanism of penile erection

The body of the human penis consists of three elongated erectile masses known as the right and left corpora cavernosa and the median corpus spongiosum. The corpora cavernosa forms most of the body of the penis and is composed of an array of sinusoidal spaces. They are enveloped by a common fibrous tunica albuginea and separated by a median fibrous septum. On the ventral urethral aspect lies a wide median groove containing the corpus spongiosum. The function of the corpus cavernosa is purely erectile. In the flaccid penis, cavernous arteries are contracted and vascular resistance is high. Relaxation of the muscle tone reduces resistance to arterial inflow and brings about tumescence. The cavernous smooth muscle compliance increases and sinusoids distend with blood, increasing their dimensions and rigidity. This increase in size compresses the venous plexus against the tunica albuginea which restricts venous outflow, resulting in erection[33]. The rigidity of the tunica albuginea limits the distensibility. This results in an increase in length and circumference of the pendulous portion of the penis known as an erection. The penile volume increases by approximately 100ml on average. The erection is a manifestation of the balance between arterial inflow, venous outflow and dilatation of lacunar spaces. Tumescence involves a complex interaction between neuronal stimulation of corporal smooth muscle, neurohumoral release of specific endothelial contractile and relaxant factors and secondary modulation by a variety of neuropeptides and vasoactive modulators[34].

Adrenergic neurotransmission mediates the contraction of cavernosal smooth muscle (CSM) through stimulation of postjunctional α-adrenoceptors and maintains the penis in a flaccid state[35]. The role of β-adrenoceptors in CSM is still controversial, though its presence has been established[36]. Autonomic nervous system (ANS) control of penile erection is attributed to the vasodilator effects of acetylcholine (ACh) and vasoactive intestinal polypeptide (VIP) released from parasympathetic nerves[37], known to initiate erections in man. Nitric oxide synthase

(NOS) has been identified in the same nerve terminals, suggesting a vasodilator role of NO. CSM tone is mediated from outside the corpora by specific transmitters. From within the corpora, NO mediates cavernosal smooth muscle relaxation and endothelin mediates contraction[38,39].

Erectile dysfunction

It is hypothesized that a failure of the cavernosal smooth muscle to relax leads to reduced arterial blood flow into the sinusoids, failing to bring about penile erection. Impotence may have several causes with treatments in high demand in our current society. Erectile dysfunction (ED) is common and its incidence increases with age, with a prevalence of 70% aged 70 or more[40]. ED shares risk factors with vascular diseases including smoking, hypertension, diabetes, hyperlipidaemia, obesity and a lack of exercise[41]. Phosphodiesterase type 5 inhibitor (PDE5 inhibitor) drugs (sildenafil, tadalafil and verdenafil) are currently available to treat ED. PDE5 hydrolyses cyclic GMP (cGMP), reversing the relaxation of CSM and producing detumescence[42]. PDE5 inhibitors help maintain intracellular concentrations of cGMP and thus maintain erection.

Alternative treatments for ED include a vacuum constriction device. This applies negative pressure to the penis that brings on an erection. This is maintained by an elastic band at the base of the penis for 30mins. Another treatment is intracavernosal injections of prostaglandin E_1 (alprostadil) which relaxes arterial and trabecular smooth muscle, resulting in erection.

Lower urinary tract symptoms (LUTS) describe the clinical presentation of bladder outlet obstruction (BOO). Symptoms include urinary frequency and nocturia, postmicturition dribbling, weak urinary stream and hesitancy. LUTS is highly prevalent in ageing men, along with ED, and the two are strongly linked[43]. Whilst epidemiological studies show evidence that LUTS and ED are linked, pathophysiological mechanisms exist to support this[44]:

Aetiology of LUTS and ED

Nitric Oxide

- As stated earlier, NO is important in the relaxation of CSM. Neurogenic NO is thought to be responsible for immediate relaxation of the CSM, initiating erection, and endothelial NO is essential for maintaining relaxation[45]. Conditions associated with decreased nerve and endothelium function include ageing, hypertension, smoking, diabetes and hypercholesterolaemia are all risks factors for ED.
- NO modulates smooth muscle tone in the prostate and bladder. NO levels are reduced in the hyperplastic prostate and affects voiding. NOS gene expression is reduced with age and in hyperplastic prostates[46]. The NO theory suggests that reduced NO results is SMC proliferation leading to structural changes in the prostate and increased prostatic contraction, increasing outlet resistance.

Autonomic hyperactivity

- Glucose intolerance, insulin resistance, obesity, dyslipidaemia and hypertension are part of a metabolic syndrome and are risk factors for ED[47]. Increased ANS activity induces benign prostatic hypertrophy (BPH) in rats, and ED. Observations have noted that rats with autonomic hyperactivity also have prostatic hyperplasia, ED and increased voiding frequency[48].
- ANS hyperactivity is strongly related to LUTS, secondary to BPH[49]. It is thought that there is a relationship between the increased sympathetic activity associated with LUTS and with ED.

Rho-Kinase activation

- Rho-kinase is thought to be a calcium sensitizing mechanism in smooth muscle. Smooth muscle contraction is a result of increased intracellular Ca^{2+} concentration and mechanisms exist to modify the sensitivity to calcium. Rho-kinase activation leads to contraction of smooth muscle.

Rho-kinase inhibitors reduce prostatic SMC proliferation. Increased Rho-kinase activity is found in detrusor muscle in rabbits with BOO[50], in the CSM of rabbits with BOO[51], diabetics and vascular smooth muscle of hypertensives. A link between LUTS and ED has been speculated as an increase in Rho-kinase activity.

Pelvic atherosclerosis

- It is thought that atherosclerosis has a role in the development of BPH die to the similar risk factors the two conditions share (hypertension, diabetes, hypercholesterolaemia and smoking).
- Chronic ischemia in rabbits results in CSM fibrosis, suggestive of ED[52]. Chronic fibrosis also results in fibrosis and smooth muscle atrophy of the bladder[53]. With chronic ischaemia, the increased production of TGF-β1 correlates with the severity of fibrosis[53]. The ischaemia also impairs NO mediated relaxation of the prostate and may result in loss of elasticity and increase in smooth muscle tone of the prostate[54].
- Atherosclerotic pelvic ischaemia may be associated with all the theories mentioned here, as it induces hyperactivity of the ANS, reduces NOS expression and up-regulates Rho-kinase[55].

Both LUTS and ED are highly prevalent in ageing men and there are strong associations between the two conditions. Treating LUTS may help improve ED.

C) Purinergic Signalling

Purines and purinoceptors

A seminal paper describing the potent extracellular actions of adenine compounds was published by Drury and Szent-Györgyi[56] in 1929. Buchthal and Folkow[57] found that ACh-evoked contraction of skeletal muscle was potentiated by exposure to adenosine 5'-triphosphate (ATP) and in 1959, Holton[58] demonstrated the release of ATP during antidromic stimulation of sensory nerves supplying the ear artery, hinting at a transmitter role of ATP in the nervous system. The concept of purinergic neurotransmission was first put forward by Burnstock in 1972[59]. Purines and pyrimidines have been shown to play important roles within mammals, in particular within the cardiovascular system[60].

Implicit in the concept of purinergic neurotransmission was the existence of postjunctional purinergic receptors[61]. Burnstock[62] proposed a basis for distinguishing two types of receptor: P1 and P2. Adenosine acts on the G protein-coupled P1 receptors, of which there are four subtypes: A_1, A_{2A}, A_{2B}, A_3. Adenosine is an ectoenzymatic breakdown product of ATP.

In 1985, a basis for distinguishing two types of P2 purinoceptor, P2X and P2Y, was proposed[63]. A new nomenclature system was put forward in 1994 which is now widely accepted[64,65]. P2 receptors are activated by the extracellular nucleotides ATP, adenosine diphosphate (ADP), uridine 5'-triphosphate (UTP) or uridine diphosphate (UDP). P2 receptors have now been subdivided into seven $P2X_{(1-7)}$ ligand-gated ion channel receptors and eight $P2Y_{(1,\ 2,\ 4,\ 6,\ 11,\ 12,\ 13,\ 14)}$[66] receptors. P2X and P2Y receptors are often expressed in the same cells.

P2X receptors are characterized by 2 transmembrane domains with a large extracellular loop where 10 cysteines are preserved; both N and C terminals are intracellular. A major property of the $P2X_1$ receptor is its mimicry of the agonist actions of ATP by α,β-methylene ATP (α,β-meATP). The $P2X_1$ receptor is expressed predominantly in smooth muscle. $P2X_1$ and $P2X_3$ receptors desensitize in milliseconds, the continued presence of ATP eliciting a reduction in current. In

contrast, $P2X_2$ and $P2X_4$ receptors desensitize slowly[67]. There are no known selective $P2X_2$ receptor agonists or antagonists. $P2X_2$, $P2X_4$ and $P2X_6$ receptors are mainly distributed within the central nervous system (CNS). 2-MethylthioATP is as potent as or more potent than ATP at $P2X_3$ receptors. $P2X_3$ is solely found in sensory neurones. ATP-evoked currents at $P2X_4$ receptors are potentiated by ivermectin[68]. ATP-evoked currents at rat $P2X_5$ receptors are small in amplitude and the receptor shows little desensitization[67]. The $P2X_6$ receptor appears to be a 'silent' subunit as no currents are evoked by ATP when it is expressed as a homomultimer; it appears to only be functionally expressed as a heteromultimer eg $P2X_{2/6}$ and $P2X_{4/6}$. One main feature of the $P2X_7$ receptor is the ability to become permeable to large cations following prolonged exposure to high ATP concentrations, and to undergo a channel to pore conversion allowing the passage of large dye molecules such as ethidium, usually leading to cell death[69]. 2',3'-0-(benzoyl-4-benzoyl)-ATP is a potent $P2X_7$ receptor agonist. The $P2X_7$ receptor is dominant within the immune system and is involved in cell death[70-72].

P2Y receptors are single protein receptors, with an extracellular N-terminus and intracellular C-terminus. Each P2Y receptor binds to a G protein. Following nucleotide activation, they either activate phospholipase C releasing intracellular Ca^{2+} ions or affect adenylyl cyclase and alter cyclic adenosine monophosphate (cAMP) levels[73]. Most of the P2Y receptor subtypes still lack potent and selective agonists and antagonists, though ADPβS is a potent $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor agonist. P2Y responses occur not only within the neuronal system, but also in non-neuronal and non-muscular cell types eg endothelial cells. P2Y receptors also mediate long term effects including DNA synthesis and cell proliferation. ADP has a greater potency than ATP for the $P2Y_1$ receptor in most species. The most potent and selective agonist is the N-methanocarba analogue of 2-Methylthio ADP (2-MeSADP), MRS2365, whilst effective selective $P2Y_1$ receptor antagonists include MRS2179, MRS2279 and MRS2500. $P2Y_1$ mRNA expression is highest in the brain, prostate gland and ovary[74,75]. The γ-thiophosphate, UTPγS, is a potent $P2Y_2$ receptor agonist and suramin a weak antagonist. $P2Y_2$ receptor activation increases the synthesis and/or release of

arachidonic acid, prostaglandins and NO. $P2Y_2$ receptors play a role in the wound healing process and receptor expression in smooth muscle cells (SMC) is up-regulated by agents that mediate inflammation[76]. UTP is the most potent activator of the human $P2Y_4$ receptor, whilst Reactive Blue 2 only partially blocks it. $P2Y_4$ mRNA is most abundant in the intestine[77]. UDP and the more selective uridine β-thiodiphosphate are both agonists of the $P2Y_6$ receptor. The $P2Y_6$ receptor is slow to desensitize and is highly expressed in the spleen, intestine, liver, brain and pituitary[78,79]. ATPγS is a more potent agonist than ATP at the $P2Y_{11}$ receptor, whilst suramin behaves as a competitive antagonist. The 5'-triphosphate derivative AR-C69931MX compound, named cangrelor, is an antagonist to both $P2Y_{12}$ and $P2Y_{13}$ receptors. The $P2Y_{12}$ receptor is present on platelets, the brain, SMC and chromaffin cells[80-82]. The $P2Y_{13}$ receptor is present in the spleen, placenta, liver, heart, bone marrow, monocytes and T-cells[83,84]. The $P2Y_{14}$ receptor, a recent discovery, has no known selective antagonists, though UDP, UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine are agonists.

Different receptor subunits can be coexpressed on the same receptor, producing a heteromeric receptor. These receptors have properties reflecting both subunit types. The $P2X_{2/3}$ receptor is potentiated by a low pH and does not desensitize rapidly, reflecting the homomeric $P2X_2$ receptor properties, whilst like the homomeric $P2X_3$ receptor, it is blocked by 2', 3'-0-(2,4,6-trinitrophenyl)-ATP, pyridoxal-phosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) and suramin[85].

Receptors on vascular endothelium

Both P2Y and P2X receptors expressed on EC mediate vasodilatation via NO[61]. The location of P2Y receptors on endothelial cells forming the innermost layer of blood vessels implies that these receptors are important as sensors and effectors of response to local changes of purines in blood. Thus endothelium dependent vasodilatation is likely to be linked to the release of purines at the intimal surface from erythrocytes and from endothelial cells. In many blood vessels $P2Y_1$

and $P2Y_2$ receptors coexist on EC in variable proportions[86]. Shear stress and hypoxia stimulate vascular EC release of ATP and UTP into the lumen, agonists to endothelial $P2Y_1$ and $P2Y_2$ receptors respectively[87,88]. Studies on human umbilical veins have identified $P2X_4$, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ on EC. It is thought they mediate the release of NO, endothelium-dependent hyperpolarizing factor (EDHF) and tissue-type plasminogen activator (t-PA). $P2Y_4$ and $P2Y_6$ were also identified but showed weak expression[89,90]. $P2X_4$ receptors are thought to be involved in cell permeability and adhesion[91]. High levels of $P2X_4$ receptors have been reported on saphenous vein EC and low levels on mammary arteries[92]. Yamamoto et al demonstrated $P2X_4$ receptor-mediated vascular dilatation in a NO-dependent manner[93]. Functional $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on EC mediate relaxation in the thoracic aorta[94]. In most blood vessels there is a continuous basal release of NO from EC which controls smooth muscle contractility and sets a basal vasodilator tone. Removal of the endothelium or blockade of endothelial cell NO formation (with NOS inhibitor) inhibits endothelium dependent vasodilatation via P2 receptors and facilitates vasoconstrictor actions mediated by P2 receptors on the underlying SMC.

Receptors on vascular smooth muscle

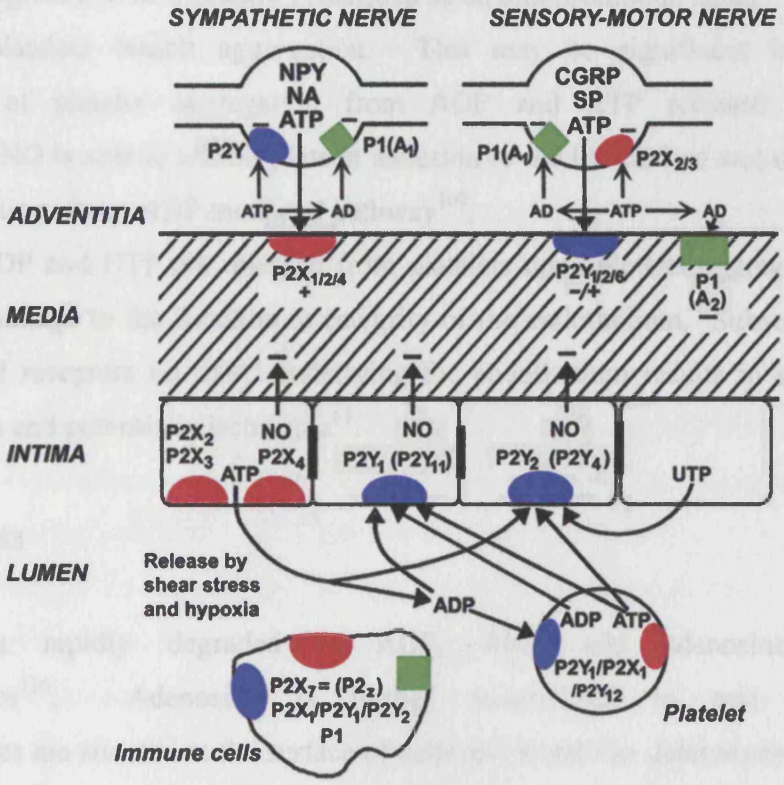
P2X receptors mediate vasoconstriction to ATP released as a cotransmitter with noradrenaline (NA) and neuropeptide Y (NPY) from sympathetic nerves (see below). $P2X_1$ receptors, potently activated by α,β-meATP, are the principal subtype[61]. This component of the sympathetic response can be blocked by purine receptor antagonists but not by adrenoceptor antagonists. Vascular smooth muscle $P2X_2$ and $P2X_4$ as well as $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors have also been identified that mediate vasoconstriction in some vessels[60,86,95,96].

Some SMC, in particular those in coronary arteries, express vasorelaxant P2Y receptors[97]. These receptors are most potently activated by 2-MeSATP and ADP, suggesting that they are likely to be $P2Y_1$ receptors.

A_2 and P2Y receptors on SMC have roles in control of cell proliferation. The change from a vascular SMC of contractile phenotype to a synthetic phenotype is a central pathophysiological process in the development of atherosclerosis and in restenosis after angioplasty. Several changes in gene expression take place, for example, the cells lose their ability to contract, synthesize ECM and express receptors for growth factors[98,99]. $P2X_1$ receptors are down regulated and $P2Y_1$ and $P2Y_2$ receptors upregulated when the vascular SMC changes from a contractile into a synthetic phenotype[100]. The upregulation of $P2Y_2$ receptor suggests it may be an important mediator of cellular growth, and have other functions such as stimulation of matrix proteins or release of growth factors[100]. Increased expression of the $P2Y_2$ receptor may mediate atherosclerosis and neointima formation after balloon angioplasty[101]. UDP stimulates mitogenesis through activation of $P2Y_6$ receptors, hence $P2Y_6$ is thought to be of importance in regulating vascular smooth muscle growth and differentiation[102].

Fig 1.1: Short term purinergic signalling controlling vascular tone. This illustration summarizes the location and action of the main purine receptors in blood vessels. Perivascular nerves in the adventitia release ATP as a cotransmitter: ATP is released with NA and NPY from sympathetic nerves to act on smooth muscle $P2X_1$, $P2X_2$ and $P2X_4$ receptors, resulting in vasoconstriction; it is released with CGRP and SP from sensory nerves during axon reflex activity to act on smooth muscle P2Y receptors resulting in vasodilatation. A_1 receptors on nerve terminals of sympathetic and sensory nerves mediate adenosine (arising from the enzymatic breakdown of ATP) modulation of transmitter release. $P2X_3$ receptors are present on a subpopulation of sensory nerve terminals. A_2 receptors on vascular SMC mediate vasodilatation. EC release ATP and UTP during shear stress and hypoxia to act on $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors leading to the production of NO and subsequent vasodilatation. Platelet aggregation releases ATP which acts on EC receptors. Platelets possess $P2Y_1$ and $P2Y_{12}$ ADP-selective receptors along with $P2X_1$ receptors. Immune cells posses $P2X_7$, $P2X_1$, $P2Y_1$ and $P2Y_2$ receptors. $P2X_2$,

$P2X_3$ and $P2X_4$ receptors have been identified on EC membranes. (Reproduced from Burnstock G. Purinergic signaling and vascular cell proliferation and death. Arterioscler Thromb Vasc Biol. 2002;22:364-373[103]).



Receptors on perivascular nerves

Blood vessel adventitia contains perivascular nerves which express purine receptors. Activation of A1 receptors prejunctionally on the terminals of perivascular sympathetic and sensorimotor nerves mediates inhibition of neurotransmitter release. ATP release and breakdown acts on A_1 as auto regulation of ATP neurotransmission. $P2Y_1$ receptors on postganglionic sympathetic neurons mediate neurotransmission by involvement in feedback inhibition of co released ATP, NA and NPY. Heteromeric $P2X_{2/3}$ receptors may be involved in nociception[61].

Receptors on platelets

$P2Y_{12}$ receptors are almost exclusively found on platelets and (along with $P2X_1$ and $P2Y_1$) mediate aggregatory properties[80,104]. Clopidogrel, a $P2Y_{12}$ receptor competitive antagonist, is now readily prescribed as an antithrombotic agent[80]. A_{2A} receptors on platelets inhibit aggregation. This may be significant in the autoregulation of platelet aggregation from ADP and ATP released from platelets[105-108]. NO is able to inhibit platelet adhesion to the EC surface and inhibit platelet aggregation via an ADP mediated pathway[109].

ATP, ADP and UTP are released from platelets upon platelet aggregation, when there is damage to the function or integrity of the endothelium. Subsequent activation of P2 receptors on SMC underlying the endothelium results in severe vasoconstriction and potentially ischaemia[61].

Ectonucleotidases

ATP is rapidly degraded to ADP, AMP and adenosine by ectonucelotidases[110]. Adenosine is further metabolized to uric acid. Ectonucleotidases are situated at the surface of cells and in the vas deferens may be coreleased with ATP. This limits the action of ATP at P2 receptors by removing it through enzymatic breakdown, but it can evoke opposite effects via the actions of the breakdown products ADP and adenosine at P2Y and P1 receptors respectively[60].

The role of purines in inflammation

Purines acting on purinoceptors have a broad range of functions including pro and anti-inflammatory actions, killing intracellular pathogens by inducing apoptosis of host macrophages, chemoattraction and cell adhesion[111,112]. ATP is involved in the development of inflammation through several actions: release of histamine from mast cells, provoking production of prostaglandins from P2Y receptors[113]; and the production and release of cytokines from immune cells[113,114].

Adenosine is released at sites of inflammation and has anti-inflammatory effects via several mechanisms. It inhibits neutrophil rolling and adhesion to vascular endothelium, it decreases oxygen free radical production by neutrophils via activation of A_{2A} receptors, and via A_1 and A_{2A} receptors it affects endothelial cell permeability[115]. Adenosine also inhibits macrophage production of the cytokine TNFα and suppresses TNFα mRNA expression[115].

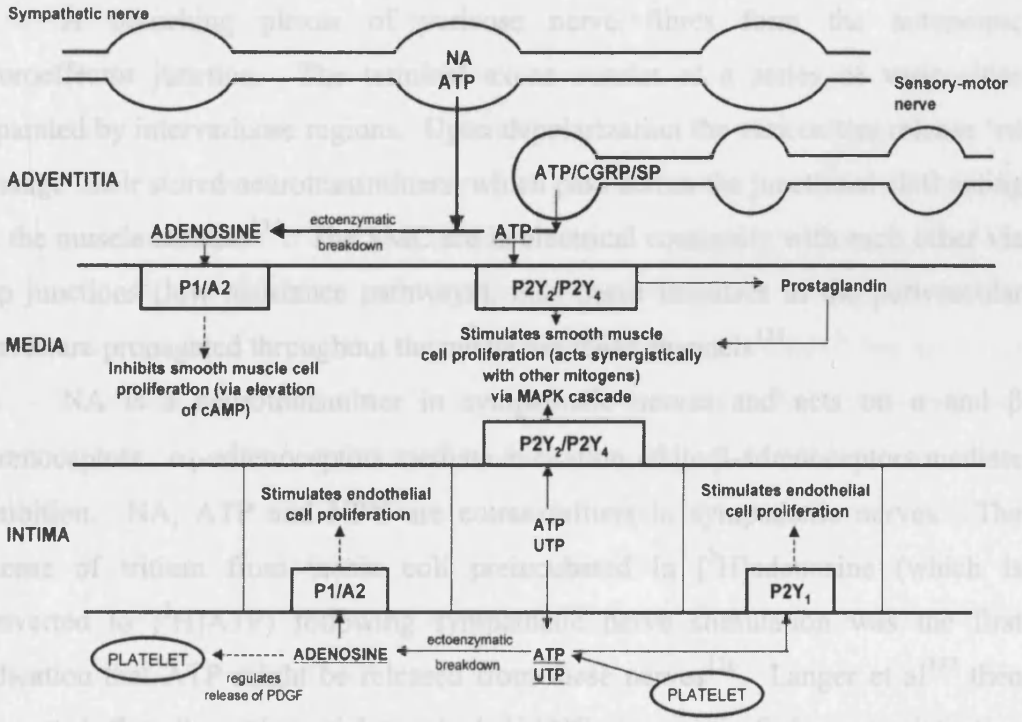
The $P2X_7$ receptor mediates cytokine release, generates NO, the killing of intracellular pathogens and cytotoxicity[116]. $P2X_7$ receptor stimulation on alveolar macrophages triggers activation of interleukin-1 (IL-1) and IL-6 cytokines and granulomatous reactions[117]. In contrast, ATP and ADP inhibit cytokine generation by human mast cells through P2Y receptors[118]. Stober et al have provided evidence for $P2X_7$ receptor mediated cytotoxic actions of ATP on macrophages and $P2Y_2$ receptor mediated bacteriocidal effects of ATP[119]. $P2X_7$, $P2Y_1$ and $P2Y_2$ receptor expression on macrophages and giant cells increase during an inflammatory reaction demonstrating a regulatory function in inflammation[120].

Atherosclerosis is considered to be an inflammatory disease. The inflammatory mediator IL-1β increases $P2Y_6$ receptor mRNA expression[102]. As the $P2Y_6$ receptor is involved in the regulation of vascular smooth muscle growth and differentiation, it is thought to play a role in the formation of atherosclerosis. Extracellular ATP inhibits the activation of $CD4^+$ T lymphocytes via P2 receptors, suggesting a possible therapeutic target for topical immunosuppression in some inflammatory diseases[121]. ATP is thought to regulate the trafficking of specific dendritic cells via $P2Y_{11}$ receptors[122]. The migration of dendritic cells to lymphoid tissue from the site of the captured antigen is required for the induction and regulation of immune responses. $P2Y_{11}$ receptor targeting may have a therapeutic role in improving migration of the dendrites[123].

Long term trophic effects of purines

It is now known that purines play a major role in cell proliferation, differentiation and death in many tissues. These changes are long term, in comparison to their fast P2X mediated vasoconstriction effects on SMC. In vascular SMC and EC, purines play an important role in the development of intimal thickening during atherosclerosis and in the growth of new vessels that occur during wound healing and in tumours[98,124,125]. Purines play important roles in the maintenance of the keratinocyte epidermal layer in skin[126]. ATP and ADP have synergistic actions with a number of growth factors eg. angiotensin II, endothelin-1, NADR, NPY, 5-hydroxytryptamine, platelet derived growth factor, insulin like growth factor and basic fibroblast growth factor[127]. Extracellular ATP has mitogenic actions with other cell types including fibroblasts and endothelial cells[128,129]. $P2Y_8$ receptors in the frog embryo (not a recognized mammalian receptor) are involved in the development of the neural plate and $P2Y_1$ receptors play role in cartilage development in limb buds and in the development of the mesonephros[130].

Fig 1.2: Schematic of long term (trophic) actions of purines released from nerves, platelets and endothelial cells (which also release UTP) acting on P2 receptors to stimulate or inhibit cell proliferation. ATP release as a cotransmitter from sympathetic nerves and sensory-motor nerves (during reflex axon activity) stimulates smooth muscle proliferation via $P2Y_2$ and/or $P2Y_4$ receptors via a MAPK cascade, while adenosine resulting from enzymatic breakdown of ATP acts on A_2 receptors to inhibit cell proliferation (via elevation of cAMP). ATP and UTP released from endothelial cells stimulate both endothelial and smooth muscle cell proliferation via $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors. Adenosine resulting from ATP breakdown acts on A_2 receptors to stimulate endothelial cell proliferation and regulate release of PDGF from platelets. (Reproduced from Burnstock G. Purinergic signaling and vascular cell proliferation and death. Arterioscler Thromb Vasc Biol. 2002;22:364-373[103]).



Purinergic cotransmission and neuromodulation

The concept that one neuron releases only a single transmitter, known as 'Dale's principle', was suggested in 1935[131] and governed our understanding of neurotransmission at the time. Chemical transmission was thought to consist of adrenergic nerves releasing NA and cholinergic nerves releasing ACh. In the 1950's hints of acceptance to this theory appeared and in 1976 Burnstock[132] challenged the principle with an article entitled 'Do some nerve cells release more than one transmitter?'. The concept of cotransmission thus materialised, where more than one transmitter is synthesized, stored and released by one nerve eg amino acids, purines, NO, peptides and monoamines. Further research has demonstrated the role of ATP as a cotransmitter in sympathetic, parasympathetic, sensory-motor and enteric non-adrenergic, non-cholinergic (NANC) inhibitory nerves[133]. Not all colocalised substances are necessarily cotransmitters; instead they can act as pre- and/or post-junctional neuromodulators of the release and actions of the neurotransmitters[133].

A branching plexus of varicose nerve fibres form the autonomic neuroeffector junction. The terminal axons consist of a series of varicosities separated by intervaricose regions. Upon depolarization the varicosities release 'en passage' their stored neurotransmitters, which pass across the junctional cleft acting on the muscle bundles[134]. The SMC are in electrical continuity with each other via gap junctions (low resistance pathways), thus nerve impulses in the perivascular nerves are propagated throughout the media via these channels[135].

NA is a neurotransmitter in sympathetic nerves and acts on α and β adrenoceptors. α_1-adrenoceptors mediate excitation while β-adrenoceptors mediate inhibition. NA, ATP and NPY are cotransmitters in sympathetic nerves. The release of tritium from taenia coli preincubated in [^{3}H]adenosine (which is converted to [^{3}H]ATP) following sympathetic nerve stimulation was the first indication that ATP might be released from these nerves[136]. Langer et al[137] then suggested that the substantial residual NANC response of the cat nictitating membrane observed after depletion of NA by reserpine was due to the release of

ATP remaining in sympathetic nerves. Early studies demonstrated cotransmission of NA and ATP in vas deferens, a tissue with a high density of sympathetic nerves[138,139]. Cotransmission of NA and ATP in perivascular sympathetic nerves has now been demonstrated in rat tail artery[140], rabbit saphenous[141] and pulmonary arteries[142], and dog basilar[143] and mesenteric arteries[144]. Studies have demonstrated a biphasic response on sympathetic stimulation; an initial fast, transient depolarization or excitatory junction potential (EJP) and contraction of vascular smooth muscle is followed by a slow, prolonged depolarization and slow relaxation. The EJP and slow depolarization are mimicked by the effects of ATP and NA, respectively. Variations in the proportions of NA and ATP utilized by sympathetic nerves exist. The purinergic component is optimal with short bursts of low frequency stimulation, whereas longer durations of higher frequency favour adrenergic transmission[145]. NPY is often co-stored with NA in large granular vesicles in sympathetic nerves. NPY has few postjunctional actions, although at high concentrations it can produce contraction. Neuromodulation is the main function of NPY; pre-synaptically it reduces the release of NA and ATP, post-synaptically it augments their actions[146].

ACh, the main post-ganglionic neurotransmitter in parasympathetic nerves, acts on muscarinic receptors. In 1981, Lundberg[147] demonstrated cotransmission of ACh and VIP in parasympathetic nerves on cat salivary glands. Low frequency nerve stimulation releases ACh which increases salivary secretion from acinar cells and elicits minor dilatation of the gland's blood vessels. High frequency stimulation releases VIP causing marked vasodilatation, and whilst it has no direct effect on acinar cells, it acts as a neuromodulator enhancing both the postjunctional effect of ACh on acinar cell secretion and increasing ACh release from nerve varicosities via prejunctional receptors. Parasympathetic nerves supplying the urinary bladder utilize ACh and ATP as cotransmitters in variable proportions in different species[148,149]. ATP acts through P2X receptors to produce a fast contraction, while ACh mediates a slow response[150].

Calcitonin gene-related peptide (CGRP) and substance P (SP) have been shown to coexist in sensory nerve terminals in perivascular nerves[151] and in large

granular vesicles[152]. CGRP release from sensory-motor efferent nerves in rat mesenteric arteries mediates vasodilatation while SP is not co-relased[153]. In guinea pig skin, unmyelinated sensory neurons contain cholecystokinin, CGRP and SP[154]. There is evidence that ATP coexists in some sensory nerve terminals with SP and CGRP[155]. The role of these nerves during axon reflex to many organs is motor rather than sensory[156].

Intrinsic neurons exist in most of the major organs of the body. In the gut and heart, some of these are derived from neural crest tissue and thus differ from the parasympathetic and sympathetic nervous system. They appear to participate in local reflex actions independent of the CNS. A subpopulation of this enteric nervous system provides NANC inhibitory innervation of the gastrointestinal smooth muscle. Three major cotransmitters are released from these nerves. ATP produce fast inhibitory junction potentials (IJP), NO produces slower IJPs, while VIP produces slow tonic relaxations[157]. Intrinsic cardiac neurons have identified subpopulations that contain and/or release the cotransmitters ATP, NO, NPY ACh and serotonin, many of which produce potent vasomotor actions[158-161].

Although single presynaptic action potentials release small molecule neurotransmitters, trains of impulses are needed to release neuropeptides. In sympathetic and parasympathetic cotransmission, ATP is released at low frequency stimulation, whereas the larger molecules of NA, ACh and neuropeptides are released at high frequencies [162,163]. Neurons containing several neurotransmitters may have more than one target. In the acinar cells of the salivary gland, ACh release, following low frequency stimulation, produces saliva secretion and minor vasodilatation, whereas high frequency stimulation releases VIP causing powerful vasodilatation and enhancing ACh-induced saliva secretion[164]. Cotransmitters can feed back on presynaptic receptors and neuromodulate their own release. Both NA, via α_2-adrenoceptors, and ATP, via P1 receptors following its breakdown to adenosine, can prejunctionally modulate sympathetic cotransmission[165,166]. Postjunctionally cotransmitters may work synergistically eg ATP and NA on smooth muscle in the vas deferens[167], and antagonistically eg nicotinic $\alpha 3\beta 4$ and $P2X_2$ in Xenopus oocytes[168]. Some cotransmitters have opposing actions on their

postjunctional cells. Brain-derived neurotrophic factor (BDNF) alters neurotransmitter release in cardiac sympathetic neurons. BDNF increases the release of ACh and reduces NA release to cause a rapid shift from excitatory to inhibitory transmission[169].

There is increasing evidence that the expression of autonomic transmission can alter in disease. ATP is a significant cotransmitter in sympathetic nerves supplying hypertensive blood vessels and this purinergic component is increased in spontaneously hypertensive rats[61]. The parasympathetic purinergic nerve-mediated component of contraction on the human bladder is increased to 40% in pathophysiological conditions such as interstitial cystitis and outflow obstruction[170,171]. Perivascular nerves in penile vessels containing the vasodilator VIP are reduced in diabetic man[172], whilst VIP expression is increased in the diabetic rat gut[173]. In Crohn's disease there is a transmural increase in VIP in the diseased gut[174]. The ANS shows marked plasticity. The expression of cotransmitters and receptors shows dramatic changes during development and ageing, in nerves that remain after trauma and in disease conditions[130].

D) Purinergic signalling in the long saphenous vein, skin and the penis

Purinergic signalling in the LSV

Perivascular nerve fibres lie at the media-adventitia border forming the neuroeffector junction. There is an extensive network of branching terminal fibres that lack a Schwann cell covering. These contain varicosities (1-2μm diameter), separated by intervaricose regions (0.1-0.3 μm diameter)[175], where neurotransmitters are stored and released by the depolarizing effect of nerve impulses along the axons. For many years, ACh and NA were thought to be the only neurotransmitters. Many neurotransmitters have now been identified including ATP as wells as Substance P, CGRP, NO and serotonin[176]. The SMC are in electrical continuity with each other via gap junctions, there is no postjunctional specialization, thus they differ from synapses at motor end plates in striated muscle and within ganglia[27,177].

SMC are specialised for continuous contraction of low force, in comparison to skeletal muscle, which is specialized for greater force of shorter durations. The muscle contracts as a whole mass rather than individual motor units. Muscle contractions occur from stimulation via the ANS, hormones and local metabolites. Contractions can be independent of neurological innervations. SMC are relatively small with a single nucleus. The fibres are bound together in irregular branching fasciculi[178].

Purinergic cotransmission has previously been demonstrated in the LSV. ATP and α,β-meATP contract the circular smooth muscle in vein rings. This response is markedly inhibited in the presence of P2X receptor antagonists[179,180]. On control LSV circular rings, Borna et al demonstrated contractile effects of $P2X_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ and detected mRNA for $P2X_1$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$[181]. Ray et al[92] identified $P2X_{(1-7)}$ and $P2Y_2$ receptors on LSV

endothelial cells[92] while in addition Conant et al[182] detected $P2Y_1$ receptors on endothelial cells. Northern blot analysis of total RNA from LSV smooth muscle detected the $P2X_7$ receptor, where its activation formed membrane pores permeable to large molecules[183].

Ziganshin et al compared purine-mediated contractions in varicose vein rings with veins obtained from atherosclerotic legs, showing stronger contractions to ATP, α,β-methylene ATP and UTP in the atherosclerotic veins[184]. No comparison has been made of purinergic signalling between varicose and healthy LSV. It is also interesting to note that pharmacological studies on the LSV have studied the circular muscle; none have studied the medial longitudinal smooth muscle present.

Analysis of aortocoronary LSV grafts showed intimal hyperplasia results from the migration and proliferation of de-differentiated SMC originating from the media[185] in response to mechanical injury and haemodynamic disturbances[186,187]. Adenosine is known to inhibit vascular SMC via A_{2B} receptors. Targeting this receptor may reduce the SMC hypertrophy seen in LSV grafts, thereby improving its lifespan. Studies of adenosine on vein grafts in the arterial circulation revealed unique properties[188]:

- Vein graft adenosine-mediated relaxation is NO and prostanoid dependent.
- A_1-receptor activation in the vein graft produces an endothelium dependent contractile response, rather than an expected relaxant response.
- A_2-receptor mediated responses in the vein graft appear independent of the endothelium.

These findings suggest the vein graft has neither a venous nor arterial phenotype. ACh usually relaxes vein smooth muscle, but induces contraction in vein grafts, perhaps due to alteration of the muscarinic receptors. Davies et al[188] suggest that these findings are a result of functional changes of the EC, possibly a phenotype change, that may contribute to the intimal hyperplasia seen in vein grafts.

Purinergic signalling in skin

Purine receptor activity has been identified in skin epidermis. A $P2X_3$-like receptor is thought to reduce keratinocyte repair and mediate epidermal hyperplasia following injury[189]. The roles of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors in different layers in human skin epidermis has been described previously[190]. $P2Y_1$ and $P2Y_2$ receptors are involved in keratinocyte proliferation in the stratum basale, while $P2X_5$ receptors are associated with keratinocyte differentiation in the stratum basale, spinosum and granulosum and $P2X_7$ receptors with keratinocyte cell death in the stratum corneum[190,191].

Inoue et al[192] identified increased levels of $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_7$ receptor mRNA in differentiating keratinocyte cells. mRNA levels of $P2X_1$ receptor fell whilst that of $P2X_4$ remained unchanged during differentiation. Burell et al[193] identified mRNA for $P2Y_4$, and $P2Y_6$ receptors on keratinocytes and concluded that $P2Y_4$ receptors played a regulatory role in proliferation. UTP stimulation of P2Y receptors leads to IL-6 production from keratinocytes[194].

To date, the role of purine receptors on keratinocytes in CVI skin and venous ulcer formation has not been reported.

Purinergic signalling in the penis

Penile erection involves cholinergic, adrenergic and purinergic pathways[195]. Adenosine has been shown to mediate CSM relaxation via A_{2A} (pathway independent of NO) and A_{2B} (partially endothelium dependent) receptor subtypes[196-198]. Adenosine also induces cavernosal peak blood flow velocity and tumescence[199,200].

ATP released neuronally or from endothelium, has been shown to induce CSM relaxation[34,198,200-202]. ATP-mediated relaxation is more pronounced on CSM at high basal tension (when precontracted with phenylepherine)[203]. At low CSM basal tension, ATP causes contraction. It may be that ATP is involved as part of a regulatory mechanism in maintaining physiological CSM basal tone at rest. ATP

mediated contractions are partially attributable to its metabolic breakdown to adenosine, which acts directly on A_{2A} and A_{2B} receptor subtypes. However P2Y receptor-mediated relaxation may be accountable too, leading to NO release from the endothelium[204,205]. The $P2Y_1$ receptor has been identified on endothelial cells lining lacunar spaces but was not identified on the CSM in the rat[206]. $P2Y_1$ and $P2Y_2$ receptors have been identified on hamster urethral smooth muscle and relaxation to ATP is thought to be $P2Y_1$ receptor mediated[207]. The role of the $P2Y_2$ receptor may be to mediate trophic effects, as it is known to mediate the proliferation of keratinocytes and rat aortic smooth muscle cells[208,209]. P2X receptors induce contractions of CSM[205]. Lee et al demonstrated the presence of strong $P2X_1$ and weaker $P2X_2$ immunoreactivity in rat CSM, which suggests their possible involvement in the process of detumescence[210].

CHAPTER 2

Alterations In Purinoceptor Expression In Human Long Saphenous Vein During Chronic Venous Insufficiency

Abstract

Varicose veins are dilated tortuous veins of varying tone. Purinergic signalling is important in the control of tone and in mediating trophic changes in blood vessels. The expression of P2 receptors in control and varicose veins was examined. Purinergic signalling in circular and longitudinal smooth muscle of the human long saphenous vein was studied in control and varicose tissues using immunohistochemistry, organ bath pharmacology and electron microscopy. $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were present on circular and longitudinal smooth muscle. Purine-mediated circular and longitudinal muscle contractions were weaker in varicose veins. Electron microscopy and immunohistochemistry findings support the view that smooth muscle cells change from the contractile to synthetic phenotype in varicose veins, associated with an upregulation of $P2Y_1$ and $P2Y_2$ receptors and a down regulation of $P2X_1$ receptors. Down regulation of $P2X_1$ receptors on the smooth muscle of varicose veins is associated with loss of contractile activity. Upregulation of $P2Y_1$ and $P2Y_2$ receptors is associated with a shift from contractile to synthetic and/or proliferative roles. The phenotype change in smooth muscle is associated with weakening of vein walls and may be a causal factor in the development of varicose veins.

Introduction

As our life expectancy continues to increase along with our population size, the incidence of CVI is predicted to increase. Incompetent valves in varicose veins requiring stripping of the LSV is a common surgical intervention aimed at inhibiting disease progression. As health services attempt to cut costs, stricter criteria exist for varicose vein surgery. Cheaper alternatives for the management of CVI are an attractive welcome. A more detailed understanding of the disease process would help in the development of new treatments.

Structural changes between healthy and varicose LSV have been well described in the literature, with collagen deposition and muscle fibre disorganization a characteristic feature[26]. Contractile strength weakness of the varicose circular smooth muscle is also reported[24]. SMC are known to play a role in the pathophysiology of vascular remodelling that occurs in hypertension, atherosclerosis and restenosis. Their roles include proliferation, migration and deposition of ECM eg collagen. SMC show plasticity, changing from a contractile to a synthetic phenotype, which is central to these processes[127].

Purinergic signalling has been demonstrated in the LSV[179,180] as discussed earlier, with one study comparing purine-mediated contractions on varicose veins with veins obtained from atherosclerotic legs[184]. No comparison has been made of its role between varicose and healthy LSV. Aside from the short term actions of purines on blood vessels, purines have long term proliferative actions too[125].

This study was aimed at examining the expression of different P2 receptor subtypes present in healthy and varicose human LSV and comparing the contractile effects mediated by these receptors in both circular and longitudinal smooth muscle. Vein wall ultrastructure, with particular emphasis on smooth muscle phenotype was also studied.

Methods

Patients

Proximal end varicose vein segments were obtained from 31 patients (19 female, 12 male, aged 21-77, mean age 46.8 ± 2.6 years) undergoing stripping of their LSV. The most proximal end of the sample was obtained prior to insertion of the stripper. Reflux, had been confirmed by either hand held doppler or venous duplex scanning by the vascular team prior to surgery. Healthy control vein was obtained from 34 patients (6 female, 28 male, aged 41-84, mean age 63.6 ±1.6 years) undergoing coronary artery bypass surgery where the LSV was harvested for a graft. A segment from the proximal end of the exposed LSV was excised for the study prior to distension. Reflux was excluded in control patients by hand held doppler prior to surgery. Diabetic patients were excluded from the study. Ethics approval was obtained by the joint UCL/ULCH Ethics Committees on Human Research and by the Royal Free Hampstead Research Ethics Committee.

Immunohistochemistry

Vein segments were collected in Hanks balanced salt solution (HBSS; Invitrogen, Paisley, UK) and frozen in isopentane, precooled in liquid nitrogen. Segments were sectioned at 10μm on a cryostat (Reichert Jung CM1800), collected on gelatine-coated slides and air dried at room temperature. Slides were stored at -20°C. Sections were fixed for 4 min in 4% formaldehyde in 0.1M phosphate buffer solution (PBS) containing 0.2% picric acid, then washed three times for 5 min with PBS. Sections were primarily blocked for 60 min in 10% normal horse serum (NHS) in 0.1M phosphate buffer, containing 0.05% merthiolate. Sections were incubated overnight with two primary antibodies: polyclonal P2X ($P2X_{1-6}$) (Roche Palo Alto, CA, USA) or P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) (Alomone

Laboratories, Jerusalem, Israel) antibodies at concentrations of 1:50 to 1:200, and monoclonal anti α-smooth muscle actin antibody (Sigma Chemical Co., Poole, UK) at 1:400 in 10% NHS in PBS with 0.05% merthiolate. On the second day, sections were washed three times for 5 min in PBS and then stained with the secondary antibodies: donkey anti-rabbit Cy3 (Jackson Immunoresearch Laboratories, West Grove, USA) at 1:300 and donkey anti-mouse FITC (Jackson) at 1:200 in PBS-merthiolate for 60 min. Sections were washed three times for 5 min before being mounted in Citifluor (Citifluor Ltd, London, UK). Control experiments were performed by omitting the primary and secondary antibody, and by preabsorbing the primary antibody with its corresponding peptide. Preabsorption was carried out by adding the peptide at a ratio of 1:1 in 10% NHS in PBS with 0.05% merthiolate, leaving for 12 hours at 4°C, passing through a syringe filter (4mm with a 0.2μm PPmembrane) then centrifuged at 13,000rpms for 5 min using only the supernatant.

Semi-quantitative assessment of the changes in immunofluorescent intensity was performed by an independent observer, blinded from the patient group from which samples were taken.

Haematoxylin and Eosin (H&E) slides were prepared by fixing in 4% paraformaldehyde in PBS for 10 min. Sections were washed in distilled water then stained for 20 min in Ehrlichs Haematoxylin. Following washing in running tap water, slides were dipped in acid alcohol and washed again for 15 min. Sections were then stained in Eosin for 5 min, dipped in tap water, then washed for 1 min in 70% alcohol, 3 min 100% alcohol, another 3 min 100% alcohol, dried for 3 min in xylene, and finally another 5 min in xylene. Sections were mounted in eukitt.

The results were photographed using a Zeiss Axioplan, high definition light microscope (Zeiss, Oberkocken, Germany) mounted with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland).

Electron microscopy

For electron microscopy vein segments were collected in HBSS and transported back to the laboratory and fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1M phosphate solution. Tissue was washed in phosphate buffer, post-fixed in 1% osmium tetroxide in phosphate buffer, en-block stained with a 2% solution of uranyl acetate in distilled water, dehydrated in graded ethanols and embedded in an agar resin. 80nm thick sections were cut and collected on thin films, counterstained with lead citrate and viewed in a Jeol 1010 TEM.

Pharmacology

For functional pharmacology experiments tissues were collected in Krebs solution (pH 7.2) of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8. Tissues were cleaned of adherent connective tissue and set up in 10ml organ baths containing the above Krebs solution, gassed with 95% O_2-5% CO_2, and maintained at 37±1°C within 2 hours of collection.

Circular muscle was tested by cutting ring vein segments of 5mm length, mounted with tungsten wires to an L-shaped stainless steel rod. Isometric tension was recorded with a Grass FT03C force-displacement transducer. Rings were placed under an initial tension of 2g and allowed to equilibrate for 60mins prior to experiments starting. Longitudinal muscle was tested on 15mm lengths of vein, suspended with silk thread, between an L-shaped rod and a Grass FT03C transducer. A resting tension of 2.5g was applied and allowed to equilibrate for 60min prior to isometric tension recordings. Mechanical activity was recorded using the software PowerLab Chart for Windows (version 4; ADInstruments, Australia). Contractions along the longitudinal axis (along the length of the vein) were measured, which recorded activity of both the longitudinal smooth muscle and the longitudinal component of disorganised fibres.

NA was applied accumulatively to the organ baths at increasing concentrations (10^{-8} to 10^{-3} M). Baths were then washed and the vein allowed to return to its resting tension. Not all vein segments were exposed to the complete range of concentrations, some segments received a one off concentration (10^{-5} M), were washed, and tension readings obtained for the remaining experiments. This did not significantly affect later results. ATP was applied non-cumulatively (10^{-6} to 10^{-3} M) separated by 15 min intervals, washing in between. After a further 30 min rest, α,β-meATP (10^{-8} to 10^{-5} M) was added in the same manner. Vein segments were again washed and upon returning to their resting tension (approx 30min) were contracted to increasing concentrations of KCl (10^{-5} to $3x10^{-1}$ M).

On separate vein segments the effect of one of the following P2Y agonists was examined. 2-MeSADP (10^{-8} to 10^{-5} M) was applied cumulatively and a concentration-response curve was constructed. Following wash-out, the curve was repeated in the presence of the antagonist MRS2179 (10^{-6} M). On a separate vein segment, a concentration-response curve was constructed to UDP (10^{-6} to 10^{-3} M); this was repeated in the presence of the antagonist cibacron blue 3GA (10^{-4} M). Finally, a concentration-response curve was constructed to UTP (10^{-6} to 10^{-3} M) and repeated in the presence of the antagonist suramin (10^{-4} M). All antagonists were incubated for 45mins prior to the addition of the agonist.

With the exception of NA, full concentration-response curves could not be constructed since the maximum concentration of agonist that could be applied to the organ bath was less than that required to obtain a maximum contraction.

Electrical field response of longitudinal muscle was tested using platinum wire electrodes, one placed in the lumen and one outside of the vessel. Transmural nerve stimulation was delivered for 1min by an electronic stimulator (Grass SD9) at 1-16Hz (100V, 0.1ms duration) with rest periods of 10-20min. Frequency response curves were repeated firstly in the presence of an adrenergic antagonist (prazosin 10^{-6} M), then in the presence of both prazosin and the purinoceptor antagonist PPADS ($3x10^{-5}$M) and finally in the presence of the nerve conduction blocker tetrodotoxin (TTX; 10^{-6}M). All agonists were allowed to equilibrate for 30mins before stimulation commenced.

The integrity of the endothelium was examined in control and varicosed samples by their ability to relax to ACh. Vessels were precontracted with NA (EC_{50} concentration) and increasing concentrations of ACh were applied ($10^{-7} - 10^{-3}$M).

Statistical Analysis

All concentration-response curves were expressed as –log of the molar concentration of the agonist. Concentration-response curves to NA were prepared using the software Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA). For each curve the software calculates the lower and upper plateau, the slope and the EC_{50} value ±SE of the mean by means of a linear regression analysis. Significance for all concentration response curves was tested using a two-way analysis of variance (ANOVA) followed by a *post hoc* test (Bonferroni's). A probability of $P<0.05$ was taken as significant.

Results

An un-paired two-tailed *t*-test revealed a significant age difference between the two groups ($P<0.0001$), the control group being older.

Histology

Sections stained with H&E confirmed previously published findings of smooth muscle variation in varicose sections between atrophic and hypertrophic segments[26]. Atrophic sections consisted of reduced ECM and SMC content resulting in a thin vein wall where individual layers could not be distinguished. In hypertrophic segments an increase in extracellular matrix broke up the smooth muscle bundles. The intima was often thickened with hyperplasia and hypertrophy of the longitudinal SMC, consistent with the earlier report by Wali et al[211].

Using an antibody to smooth muscle actin, our double staining fluorescent immunohistochemistry identified $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on longitudinal and circular smooth muscle. There was no immunostaining for $P2X_{2\text{-}6}$ or $P2Y_{11}$ receptors. There was a reduction in intensity of the $P2X_1$ receptor staining in the varicose vein sections (Fig. 2.1b) when compared to the control vein (Fig. 2.1a). Conversely, there was an increase in intensity of the $P2Y_1$ receptor staining in the varicose sections (Fig. 2.2b) when compared to the control vein (Fig. 2.2a). $P2Y_2$ receptor staining (Fig. 2.3a and 2.3b) was weak in both tissue groups, but its intensity was increased on intimal longitudinal muscle in most of the varicose veins. $P2Y_4$ (Fig. 2.4a and 2.4b) and $P2Y_6$ (Fig. 2.5a and 2.5b) receptors stained with similar intensities in control and varicose sections. Preabsorption of the primary purinoceptor antibody with its corresponding peptide showed reduced immunofluorescence for all receptors in both control and varicose vein (Fig. 2.1c, 2.1d, 2.2c, 2.2d, 2.3c, 2.3d, 2.4c, 2.4d, 2.5c and 2.5d). **For each P2 receptor, control and varicose tissue from a minimum of 10 patients each were immunostained and compared.**

Colocalisation of purinoceptor and smooth muscle actin staining of the boxed areas in figure 2.1a and 2.2b are represented in figures 2.6 and 2.7 at increased magnification. The red $P2X_1$ staining (Fig. 2.6a) and the green smooth muscle actin staining (Fig. 2.6b) on the same section are shown. When the images are overlapped, the smooth muscle cells that contain actin and $P2X_1$ receptors appear yellow (Fig 2.6c), confirming the location of the purinoceptor on the SMC. Similar findings are shown for $P2Y_1$, confirming the presence of the purinoceptor on the SMC (Fig. 2.7a, 2.7b and 2.7c).

Electron Microscopy

At high magnification, structures could be identified in healthy (control) veins that are characteristic of a smooth muscle contractile phenotype. These include numerous myofilaments that attach to dense bodies within the cytoplasm and in dense areas of the plasma membrane that alternate with caveolae. Organelles (including mitochondria and golgi complexes) are located in the perinuclear region (Fig. 2.8a). SMC were tightly packed together in organised bundles and surrounded by fibrous tissue (Fig. 2.9a).

High magnification of varicose SMC revealed properties that are characteristic of a synthetic phenotype. Whilst some varicose SMC showed characteristics similar to that of control SMC, other varicose SMC contained an increased volume of organelles (including vesicles and dilated rough endoplasmic reticulum) and these were located at the periphery of the cell (Fig. 2.8b). These cells, though synthetic in their appearance, were confirmed as being a SMC by the presence of a continuous basal lamina with caveolae and a limited appearance of myofilaments and dense bodies. The varicose SMC showing synthetic properties were located around the intimal and inner medial layers. Due to the disorganisation of muscle fibres seen in varicose veins, it was not possible to distinguish whether they were longitudinal or circular fibres. No synthetic phenotyped SMC was seen in the adventitia. Low magnification of the varicose sections revealed an increase in

collagen and elastic tissue separating the muscle bundles, when compared to control sections (Fig. 2.9b).

Functional Experiments on Circular Smooth Muscle

The LSV circular muscle contracted to NA and KCl, and contractions were reduced in the varicose tissue ($P<0.0001$ and $P=0.0338$, respectively) (Fig. 2.10a). Circular muscle contracted to both $P2X_1$ receptor agonists; contractions to ATP ($P=0.0178$) and α,β-meATP ($P=0.0292$) (Fig. 2.10b) were significantly reduced in the varicose tissue.

ACh failed to induce relaxation on NA (EC_{50} concentration) precontracted control or varicose vessels. At times, contractions were seen instead. This suggests that the endothelium in the two vessel groups was significantly disrupted (figure not shown). ACh caused small contractions instead due to its muscarinic actions directly on the smooth muscle cells.

Functional Experiments on Longitudinal Smooth Muscle

Longitudinal muscle in both healthy and varicose vein contracted to purinoceptor and adrenoceptor agonists. There was a significant reduction in contractions of the varicose tissue to NA ($P<0.0001$), KCl ($P=0.0324$), ATP ($P<0.0001$) and α,β-meATP ($P=0.0033$) (Fig. 2.10c and 2.10d).

Unlike circular muscle, the longitudinal muscle contracted to several P2Y receptor agonists. Significant reductions in contractions were again seen in varicose tissue to 2-MeSADP ($P=0.0091$), UTP ($P=0.0369$) and UDP ($P=0.292$) (Fig. 2.11a, 2.11b and 2.11c) compared to control tissue.

In both control and varicose tissue, contractions to 2-MeSADP were significantly reduced ($P=0.0001$ and $P=0.0125$, respectively) in the presence of the competitive $P2Y_1$ receptor antagonist MRS2179 (10^{-6}M). Contractions to UDP

were also significantly reduced in the presence of the non selective P2 receptor antagonist cibacron blue 3GA (10^{-4}M) (varicose tissue, P<0.0001 and control tissue, P=0.0008) (Fig. 2.11d and 2.11e). Contractions to UTP were inhibited with suramin (10^{-4}M). UTP is an agonist at both $P2Y_2$ and $P2Y_4$ receptors, however the antagonist suramin enables a distinction to be made between suramin-sensitive $P2Y_2$ and suramin-insensitive $P2Y_4$ receptors[212]. Significant reductions in longitudinal muscle contraction in control (P=0.0009) and varicose (P=0.0023) tissue were seen in its presence.

As with circular muscle, ACh failed to induce relaxation on NA (EC_{50} concentration) precontracted control or varicose veins. At times, contractions were seen instead (Fig 2.12). This suggests that the endothelium in the two vessel groups was significantly disrupted. ACh caused small contractions instead due to its post ganglionic parasympathetic muscarinic actions directly on the smooth muscle cells.

Electrical field stimulation induced frequency-dependent increases in tension in longitudinal muscle. The addition of prazosin significantly reduced contractility (P<0.0001) and the subsequent addition of PPADS reduced contractility even further (P=0.0481) (Fig. 2.11f). Frequency response curves, without the addition of any antagonists, acted as time controls demonstrating no smooth muscle fatigue. Increasing the stimulation frequency results in greater receptor activation and an increase in receptor effects. Our results demonstrate that longitudinal muscle contractions consist of both noradrenaline and purine-mediated responses. The addition of TTX produced a graph similar to that of PPADS and prazosin combined, indicating that all responses were due to nerve mediated stimulation. Residual responses with TTX represent direct muscle stimulation.

Contractions to UTP were thought to be $P2Y_2$ receptor mediated due to the significant contraction reduction seen with the addition of the antagonist suramin. Contractions to UTP, in the presence of suramin, could be $P2Y_4$ receptor mediated, but as they were small and due to the lack of specific $P2Y_4$ receptor antagonists, this was not further investigated.

Discussion

There was a significant age difference between the control and varicose vein groups. There was also a greater proportion of females in the varicose group. Oestrogen has been considered to be a causal factor of varicose veins[213], perhaps explaining the increased prevalence amongst females. During pregnancy, varicose vein presentation increases along with plasma oestrogen levels. Post partum, when oestrogen levels fall, varicose veins commonly resolve. However other factors during pregnancy must not be ignored, such as the enlarged uterus impairing venous drainage from the lower limbs. Oestrogen is thought to affect vascular wall strength by relaxing smooth muscle and softening collagen fibres. Oestrogen receptors have been identified on varicose and control LSV[214]. Oestrogen not only affects the smooth muscle in vessel walls, but also affects the collagen in regulating the wall strength. Haynes et al[215] showed that oestrogen increases $P2X_1$ and $P2X_7$ receptor-mediated contractions in uterine arteries. Further, UTP was shown to be more potent in oestradiol-treated animals (low progesterone levels), and an upregulation of a UTP-specific pyrimidine receptor subtype was thought to occur, although no differences were seen in pregnant animals.

The findings of competent valves in patients with a 20 year history of gross varicosities[23] and of the presence of varicosities below competent valves, supports the theory of vein wall weakness and is contradictory to that of valve insufficiency. A saphena varix, where a saccular dilatation occurs laterally out of normal vein wall, suggests a localised weakness that progresses. If back pressure was the source, then the whole wall would be expected to be evenly affected. When a muscular tube sustains damage due to chronic pressure, it responds with hypertrophy (eg LSV hypertrophies at arterial pressures in bypass surgery). Exposed to increased chronic venous pressure the LSV could be expected to hypertrophy rather than respond by passive dilatation, suggesting an underlying vein wall weakness.

Hypertrophy of the intimal longitudinal muscle layer in varicose veins is consistent with previous studies[29]. An increase in cell size with no changes in cell number, suggesting hypertrophy but not hyperplasia has been reported in

hypertensive rat portal vein[216]. Theories for the intimal changes include hypoxia of the endothelial cells[217,218] and endothelium disruption causing SMC exposure to blood flow leading to modulation of its function[219,220]. The possibility of modulating the SMC function by an increase of the extracellular matrix was suggested by Lee et al[221], and is supported by the increase in SMC and ECM (collagen) in the LSV[222]. It has been previously proposed that varicose changes are not due to vein wall deficiencies but rather to modulation of their normal function[29]. The separation of the SMC by increased ECM in the hypertrophic intima suggests they have proliferative and synthetic functions and have adopted a different phenotype[26]. Analysis of aortocoronary saphenous vein grafts showed intimal hyperplasia results from the migration and proliferation of de-differentiated SMCs originating from the media in response to mechanical injury and haemodynamic disturbances[26]. A similar physiological exposure occurs in the LSV due to the increased venous pressure. This could promote the SMC to change phenotype. Buján et al[223] studied elastin expression in LSV and their findings indicated a higher metabolism of the elastic component in varicose veins. They concluded that varicose pathology involved a restructuring of the elastic component of the vein wall, which could be a consequence of alterations in the transcription mechanisms of muscle cells.

The electron microscopy findings in control veins were characteristic of contractile SMC as previously reported[28]. Populations of poorly differentiated SMC with an increase in secretory cytoplasmic organelles and a reduction in filament bundles, characteristics seen in synthetic phenotyped SMC, have been previously noted in LSV used in surgery for critical ischaemia, where no history of venous disease was given but tests to exclude varicose veins were not performed[28]. Phagocytic and secretory properties of SMC in varicose vein have been previously suggested[23,211]. The ECM seen in varicose veins could be synthesised via synthetic phenotyped SMC.

In the varicose vein wall, a significant increase in transforming growth factor β1 (which stimulates the synthesis of ECM components, especially collagens and elastin, reduces the expression of matrix metalloproteinases and increases

expression of tissue inhibitors) and an increase in the cytokine basic fibroblast growth factor (known to be chemotactic and mitogenic for SMC) has been reported, with no variation in vascular endothelial growth factor[26]. Inflammatory cells could not account for the cytokine modulation as they were not present. These three mechanisms could increase the ECM in varicose veins.

Previous studies on animal and human LSV have shown reduced contractility to angiotensin II, NA, endothelin-1 and KCl of the circular muscle in the varicose state[24,224]. $P2X_1$, $P2Y_2$, $P2Y_6$ receptors have been identified on the circular smooth muscle in previous studies[179-181,183], however for the first time we show a reduction in contractility through $P2X_1$ receptors in the varicose tissue. Our findings of reduced contractions to NA and KCl in varicose tissue is consistent with those of others[24,224]. Reduced contractions may result from a combination of both decreased muscle volume and weaker contractile muscle cells in the varicose vein.

For the first time we have recorded contractions of the longitudinal smooth muscle on the human LSV, showing contractions mediated by $P2X_1$, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors. mRNA of $P2Y_1$ receptors has been detected on endothelium-denuded healthy LSV[181] and on LSV endothelial cells[182], but no contractile property has been demonstrated. $P2Y_1$ receptors on endothelial cells mediate vasodilatation, but for the first time we have shown stimulation of $P2Y_1$ receptors induces contraction of the longitudinal muscle. Our results also show that longitudinal muscle contractions to both adrenoceptors and purinoceptors are reduced in varicose tissue.

ACh is known to cause vasodilatation by stimulation of the muscarinic receptors located on endothelial cells, triggering a release of NO, initially known as 'endothelium-dependent relaxing factor' (EDRF). In our experiments it failed to cause relaxation, suggesting that EC had been damaged during the tissue preparation. At higher ACh concentrations, vasoconstriction was noted, a result of the direct actions of ACh on muscarinic receptors on smooth muscle.
Whilst we did not actively denude endothelial cells from the vein preparations, they were inactive when it came to our functional experiments. Endothelium-mediated relaxation of vein wall tone did not interfere with our experimental results.

Contractile properties of longitudinal muscle in both the media and intima of the human internal coronary artery[225] has been previously demonstrated. In the rat portal vein, functional studies demonstrated P2X receptor-mediated contractions of longitudinal muscle[226,227] and histological studies showed that in hypertension, the outer longitudinal muscle cells have an irregular outline and hypertrophy[228]. Similar histological results have been seen in hypertensive rat arteries[229]. LSV has both intimal and outer medial longitudinal muscle bundles, and we have shown their combined purinergic and adrenergic contractile properties. We were unable to separate the two layers as they are often difficult to identify in the varicose tissue.

SMC and endothelial cell proliferation, death and secretory properties play important roles in both new vessel growth during wound healing and intimal thickening during arterial diseases[124,125]. Purinoceptors, aside from their role in vessel tone control, are known to play important roles in the signalling pathways of these events[103,127]. Selective agonists have shown the contractile effects in blood vessels are mediated mainly by $P2X_1$ receptors with smaller effects by $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors[60,230], while the mitogenic effects of smooth muscle cells are mediated by $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors[60,127]. The transition from contractile to synthetic SMC phenotypes has been shown in atherosclerosis, restenosis after angioplasty and during cell culture *in vitro*[127,231]. mRNA levels of $P2Y_1$ and $P2Y_2$ receptors increase 342-fold and 8-fold, respectively, in cultured rat aortic smooth muscle cells that show the synthetic phenotype when compared to phenotypic contractile cells in freshly dissociated muscle[100]. $P2X_1$ receptor mRNA, present in the contractile cells, was not detected in the synthetic cells. mRNA levels of $P2Y_4$ and $P2Y_6$ receptors were similar in both cell phenotypes. $P2Y_4$ and $P2Y_6$ receptor activation stimulates mitogenesis in SMC[102,232]. The smooth muscle cells in varicose LSV show similar changes in expression, ie. reduced $P2X_1$ receptor immunostaining and reduced $P2X_1$-mediated contraction. There is also increased immunostaining intensity of $P2Y_1$ and $P2Y_2$ receptors, associated with increased synthetic and proliferative activity, and loss of contractile activity. Immunostaining intensity remained constant for $P2Y_4$ and $P2Y_6$ receptors, although there was a reduction in $P2Y_6$-mediated contractions in varicose veins.

Activation of endothelial cell production of nitric oxide and increased sensitivity of the smooth muscle is thought to lead to increased synthesis of cyclic GMP and heighten vascular relaxation[233]. A mitogenic phenotype may account for this altered sensitivity. In aortic SMC, A_{2B} receptor activation inhibits growth[234], and either $P2Y_2$ or $P2Y_4$ receptor activation stimulates cell proliferation[129]. In atherosclerosis there is an upregulation of $P2Y_2$ receptors by a mitogen-activated protein kinase (MAPK)-dependent growth factor. Suramin inhibits platelet derived growth factor (PDGF) receptor activation and signalling through the MAPK-activator protein 1 pathway. PDGF is a growth factor antagonist inhibiting cell proliferation and reducing neointimal thickness in LSV grafts in mice[31]. These two findings suggest an increased $P2Y_2$ receptor expression in a hypertrophied intima, similar to our theory of increased $P2Y_2$ receptor expression in varicose intima.

Intimal longitudinal smooth muscle is more prominent in hypertrophied varicose vein segments. It is visible in Figure2.9b, but is more clearly visible in Figure 2.2b. $P2Y_1$ and $P2Y_2$ receptors stain to a bright intensity on the intimal muscle in varicose sections. However due to the disorganisation of muscle bundles in varicose tissue, longitudinal and circular muscles are not always clearly identifiable. We have contracted, for the first time, intimal and outer medial longitudinal muscle together in LSV. We propose that intimal longitudinal smooth muscle undergoes a change from contractile to synthetic phenotype in varicose veins. This is supported by the increase in intimal smooth muscle volume, a reduction in its contractile strength, increases in intimal extracellular matrix, increased intensity of intimal $P2Y_1$ and $P2Y_2$ receptor staining and reduced intimal $P2X_1$ receptor staining.

What is the source of the ATP that acts on the P2 receptors expressed on the smooth muscle of LSV? One possibility is that ATP is released as a cotransmitter with NA from perivascular sympathetic nerves[235]. Another is that ATP is released from endothelial cells during changes in flow (shear stress) and hypoxia[88]. A further question that will need to be resolved is whether the changes in smooth muscle phenotype and associated changes in purinergic signalling are causal or consequential in varicose vein development.

An understanding of these changes occurring within the purinergic signalling pathway may identify targets for therapeutic intervention. Modulation of vein muscle phenotype may be a useful approach for treating varicose veins and subsequent chronic venous insufficiency. It could potentially optimise the role of the long saphenous vein as a bypass graft.

Figure 2.1: Immunofluorescent staining of transverse sections of LSV. $P2X_1$ receptors on control (a) and varicose (b) veins. Reduced immunofluorescence is seen when the antibody is blocked with its peptide in control (c) and varicose (d) veins for $P2X_1$ receptors. (L = lumen, I = intima, M = media, A = adventitia). Boxed areas in (a) represent areas magnified in Fig 2.6 (a, b and c).

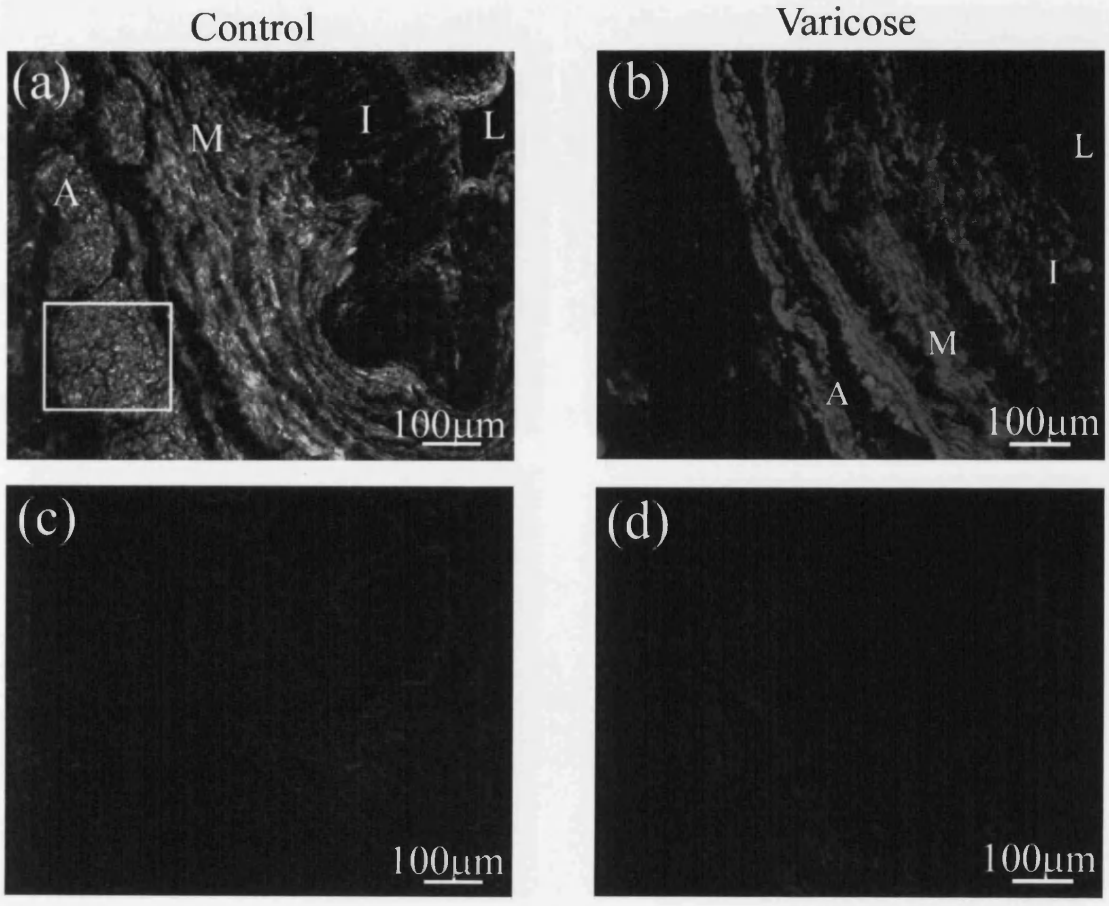


Figure 2.2: Immunofluorescent staining of transverse sections of LSV. $P2Y_1$ receptors on control (a) and varicose (b) veins. Reduced immunofluorescence is seen when the antibody is blocked with its peptide in control (c) and varicose (d) veins for $P2Y_1$ receptors. (L = lumen, I = intima, M = media, A = adventitia). Boxed areas in (b) represent areas magnified in Fig 2.7 (a, b and c).

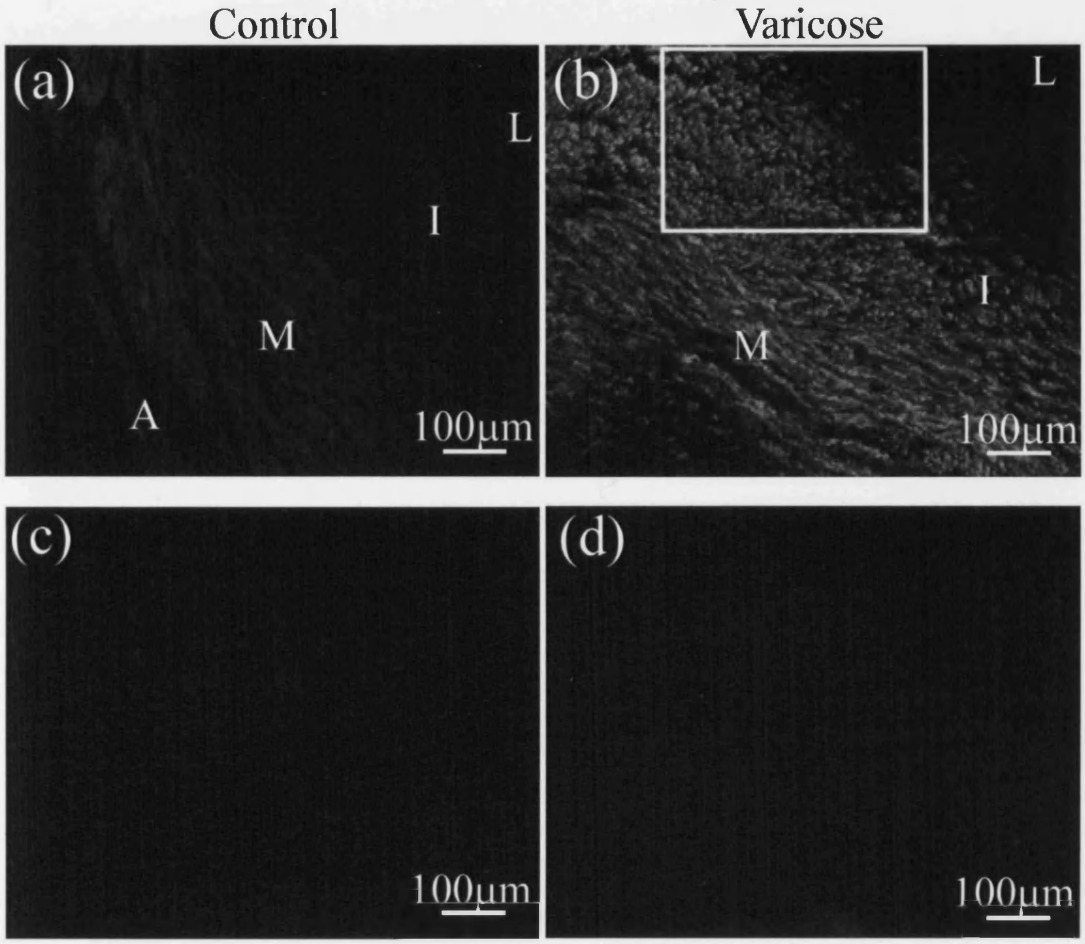


Figure 2.3: Immunofluorescent staining of transverse sections of LSV. $P2Y_2$ receptors on control (a) and varicose (b) veins. Reduced immunofluorescence is seen when the antibody is blocked with its peptide in control (c) and varicose (d) veins for $P2Y_2$ receptors. (L = lumen, I = intima, M = media, A = adventitia)

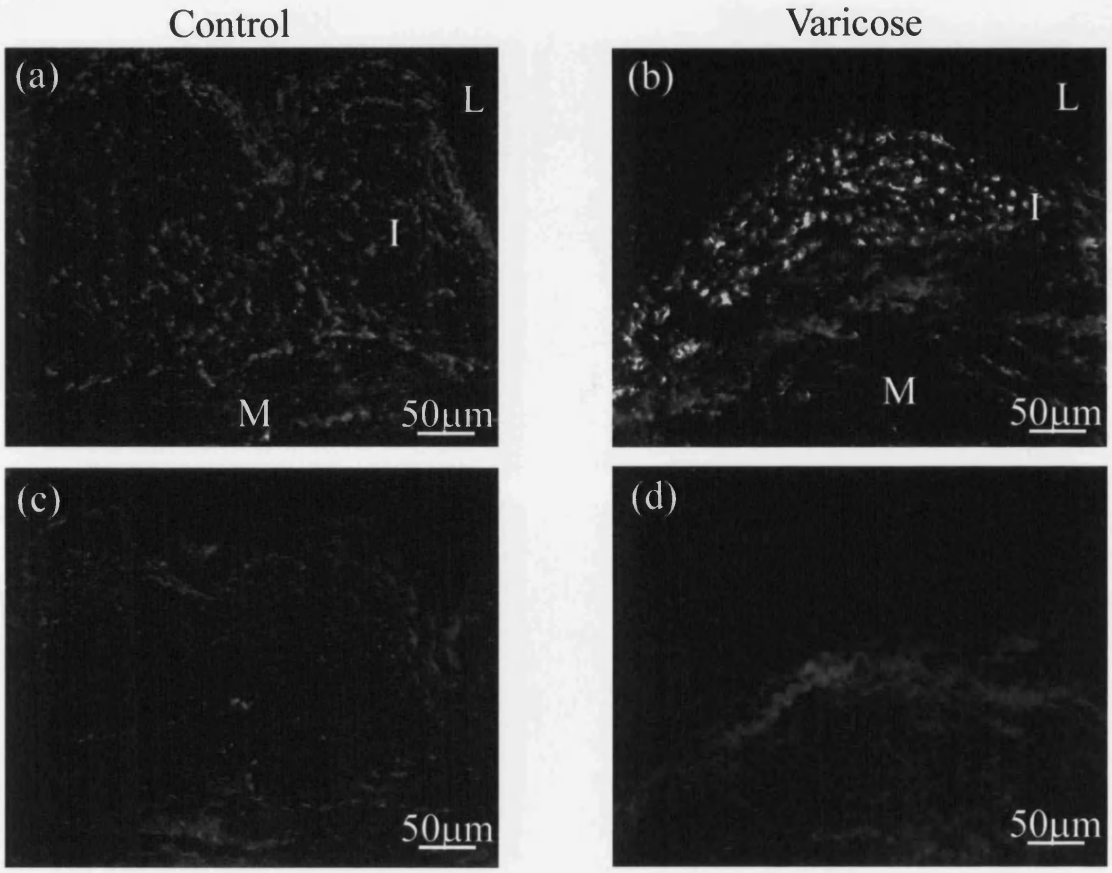


Figure 2.4: Immunofluorescent staining of transverse sections of LSV. $P2Y_4$ receptors on control (a) and varicose (b) veins. Reduced immunofluorescence is seen when the antibody is blocked with its peptide in control (c) and varicose (d) veins for $P2Y_4$ receptors. (L = lumen, I = intima, M = media, A = adventitia)

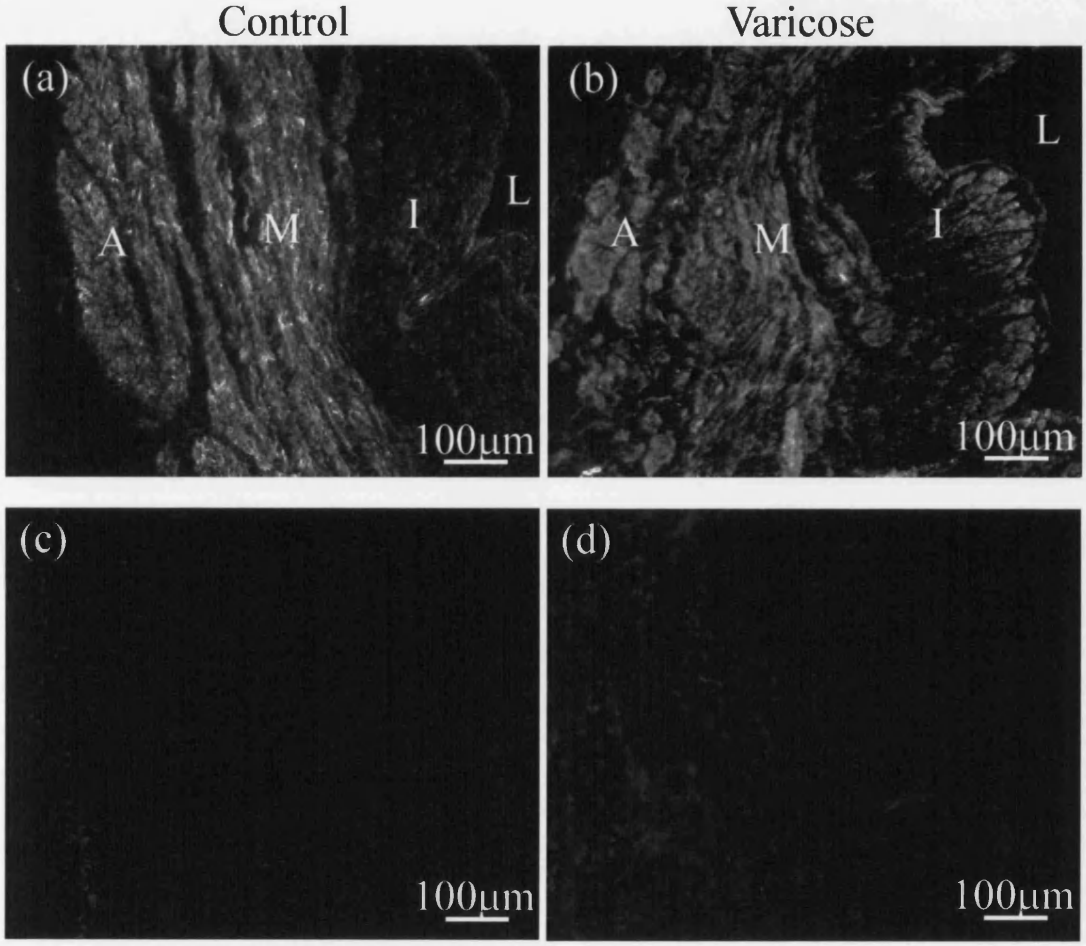


Figure 2.5: Immunofluorescent staining of transverse sections of LSV. $P2Y_6$ receptors on control (a) and varicose (b) veins. Reduced immunofluorescence is seen when the antibody is blocked with the $P2Y_6$ peptide in control (c) and varicose (d) veins. (L = lumen, I = intima, M = media, A = adventitia)

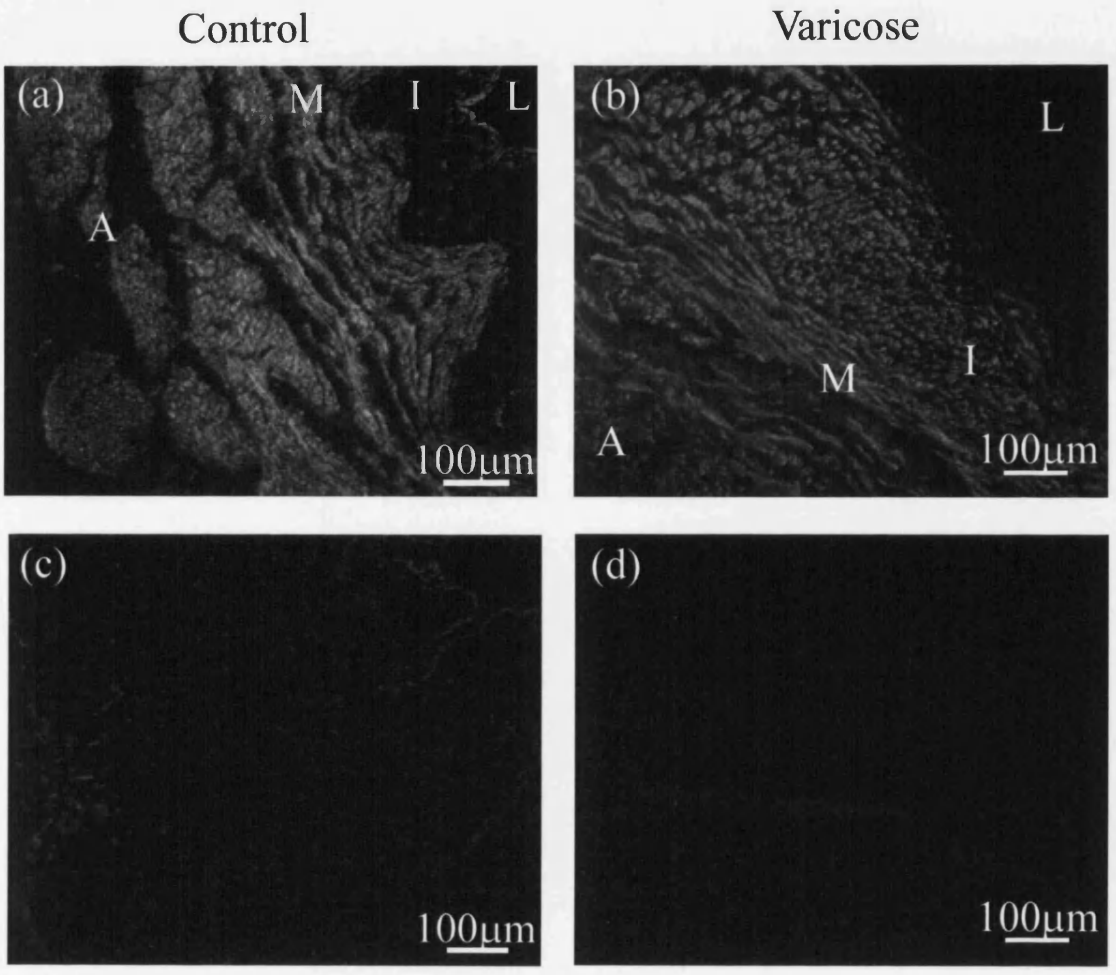


Figure 2.6: Immunostaining of the transverse sections of LSV, outlined by the box in Fig 2.1(a). Red immunostaining of the $P2X_1$ receptor in control vein (a). The same section simultaneously stained green for smooth muscle actin (b). Combining both images for each section shows the colocalisation (yellow/orange) of $P2X_1$ and actin on SMC (c).

Figure 2.6

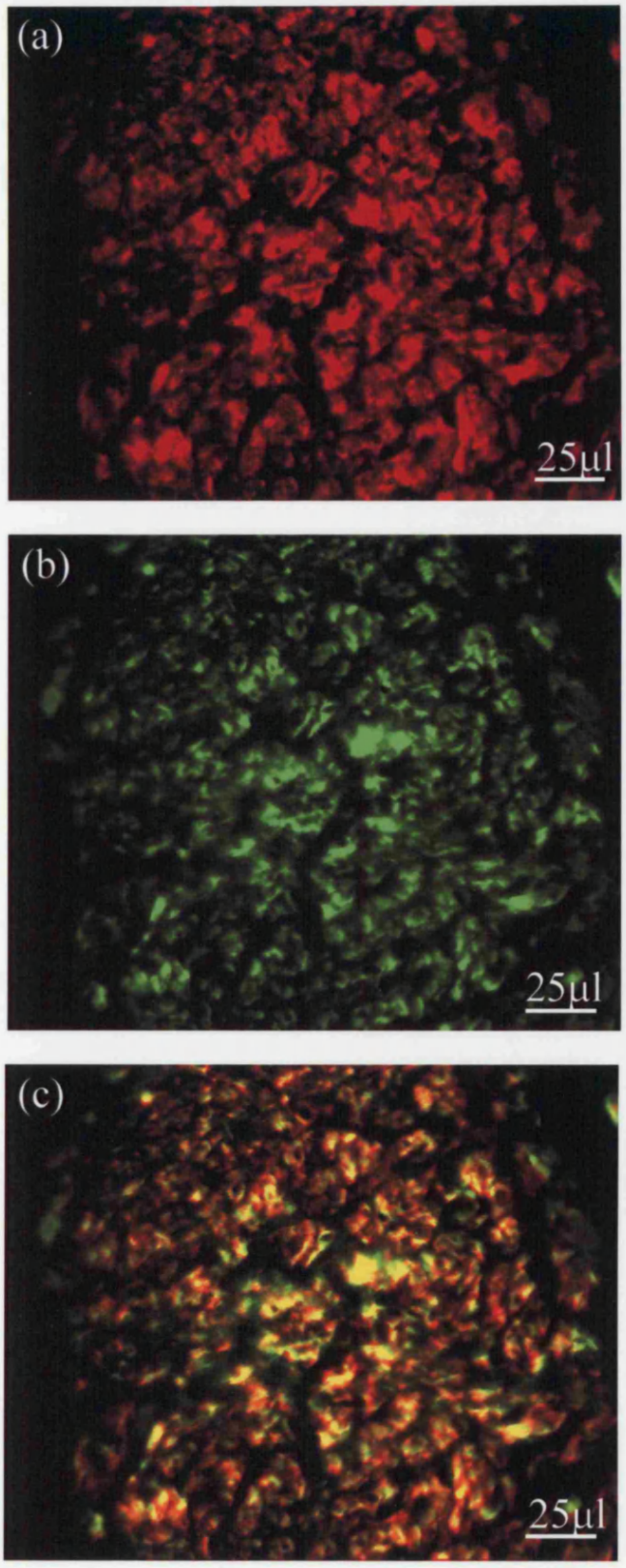


Figure 2.7: Immunostaining of the transverse sections of LSV, outlined by the box in Fig 2.1(a). Red immunostaining of the $P2Y_1$ receptor in varicose vein (a). The same section simultaneously stained green for smooth muscle actin (b). Combining both images for each section shows the colocalisation (yellow/orange) of $P2Y_1$ and actin on SMC (c).

Figure 2.7

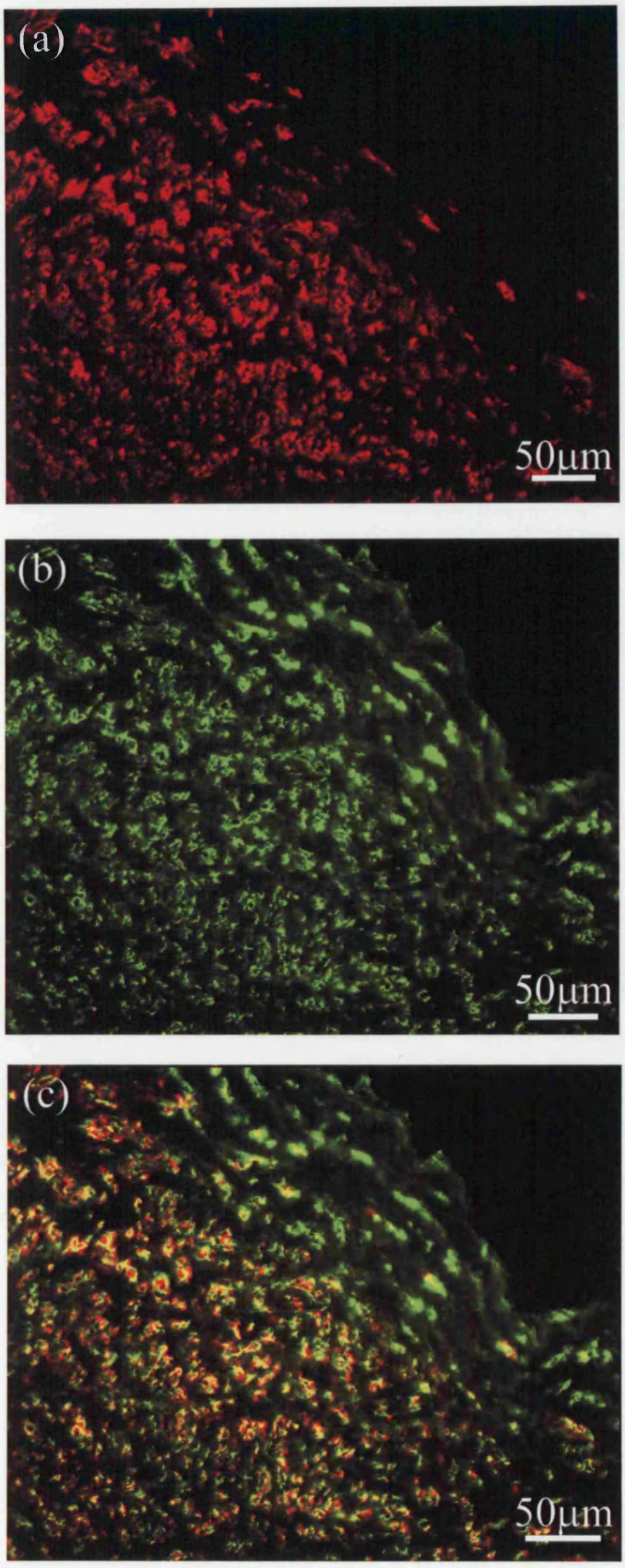
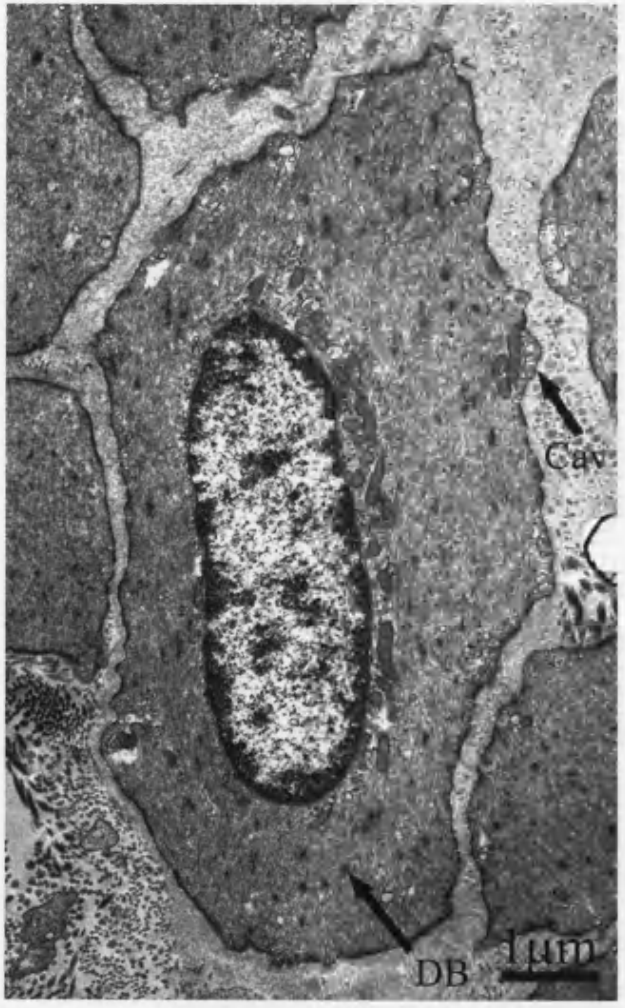


Figure 2.8: Electron microscopy of SMC demonstrating the contractile phenotype in control veins (a) and the synthetic phenotype found in varicose veins (b). Organelles appear perinuclear in the contractile phenotype, and towards the cell periphery in the synthetic phenotype. (Cav = caveolae, RER = rough endoplasmic reticulum, DB = dense bodies).

Figure 2.8

(a) Contractile phenotype



(b) Synthetic phenotype

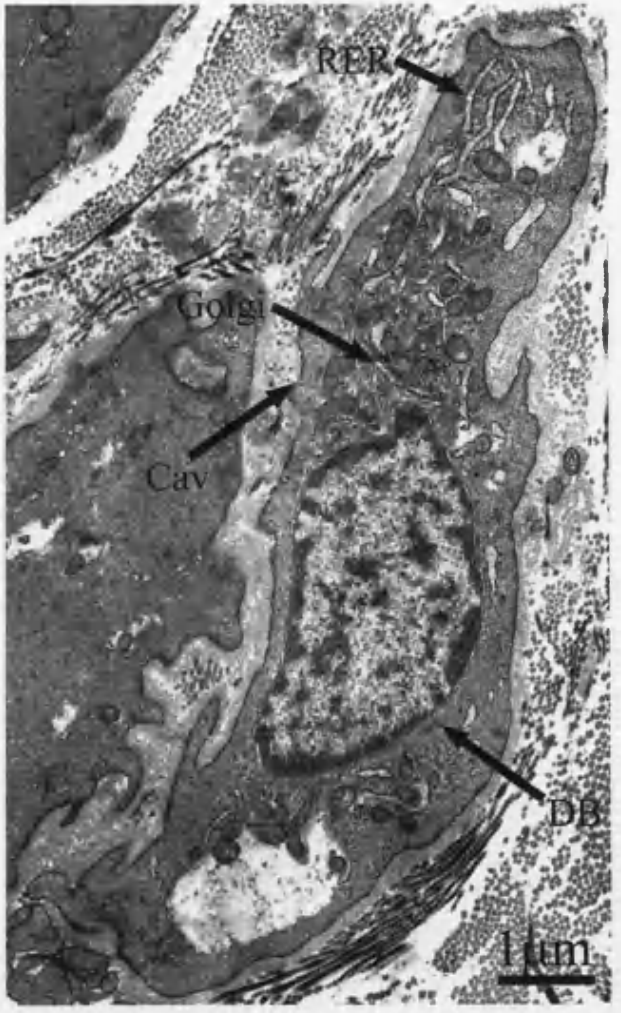


Figure 2.9: Electron microscopy of longitudinal sections of LSV comparing the closely bound muscle bundles in control vein (a) with the SMC that are separated by an increase in the extracellular matrix in varicose vein (b). (ECM = extracellular matrix, long = intimal longitudinal muscle, circ = circular muscle).

Figure 2.9

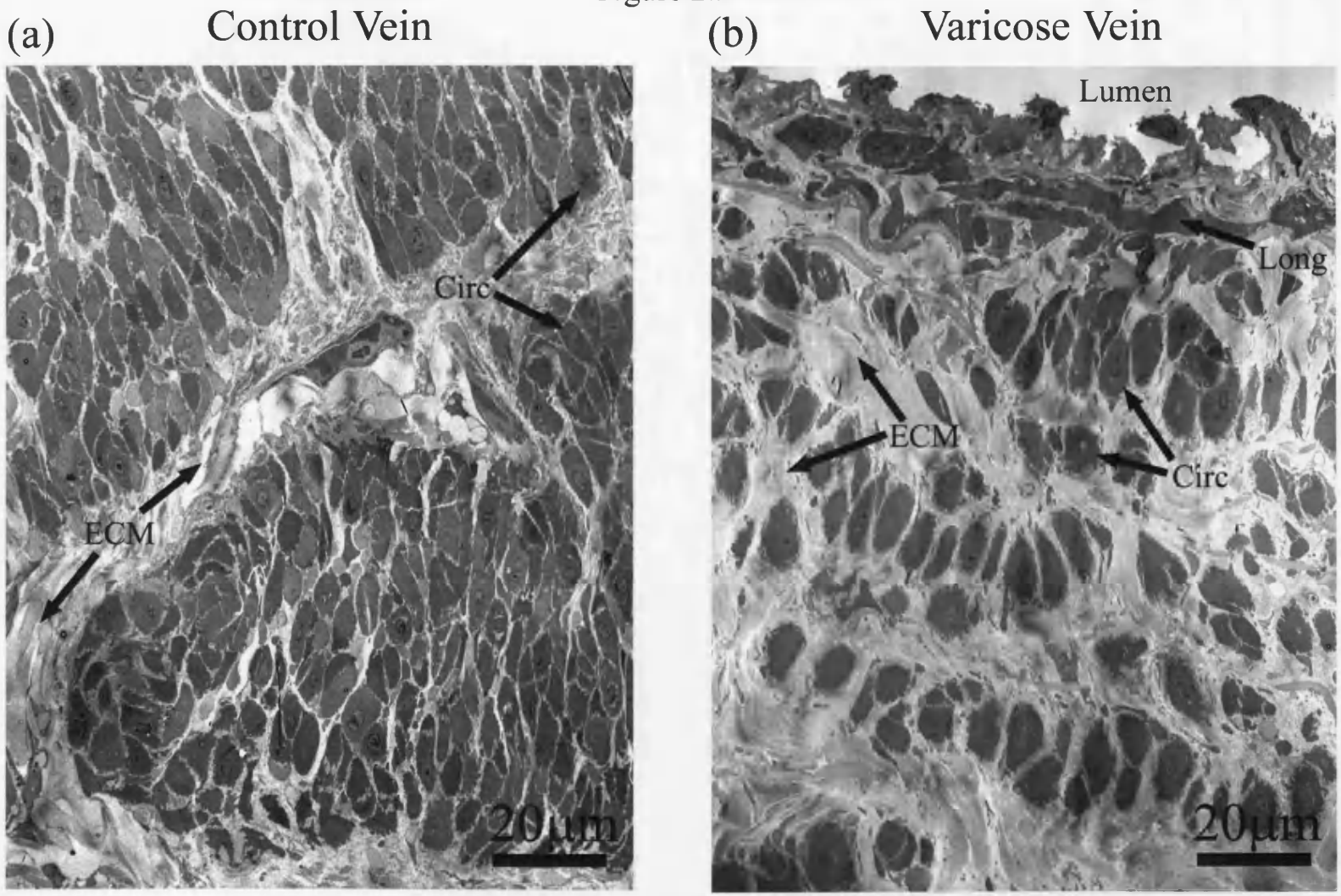

(a)
Control Vein
Circ
ECM
20µm
(b)
Varicose Vein
Lumen
Long
ECM
Circ
20µm

Figure 2.10

Concentration-response curves of circular (a, b) and longitudinal (c, d) smooth muscle of control and varicose human LSV. NA (10^{-8} to 10^{-3} M) and KCl (10^{-5} to $3x10^{-1}$ M) contractions (a,c), and α,β-meATP (10^{-8} to 10^{-5} M) and ATP (10^{-6} to 10^{-3} M) contractions (b,d) are shown. All symbols represent mean ± SE (unless masked by the symbol).

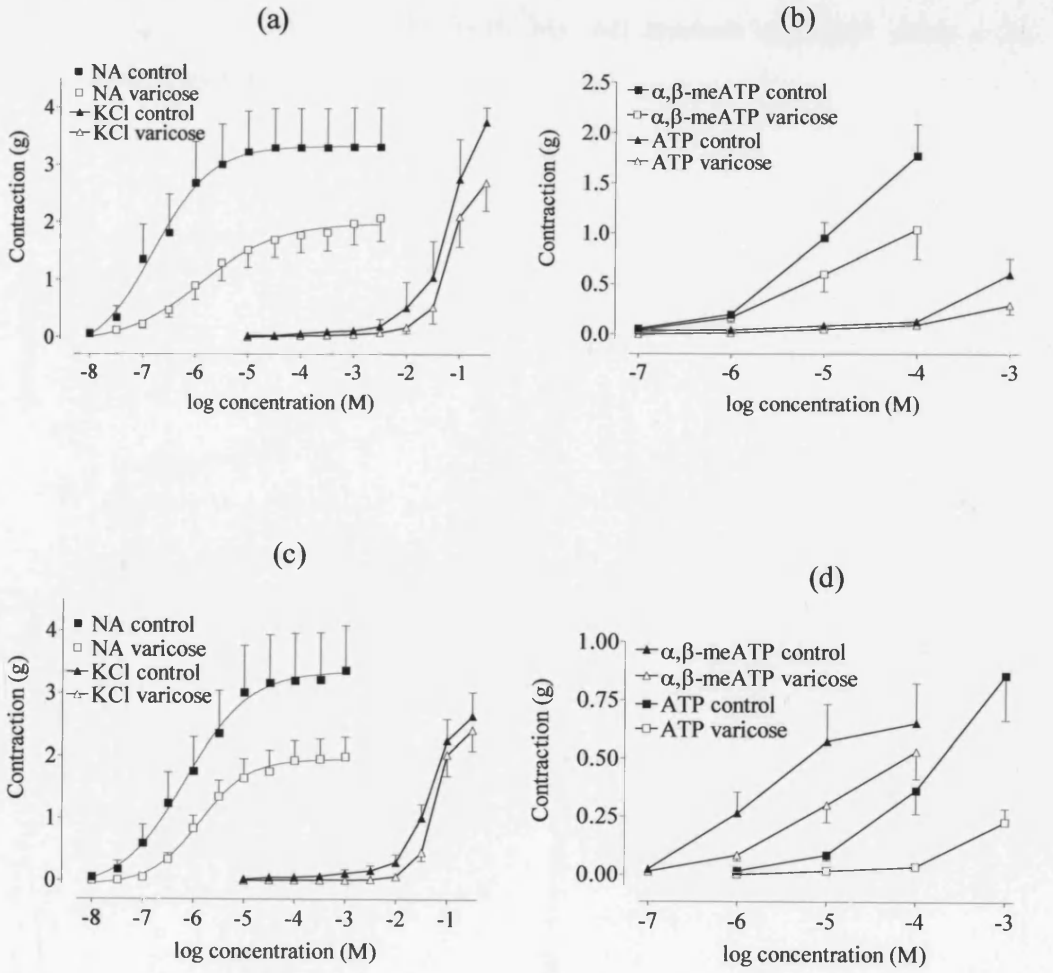


Figure 2.11

Concentration response-curves of control and varicose longitudinal smooth muscle from LSV to P2 receptor agonists, and in the absence and presence of P2 receptor antagonists. (a) 2MeSADP (10^{-8} to 10^{-5} M); (b) UTP (10^{-6} to 10^{-3} M); (c) UDP (10^{-6} to 10^{-3} M). Concentration response curves to the $P2Y_6$ receptor agonist UDP (10^{-6} to 10^{-3} M) in the absence and presence of cibacron blue 3GA (10^{-4} M) in control (d) and varicose (e) LSV. (f) Electrical field stimulation of longitudinal smooth muscle in control LSV in the absence and presence of prazosin (10^{-6} M), then in the presence of prazosin and PPADS ($3x10^{-5}$M). All symbols represent mean ± SE (unless masked by symbol).

Figure 2.11

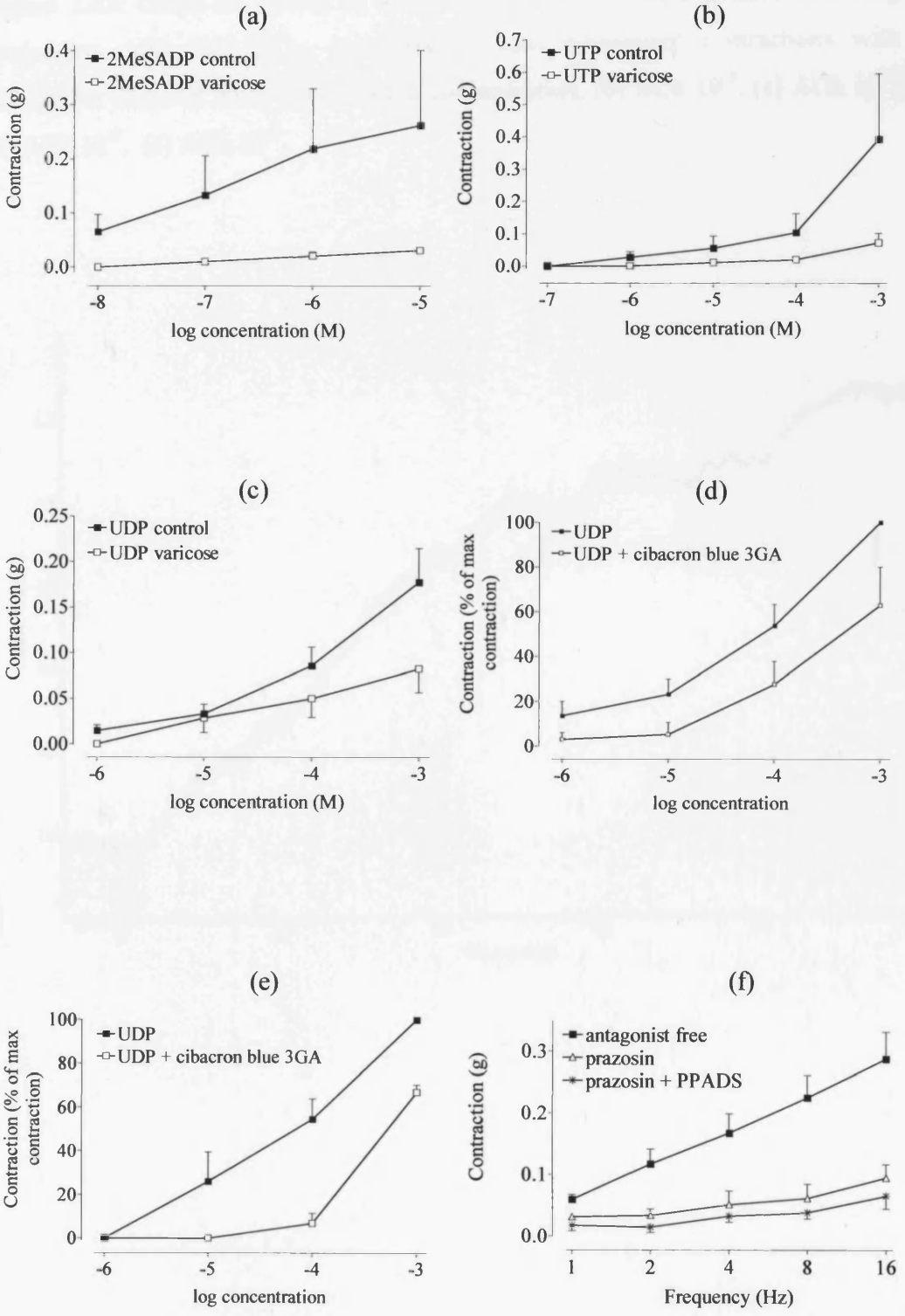
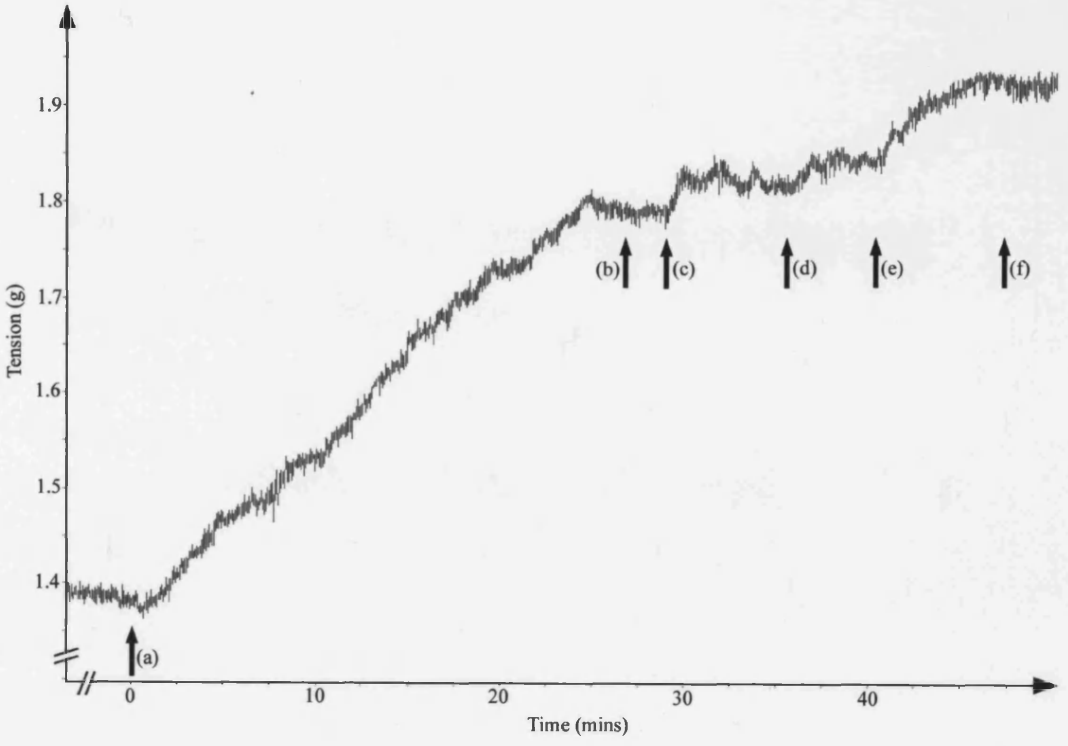

(a)
2MeSADP control
2MeSADP varicose
Contraction (g)
log concentration (M)
(b)
UTP control
UTP varicose
Contraction (g)
log concentration (M)
(c)
UDP control
UDP varicose
Contraction (g)
log concentration (M)
(d)
UDP
UDP + cibacron blue 3GA
Contraction (% of max contraction)
log concentration
(e)
UDP
UDP + cibacron blue 3GA
Contraction (% of max contraction)
log concentration
(f)
antagonist free
prazosin
prazosin + PPADS
Contraction (g)
Frequency (Hz)

Figure 2.12: Graph of tracing of longitudinal smooth muscle tension following contraction with NA (EC_{50} concentration) and subsequent contractions with cumulative doses of ACh. (a) NA EC_{50} concentration, (b) ACh 10^{-7}, (c) ACh 10^{-6}, (d) ACh 10^{-5}, (e) ACh 10^{-4}.



Chapter 3

Purinoceptor Expression On Keratinocytes Reflects Their Function On The Epidermis During Chronic Venous Insufficiency

Abstract

Purines are extracellular nucleotides that have long term effects on keratinocyte proliferation, differentiation and death through $P2Y_1$, $P2Y_2$, $P2X_5$ and $P2X_7$ receptors. This study examined changes in expression of these P2 receptors on lower leg epidermal keratinocytes in control and chronic venous insufficiency (CVI) states. Lower limb skin biopsies from CVI (CEAP classification 4a and 4b) and control skin were immunostained for the above P2 receptor subtypes and epidermal area was calculated. Our results with CVI show an increase in $P2Y_1$ and $P2Y_2$ receptor expression in basal and spinosal layers of the epidermis and an increase of $P2X_5$ receptors mainly in the spinosal layer and extending further into the stratum granulosum. In contrast $P2X_7$ receptors were reduced in the stratum corneum in CVI. In conclusion, a thinner epidermis was found in CVI, which might be the result of the changes in expression of P2Y and P2X receptors on keratinocytes: that is, increased proliferation via $P2Y_1$ and $P2Y_2$ receptors and reduced $P2X_7$ receptor-mediated cell death opposed by a dominant decrease in cell numbers as a result of increased $P2X_5$ receptor-mediated differentiation (which is in effect antiproliferative). Thus, increased keratinocyte $P2X_5$ receptor activity may, in part, be accountable for epidermal thinning in CVI.

Introduction

During the later stages of CVI, skin changes including lipodermatosclerosis may occur and lead to epidermal breakdown and ulcer formation. The healing time of venous ulcers varies greatly from months to many years and their management may include district nursing, hospital admission and surgery. Varicose vein stripping may be indicated in the prevention of ulcer formation. Due to the chronicity of the disease, its management can be expensive.

Several mechanisms have been proposed to explain epidermal loss during ulcer formation and an inflammatory process is a common underlying factor. Purine receptor activity has already been identified in skin epidermis. The roles of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors in different layers in human skin epidermis has been described previously[190]. $P2Y_1$ and $P2Y_2$ receptors are involved in keratinocyte proliferation, while $P2X_5$ receptors are associated with keratinocyte differentiation and $P2X_7$ receptors with keratinocyte cell death[190,191]. The balance between proliferation, differentiation and apoptosis of keratinocytes helps maintain an epidermis of constant thickness. Changes in the expression of these P2 receptor subtypes could alter this balance and result in either thinning or thickening of the epidermis.

Our study was to examine the expression of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors in the epidermis of human lower leg skin and the changes seen in patients with CVI. We examined pre-ulcerated CVI skin in an attempt to identify purine receptors on keratinocytes prior to their destruction and loss. If a change in P2 receptor expression occurs does it suggest functional changes of the keratinocytes in the different layers which might explain the process by which the epidermis is lost in CVI.

Methods

Tissue

Skin samples in CVI were obtained from patients undergoing stripping of their primary varicose veins. Reflux had been confirmed by either hand held doppler or venous duplex scanning by the vascular team prior to surgery. Skin biopsies at sites of CVI were taken from the medial aspect of the mid lower calf at the site of either the distal incision for stripping of the LSV or at the site of a stab incision for avulsing a prominent varicose vein. Biopsies were not taken from the ankle. Elliptical skin biopsies were obtained from 5 patients (2 males and 3 females) aged 46 to 69yrs (mean = 56.8yrs). Sites of CVI consisted of skin pigmentation in 2 patients (CEAP 4a) and lipodermatosclerosis in 3 patients (CEAP 4b), based on the Clinical-Etiology-Anatomy-Pathophysiology[16] classification. There were no co-existing medical conditions in these patients and none took any regular medications. Healthy control skin was obtained from 5 patients (3 males and 2 females) aged 56 to 68yrs (mean = 64.4yrs), undergoing coronary artery bypass surgery involving harvesting of the LSV. Elliptical skin samples were excised from an edge of the LSV incision from the medial aspect of the lower calf, corresponding to the area CVI skin samples were obtained from. Control skin showed no signs of CVI and reflux was excluded by hand held doppler prior to surgery. Patients in the control group were taking cardiac medications for ischaemic heart disease, including β blockers, diuretics, nitrates, angiotensin converting enzyme inhibitors and statins. Diabetic patients and patients with skin conditions (eg psoriasis, patients on steroids) were excluded from the study. Ethics approval was obtained by the joint UCL/ULCH Ethics Committees on Human Research and by the Royal Free Hampstead Research Ethics Committee.

Skin samples were collected in Hanks balanced salt solution (HBSS; Invitrogen Ltd., Paisley, UK) and then frozen in isopentane precooled in liquid nitrogen. Samples were sectioned at 10μm on a cryostat (Reichert Jung CM1800),

collected on gelatine-coated slides and air dried at room temperature. Slides were stored at -20°C.

Immunohistochemistry

Polyclonal $P2X_5$ and $P2X_7$ receptor antibodies (provided by Roche Palo Alto, CA, USA) and polyclonal anti-$P2Y_1$ and anti-$P2Y_2$ receptor antibodies (Alomone Laboratories, Jerusalem, Israel) were kept at -20°C.

Sections were fixed for 4 min in 4% formaldehyde in 0.1M PBS containing 0.2% picric acid. Sections were washed three times for 5 min each time with PBS. Sections were primarily blocked for 60 min in 10% NHS in 0.1M phosphate buffer, containing 0.05% merthiolate. Sections were then incubated overnight with the primary receptor antibody at concentrations of 1:100 to 1:200 in 10% NHS in PBS with 0.05% merthiolate. On the second day, sections were washed for 5 min three times in PBS and then stained with the secondary antibody donkey antirabbit Cy3 (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at 1:300 in PBS-merthiolate for 60 min. Sections were washed three times for 5 min before being mounted in Citifluor (Citifluor Ltd, London, UK).

Control experiments were performed by separately omitting the primary and secondary antibodies, and by preabsorbing the primary antibody with its corresponding peptide. Preabsorption was carried out by adding the peptide at a ratio of 1:1 in 10% NHS in PBS with 0.05% merthiolate, leaving for 12 hours at 4°C, passing through a syringe filter (4mm with a 0.2μm PPmembrane) then centrifuged at 13,000rpms for 5 min using only the supernatant.

Semi-quantitative assessment of the changes in immunofluorescent intensity were performed by an independent observer, blinded from the patient group from which samples were taken

Tissue samples from 10 patients (5 control and 5 varicose) were stained for each purinoceptor.

Haematoxylin and Eosin staining

H&E slides were prepared by fixing in 4% paraformaldehyde in PBS for 10 min. Sections were then washed in distilled water then stained for 20 min in Ehrlichs haematoxylin. Following washing in running tap water, slides were dipped in acid alcohol and washed again for 15 min. Sections were then stained in eosin for 5 min, dipped in tap water, then washed for 1 min in 70% alcohol, 3 min 100% alcohol, another 3 min 100% alcohol, dried for 3 min in xylene, and finally another 5 min in xylene. Sections were mounted in eukitt.

Photography

Slides were photographed using a Zeiss Axioplan, high definition light microscope (Zeiss, Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland). Images were converted from colour to greyscale using Photoshop (Adobe 5.0, San Jose, USA).

Area Measurement

Low magnification images of H&E stained epidermis were taken. Two sections from different areas of each skin sample from all five patients in each group were studied. The epidermal area was then calculated using a Scion Image programme and expressed as mean area (μm^2) ± standard error (n). Statistical analysis was carried out using an unpaired Student's *t*-test, $P < 0.05$ was taken as significant.

Results

Sections were cut sequentially. Low power magnification of H&E stains of skin epidermis are shown (Figures 3.1a, 3.1d, 3.2a, 3.2d, 3.3a, 3.3d, 3.4a and 3.4d), with subsequent sections stained with immunofluorescence shown below at greater magnification.

Control skin stained for $P2Y_1$ (Figure 3.1b) and $P2Y_2$ receptors (Figure 3.2b) in the basal layer of the epidermis, with $P2Y_2$ receptors also present in the stratum spinosum. In CVI skin, $P2Y_1$ receptor staining was markedly increased in the basal layer and present in the lower layers of the stratum spinosum in four of the five patients (Figure 3.1e), while $P2Y_2$ receptors were markedly increased in both stratum basale and spinosum, and extended further into the spinosal layer in all patients (Figure 3.2e).

In control skin, $P2X_5$ receptors stained throughout the stratum spinosum, but also in the basal and granulosal layers (Figure 3.3b). In CVI skin $P2X_5$ receptor staining extended further into the stratum granulosum and immunostaining intensity was increased in all layers from all patients (Figure 3.3e).

Staining in the control skin agreed with previous studies[126,191], confining $P2X_7$ receptors to the uppermost layer of dead cells, the stratum corneum (Figure 3.4b). This staining was present in CVI skin, but was markedly reduced in all patients (Figure 3.4e).

Control experiments were carried out for each P2 receptor antibody used on the subsequent section by preabsorption of the primary antibody with its corresponding peptide. For each P2 receptor antibody, preabsorption of the primary antibody resulted in no specific immunostaining (Figures 3.1c, 3.1f, 3.2c, 3.2f, 3.3c and 3.3f); for the $P2X_7$ receptor, some non-specific staining was observed (Figures 3.4c and 3.4f). Due to the limited sample size, comparisons between CEAP 4a and 4b samples in the CVI group were not made.

The mean epidermal areas were calculated for 2 sections per skin sample from the control and CVI groups. The mean epidermal areas (μm^2) for the control skin and in CVI were 295 ± 6.7 and 275 ± 5.4 respectively (Figure 3.5). The epidermal area in CVI skin was significantly (P=0.0313) reduced compared to control skin, indicating that the epidermis was thinner.

Discussion

Previous research on CVI skin has focused on changes occurring at the dermal capillaries. These pathophysiological changes occurring in capillaries may explain the skin changes seen in clinical practice. However few studies have focused on the epidermis itself. White cell infiltration[236], fibrin deposition[13] and oedema all occur within the dermis leading to a reduced delivery of nutrient and oxygen[237] to the epidermis and an accumulation of waste products from a reduced blood flow away from the site affected. Oxidative stress and white cell extravasation releases inflammatory mediators[238], which are thought to damage the epidermis[239]. It is thought that ischaemia followed by reperfusion worsens the process, leading to the chronic inflammatory state. Reperfusion to the tissue causes increased leucocyte activation, releasing reactive oxygen metabolites and hydrolytic enzymes, exacerbating the ischaemic injury[240]. Whilst dermal changes in CVI have been previously reported, little work exists to demonstrate the proliferative changes the keratinocytes undergo.

$P2Y_1$ and $P2Y_2$ receptors are known to mediate keratinocyte proliferation in the human epidermis and our controls match the findings of an earlier study of human 'leg' skin although proliferation marker staining was not repeated[190]. The increased expression of $P2Y_1$ and $P2Y_2$ receptors on the basal layer and stratum spinosum in CVI skin suggests increased proliferation. The extension of $P2Y_2$ receptor staining towards the stratum granulosum suggests an increased keratinocyte proliferation extending beyond the basal layer deep into the epidermis. CK 14 is a cytokeratin found on basal cells that is lost upon keratinocyte differentiation. It is greatly increased in suprabasal layers in venous eczema and lipodermatosclerosis[241] suggesting prolonged proliferation of the basal cells. This is consistent with our findings of increased $P2Y_1$ and $P2Y_2$ receptors in layers beyond the stratum basale in CVI. Increased keratinocyte proliferation may act as a compensatory mechanism to maintain epidermal thickness in the presence of increased differentiation, preventing epidermal thinning and ulceration.

ATP and UTP released as part of an inflammatory response promote keratinocyte proliferation and inhibit differentiation through activation of the $P2Y_2$ receptors[209]. Antagonists to $P2Y_2$ receptors are therapeutic targets in keratinocyte hyperproliferation states such as psoriasis[209]. In melanoma, a lack of $P2Y_2$ receptor expression is seen; however increased telomerase-induced proliferation has been identified which may explain the aggressive nature of the malignant lesion[242]. Hyperproliferative keratinocytes, demonstrated by an increase in integrin $\beta1$[243], at the edge of venous ulcers is consistent with increased proliferation. The inflammatory process present in CVI may increase keratinocyte proliferation through increased activity of $P2Y_2$ receptors, accounting for their increased expression.

$P2X_5$ receptors are restricted to metabolically active, differentiating cell layers of the epithelia and are not associated with mitosis and cell death[191]. $P2X_5$ receptors have been detected in spinous and granular layers, decreasing in its intensity towards the outermost layer[191]. It weakly stained the basal layer in the oesophagus and palate. Greig et al[190] showed the presence of $P2X_5$ receptors in these three layers in human 'leg' skin, with early keratinocyte differentiation occurring in the stratum spinosum, and late differentiation occurring within the upper spinosal and granular layers. These findings match our control $P2X_5$ receptor staining. In CVI skin, the intensity of $P2X_5$ receptor staining increased throughout all three layers suggesting an overall increase in $P2X_5$ receptor-mediated keratinocyte differentiation, which in effect is antiproliferative. Some cytokeratins expressed on epithelial cells are established markers of differentiation. CK 10, a marker of terminal differentiation, has been shown to increase in venous eczema and lipodermatosclerosis in suprabasal layers[241]. This is consistent with our findings of increased $P2X_5$ receptor expression representing an increase in differentiation in spinosal and granular layers, although staining with differentiation markers was not repeated.

$P2X_7$ receptors on macrophages and lymphocytes have cytotoxic functions at sites of inflammation[244]. $P2X_7$ receptors are also thought to be involved in the release of IL-1β[245], mitogenic stimulation of T lymphocytes[246], and the cytoplasmic

communication between macrophages and lymphocytes[247]. $P2X_7$ receptor levels on monocyte-macrophage lineage cells are increased in sarcoidosis, where they are associated with cytotoxicity, maturation and IL-1β release[248]. $P2X_7$ receptor staining was seen on the outer layer of dead keratinocytes, the stratum corneum, on control skin, as previously reported[191], and represents keratinocytes terminally differentiating[190]. In CVI, $P2X_7$ receptor staining was markedly reduced to small, scattered areas of the stratum corneum. $P2X_7$ receptors are involved in the induction of cell death[249,250], and might be a compensatory change, which prevents further thinning of the epidermis.

Plasma levels of endothelial leukocyte adhesion molecule-1, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 are increased in response to venous hypertension[251]. Increased expression of ICAM-1 in the capillaries and infiltration of T-lymphocytes and macrophages around the vessels are seen in patients with lipodermatosclerosis[238]. Allopurinol, a xanthine oxide inhibitor, is a free radical scavenger. It also suppresses the production of TNF-α and downregulates the expression of ICAM-1 and $P2X_7$ receptors on monocytes/macrophages[252]. ICAM-1 on macrophages attaches to T lymphocytes allowing antigen presentation and T cell activation. Thus allopurinol may suppress T cell activation and may suppress keratinocyte $P2X_7$ receptor expression and reduce keratinocyte cell death. $P2X_7$ receptors on macrophages and lymphocytes have cytotoxic functions at sites of inflammation[244]. $P2X_7$ receptors are also thought to be involved in the release of IL-1β[245], mitogenic stimulation of T lymphocytes[246], and the cytoplasmic communication between macrophages and lymphocytes[247]. Thus allopurinol may reduce the skin damage during the chronic inflammation seen in CVI. $P2X_7$ receptor levels on monocyte-macrophage lineage cells are increased in sarcoidosis, where they are associated with cytotoxicity, maturation and the release of IL-1β[248].

Epidermal mast cells are found in chronic skin inflammation with hyperproliferative epidermis and in chronic ulcers, where mast cell granules are found inside keratinocytes. The mast cell mediators histamine and heparin, and human mast cell lysate have an inhibitory effect on keratinocyte proliferation and

epithelial growth. It could be concluded that mast cells have an inhibitory effect on epidermal growth[253]. ATP induced histamine release from mast cells is mediated via $P2X_7$ receptors[114,254]. Inhibition of keratinocyte growth may be a result of the action of ATP on mast cells. Mast cells may also act directly on keratinocyte P2 receptors by releasing ATP and affecting keratinocyte growth[112].

CVI is associated with an increase in production of TGF-β1 from leucocytes[255]. This stimulates dermal fibroblasts to differentiate and secrete MMP-2. Increased MMP-2 activity results in degradation of epidermal basement membrane collagen type VI (a substrate for MMP-2) and subsequent loss of epidermal integrity[256]. Enhanced MMP-2 expression has been localised to the perivascular cuff sites, dermal fibroblasts, leucocytes and in basal layers of the epidermis[257]. $P2X_7$ receptor activation has been shown to enhance TGF-β1 mRNA expression[258]. As TGF-β1 and mRNA of MMP-2 are increased in CVI, an increase in $P2X_7$ receptor staining might therefore be predicted in the basal layer in CVI. However our results showed reduced $P2X_7$ receptor staining in the stratum corneum with no $P2X_7$ receptor staining in the basal layer in CVI.

Our results show that CVI skin is thinner than controls. If the thickness of skin decreases with CVI then over time this would lead to continuous thinning of the epidermis until it eventually breaks down, ulcerating. This would follow the clinical picture. In our study we have looked at pre-ulcerated states. Studies have shown that impaired epithelialisation of chronic ulcers is not caused by the lack of epidermal stem cells, inadequate proliferation, differentiation or apoptosis at the edge of wounds[243]. Failure of wound healing may reflect the distorted organisation of the wound bed caused by infection and impaired nutrient supply, altering keratinocyte migration at the ulcer edge[243]. Animal work has previously shown that activation of adenosine A2 receptors increase the rate of wound healing. A_{2A} and A_{2B} receptor stimulation contributes to enhanced fibroblast and endothelial cell migration[259]. Whether this is due to increased secretion of vascular endothelial growth factor or to inhibition of the secretion of cytokines or collagenase is not known. Our work has focused on the P2 receptors in non ulcerated skin, but the role

of A2 receptors on ulcerated skin may help further in our understanding of the overall function of purines in CVI skin changes.

In summary, increased expression of $P2Y_1$, $P2Y_2$ and $P2X_5$ receptors and decreased expression of $P2X_7$ receptors has been shown in CVI. Whether the thinner epidermis in CVI might be the resultant of increased keratinocyte proliferation via $P2Y_1$ and $P2Y_2$ receptors, reduced apoptosis via $P2X_7$ receptors and a dominant antiproliferative effect mediated via $P2X_5$ receptors, requires further investigation. Enhanced keratinocyte proliferation through targeting $P2Y_1$ and $P2Y_2$ receptors may increase epidermal thickening, protecting against breakdown and ulceration. Antagonising the actions mediated by $P2X_5$ receptors may reduce its antiproliferative effects resulting in a thicker epidermis. It is not clear whether these changes in purinoceptor expression found in CVI are of compensatory advantage or a secondary consequence.

Figure 3.1: H&E staining and $P2Y_1$ receptor immunostaining keratinocytes. H&E staining of control (a) and CVI (d) skin magnification, immunofluorescence shows staining on stratum control (b), with increased staining in basal and spinosal (SS Preabsorption of $P2Y_1$ receptors with its peptide immunofluorescence (c) and (f).

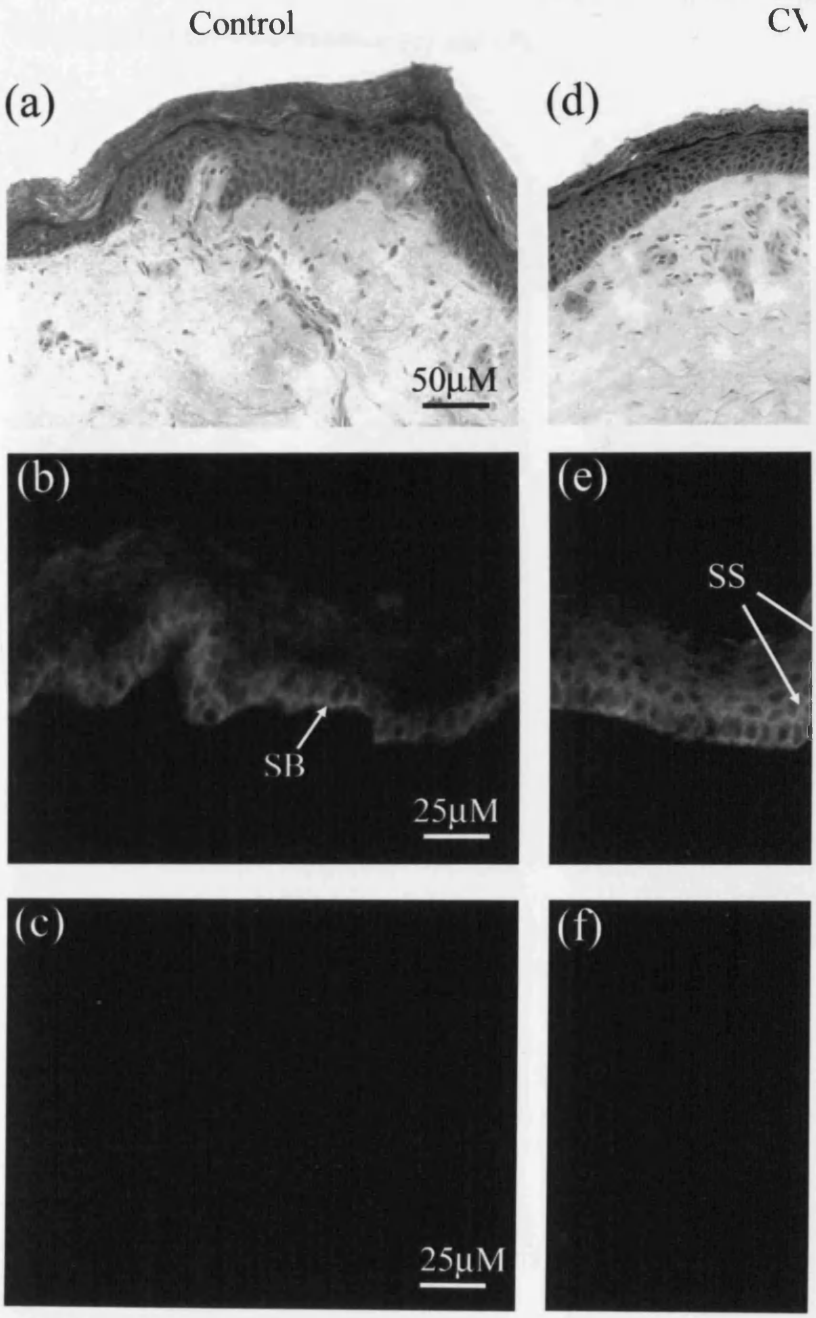


Figure 3.2: H&E staining and $P2Y_2$ receptor immunostaining of human epidermal keratinocytes. H&E staining of control (a) and CVI (d) skin sections. At higher magnification, immunofluorescence staining of $P2Y_2$ receptors is present on stratum basale (SB) and lower stratum spinosal (SS) layers in control skin (b). Immunostaining extends deeper into the spinosal (SS) layer and is of a greater intensity in CVI (e). Preabsorption of $P2Y_2$ receptors with its peptide shows minimal immunofluorescence (c) and (f).

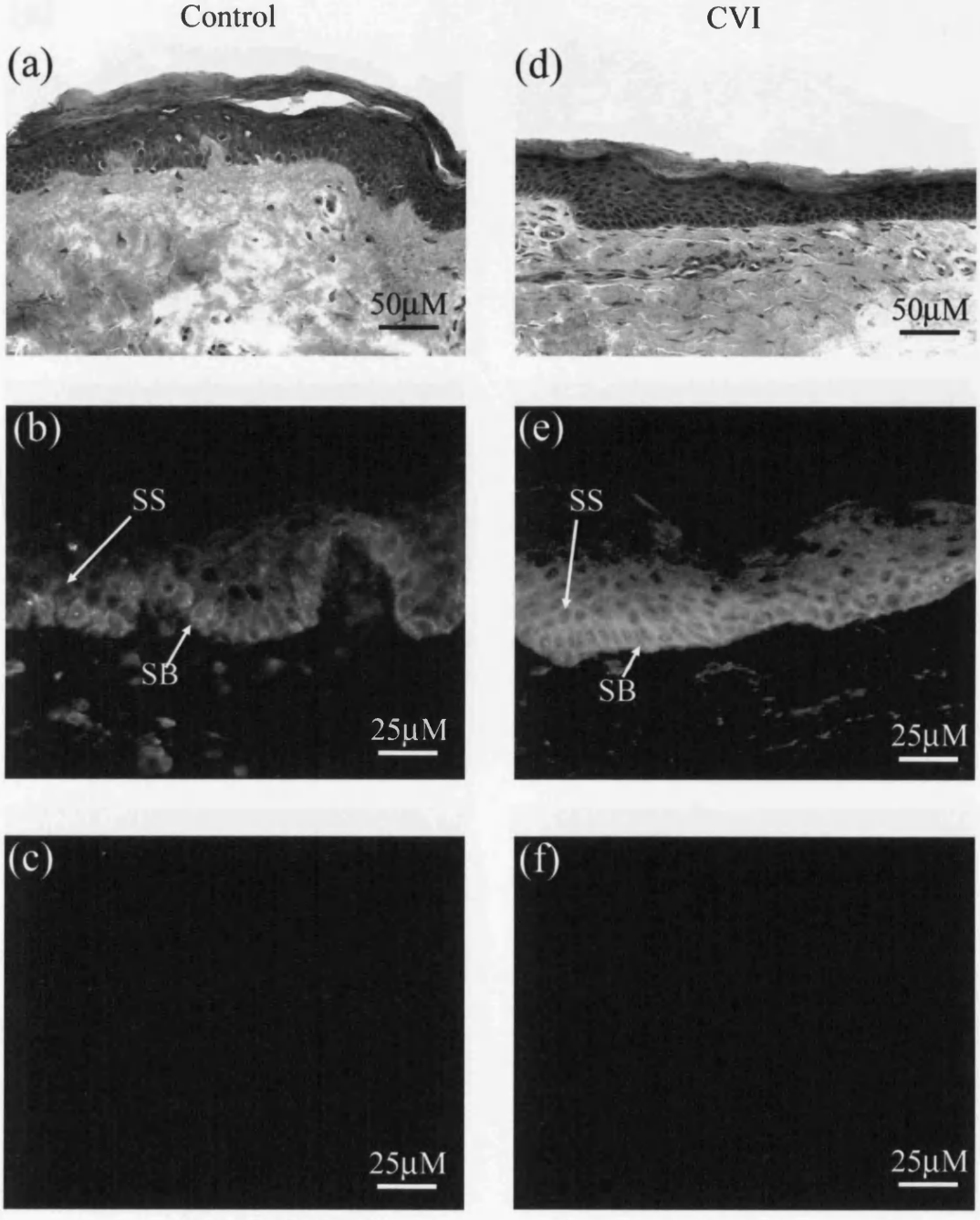


Figure 3.3: H&E staining and $P2X_5$ receptor immunostaining of human epidermal keratinocytes. H&E staining of control (a) and CVI (d) skin sections. At higher magnification, immunofluorescence staining of $P2X_5$ receptors is present on basale (SB), spinosal (SS) and stratum granulosum (SG) layers in control skin (b). Skin in CVI shows the same pattern of staining but of increased intensity (e). Preabsorption of $P2X_5$ receptors with its peptide shows minimal immunofluorescence (c) and (f).

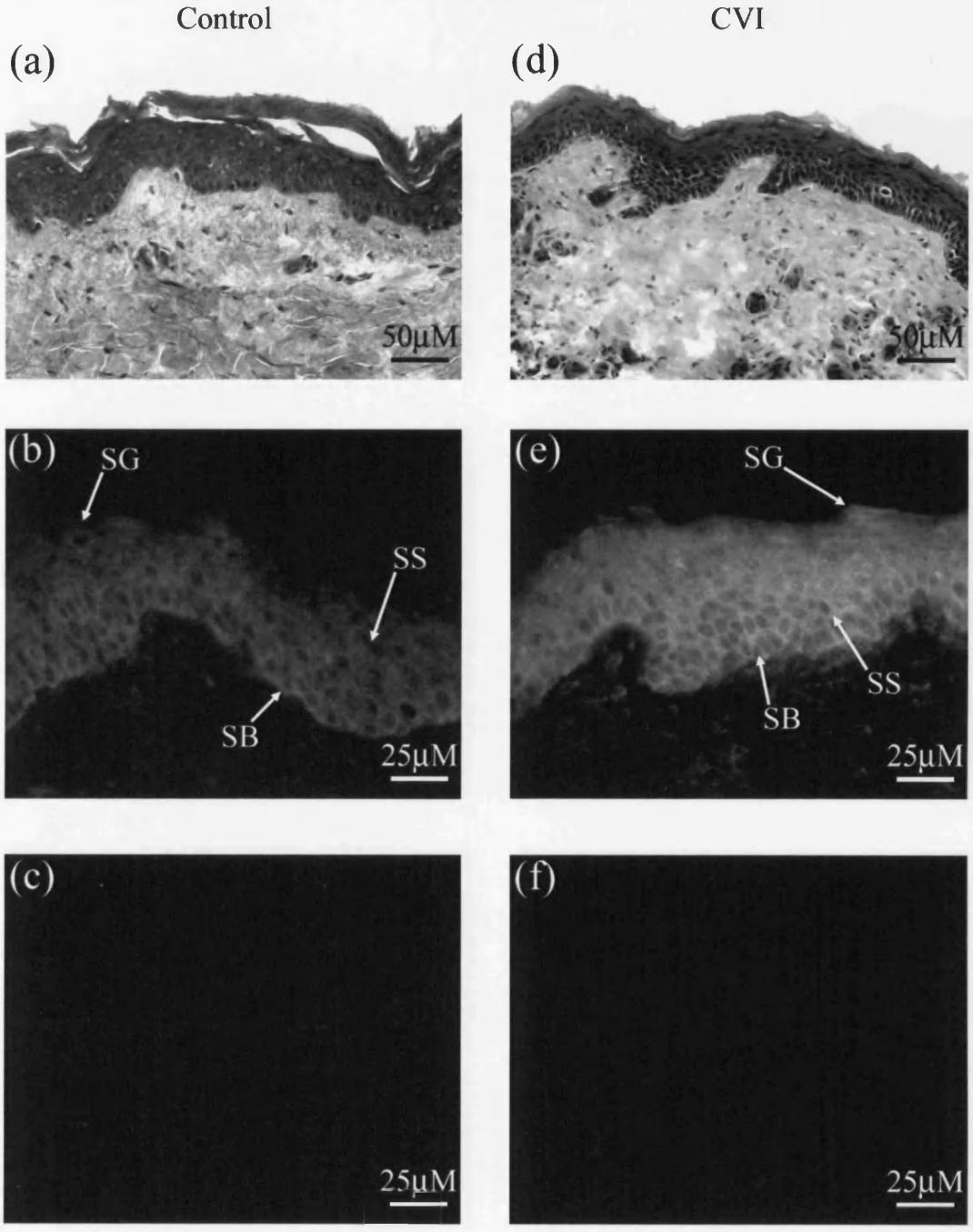


Figure 3.4: H&E staining and $P2X_7$ receptor immunostaining of human epidermal keratinocytes. H&E staining of control (a) and CVI (d) skin sections. At higher magnification, immunofluorescence staining of $P2X_7$ receptors is present on the stratum corneum (SC) in control skin (b), but staining is markedly reduced in CVI (e). Preabsorption of $P2X_7$ receptors with its peptide shows reduced immunofluorescence (c) and (f).

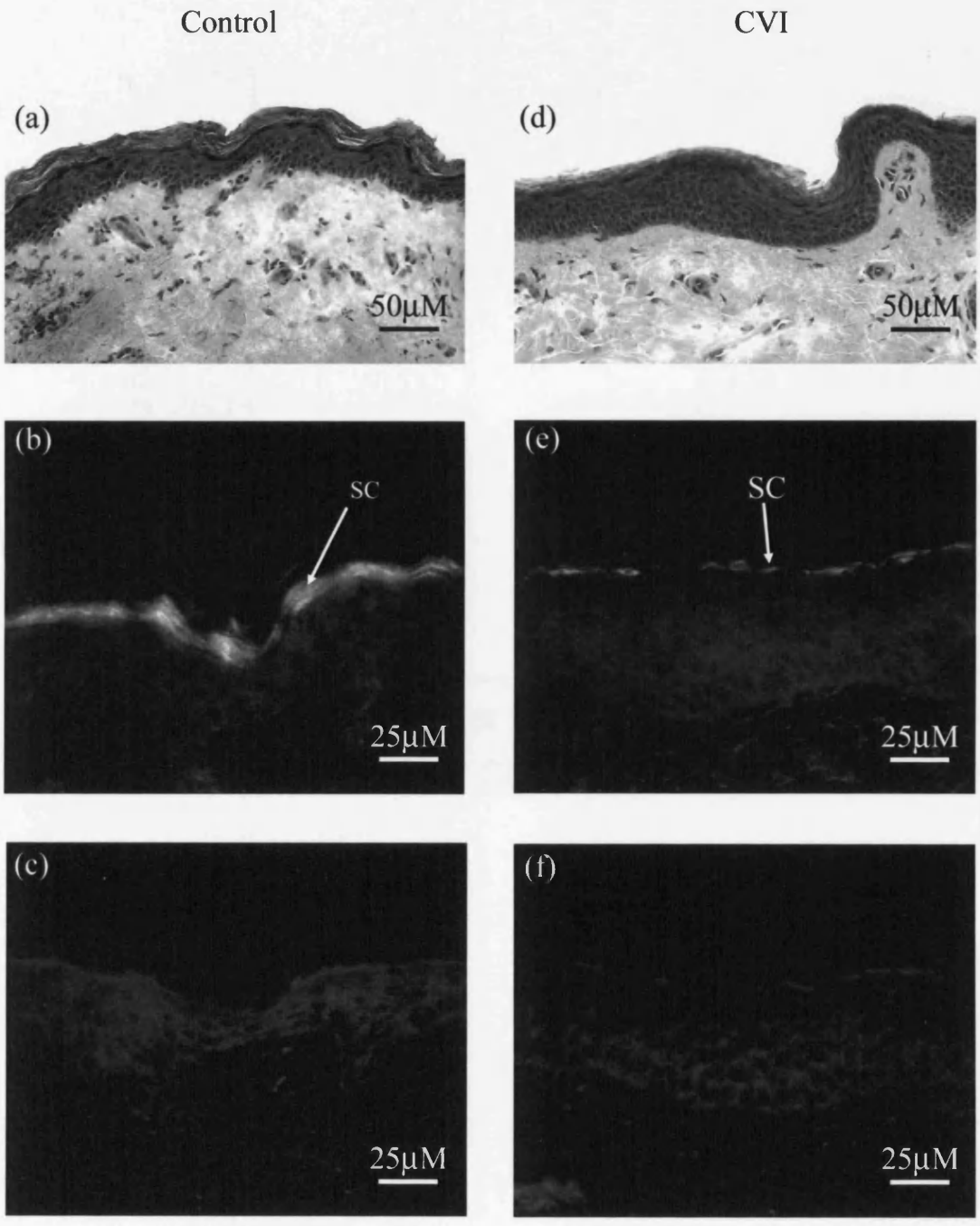
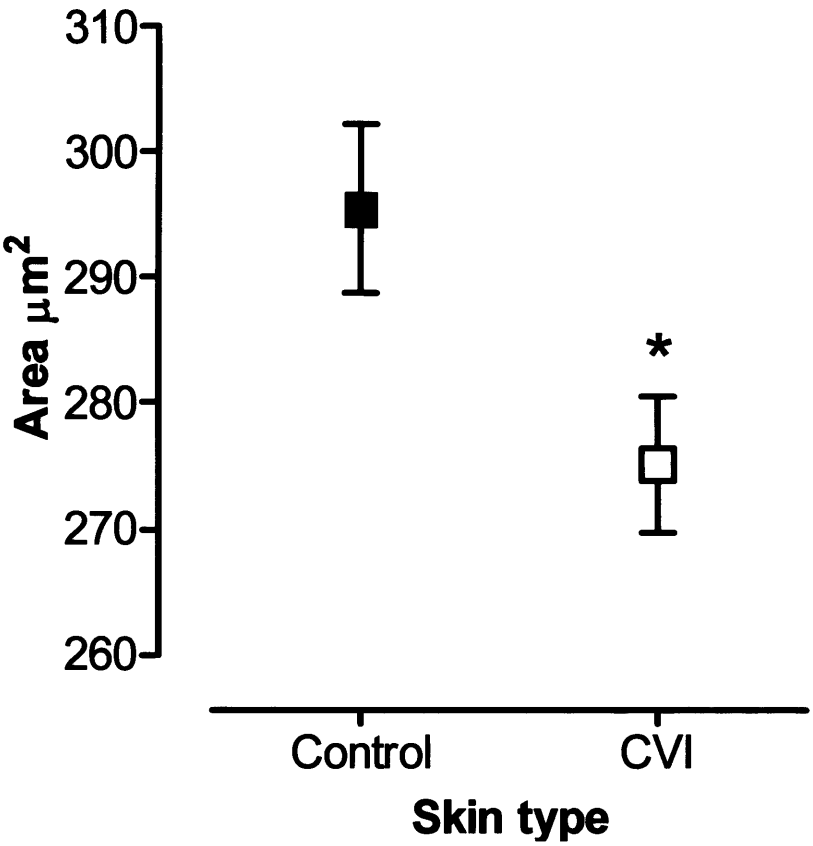


Figure 3.5: Bar chart representing epidermal area in low magnification images of control and CVI skin. Mean epidermal area (μm^2) ± standard error (n=5) of control (black square) and CVI (white square) skin are shown. The mean of the CVI skin is significantly thinner than control epidermis ($^*P=0.0313$).



Chapter 4

Purinergic Signalling In Penile Erection

Abstract

ATP and UDP are known to affect vascular tone via cell surface purine receptors, of which there are two families: P1 and P2. The activation of $P2Y_6$ receptors has been previously reported to cause vascular smooth muscle contraction. Our hypothesis was 'The $P2Y_6$ receptor plays a short term role in dilatations underlying penile erection'. Human cavernosal tissue was obtained from 23 patients undergoing gender reassignment surgery. Fluorescent immunohistochemistry and RT-PCR were used to determine the presence of $P2Y_6$ receptors in corpus cavernosal tissue. The effects of UDP, a $P2Y_6$ receptor agonist were assessed on precontracted cavernosal strips using organ baths pharmacology. Immunofluorescence and RT-PCR identified $P2Y_6$ receptors on cavernosal tissue. UDP induced a significant relaxation of precontracted cavernosal strips, which was significantly diminished by a $P2Y_6$ antagonist. We have identified the presence of $P2Y_6$ receptors on human cavernosal tissue, which when activated induces cavernosal smooth muscle cell relaxation. Purinergic modulation of human cavernosal smooth muscle cells via the $P2Y_6$ receptor subtype might play a physiological role in penile erection.

Introduction

Purines are known to mediate effects in smooth muscle and regulate blood vessel tone. In the penis modulation of smooth muscle function and blood flow regulates tumescence. Many studies have shown that the main neurotransmitter mediating relaxation of CSM is a non-adrenergic non-cholinergic neurotransmitter. Evidence exists to support the role of adenosine in the erectile process by inducing relaxant effects on CSM, increasing cavernosal blood flow and inducing tumescence[196-200]. Studies have shown ATP inducing relaxation in CSM, stimulating the erectile process[34,198,200,201]. At low CSM basal tension, ATP causes contraction, thus ATP may act via more than one pathway[203]. Possibilities include adenosine mediated effects following the breakdown of ATP, and direct effects of ATP on P2 receptors.

$P2Y_1$ and $P2Y_2$ receptors have been identified on hamster urethral smooth muscle and relaxation to ATP is thought to be $P2Y_1$ receptor mediated, leading to NO release from the endothelium[204,205,207]. P2X receptors induce contractions of CSM[205]. Lee et al demonstrated the presence of $P2X_1$ and $P2X_2$ in rat CSM[210].

Malmsjo et al found that stimulation of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors led to relaxation of the rat isolated mesenteric artery[96]. $P2Y_6$ receptor stimulation contracted the tissue. Chapter 2 demonstrates $P2Y_6$ receptor- mediated contraction of longitudinal SMC in the LSV. As CSM is akin to a modified vascular tissue, it is possible that the $P2Y_6$ receptor might have a similar bio-profile in the CSM as in the mesenteric artery. We have examined the role of the $P2Y_6$ receptor in CSM, to identify its possible role in detumescence. $P2Y_6$ receptor-mediated properties on CSM may identify targets in the treatment of impotence.

Methods

Tissue

Human cavernosal tissues were obtained (following ethical approval) from patients undergoing gender reassignment surgery at Charing Cross Hospital, London, UK.

Immunohistochemistry

Tissue was collected in Hanks balanced salt solution (HBSS from Gibco). Following dissection, cavernosal muscle was frozen in isopentane, precooled in liquid nitrogen. 10μm sections were cut on a cryostat (Reichert Jung CM1800), collected on gelatine-coated slides and air dried at room temperature. Slides were stored at -20^{0}C. Sections were fixed for 4 min in 4% formaldehyde in 0.1M PBS containing 0.2% picric acid. Sections were washed three times for 5 min each time with PBS. Sections were primarily blocked for 60 min in 10% NHS in 0.1M phosphate buffer, containing 0.05% merthiolate. Sections were then incubated overnight with the primary polyclonal anti-$P2Y_6$ antibody (obtained from Alomone Laboratories, Jerusalem, Israel) at concentration 1:200 in 10% NHS in PBS with 0.05% merthiolate. On the second day, sections were washed for 5 min three times in PBS and then stained with donkey anti-rabbit Cy3 (Jackson Immunoresearch Laboratories, USA), the secondary antibody, at 1:300 in PBS-merthiolate for 60 min. Sections were washed three times for 5 min before being mounted in Citifluor (Citifluor Ltd, London, UK). Control experiments were performed by separately omitting the primary and secondary antibodies, and by preabsorbing the primary antibody with its peptide. Preabsorption was carried out by adding the peptide at a ratio of 1:1 in 10% NHS in PBS with 0.05% merthiolate, leaving for 12 hours at 4°C, passing through a syringe filter (4mm with a 0.2μm PPmembrane) then centrifuged at 13,000rpms for 5 min using only the supernatant.

Haematoxylin and Eosin staining

H&E slides were prepared by fixing in 4% paraformaldehyde in PBS for 10 min. Sections were washed in distilled water and stained for 20 min in Ehrlichs Haematoxylin. Following washing in running tap water, slides were dipped in acid alcohol and washed again for 15 min. Sections were then stained in Eosin for 5 min, dipped in tap water, then washed for 1 min in 70% alcohol, 3 min 100% alcohol, another 3 min 100% alcohol, cleaned for 3 min in xylene, and finally another 5 min in xylene. Sections were mounted in eukitt.

Photography

Slides were photographed using a Zeiss Axioplan, high definition light microscope (Zeiss, Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA extraction: Equipment was decontaminated using an autoclave. Where this was not possible, equipment was decontaminated with RNaseZap (Ambion) following the suppliers instructions and placed in a laminar flow cabinet. Powder free gloves were worn throughout. Tissue was collected in RNAlater (Ambion) and transported to the laboratory. Unused tissue was frozen in liquid nitrogen and kept at -80°C. In an air extraction cupboard, tissue was chopped with scissors into small pieces in a glass beaker containing TRIzol Reagent (GibcoBRL), (1ml per 50mg tissue). Tissue was then homogenized at high speed for 30secs, left covered for 10min and homogenized again for 30secs. Separated out into small tubes, the solution was centrifuged at 12,000g for 10min at 4-8°C. The supernatant was pipetted out into clean tubes, chloroform added (0.2 ml /ml TRIzol) and shaken

well. Following centifugation, as before, the supernatant was pipetted out into clean tubes and isopropyl alcohol added (0.5ml/ ml Trizol). Solutions were left for 10min and centrifuged once more. The supernatant was discarded and the RNA pellet-like gel at the bottom was washed with 75% ethanol (1ml/ml TRIzol). The solution was centrifuged at 7,500g for 5min at 2-8°C. The supernatant was discarded, the RNA redissolved in 100µl distilled water and incubated for 10min at 55-60°C. Optical densitometry was used to calculate the volume of RNA present.

RNA amplification: To an RT-PCR bead (Amersham pharmacia biotech), first-strand primer, DEPC water and RNA were added totalling 49µl, following the manufacturer's instructions. Placed in an incubator, the sample was exposed to 42°C for 30mins followed by 95°C for 5min. After adding 1µl of the $P2Y_6$–specific primer, the tube was exposed to 40 cycles of 95°C for 30secs, 62°C for 1 min and 72°C for 1min. 10µl of loading buffer was added to the final solution.

Ethium bromide agarose gel was prepared by adding 4g of 2% agarose in 200mls of TAE Buffer, warming until dissolved, then adding 10µl of ethium bromide. When the gel was set, 10µl of RNA solution was placed in a well, alongside a DirectLoad wide-range DNA marker (Sigma), and 100V of 320mA was passed through for 1 hour. Ultraviolet images of the gel were taken using a multi-function camera (BIO-RAD Fluor-S™ Multilmager).

Pharmacology

Isolated human cavernosal tissues were cut into strips of approximately 2 x 5 mm length. The strips were mounted vertically in 10 ml organ baths containing Krebs solution. The Krebs solution (pH 7.2) had the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8. The tissue strips were maintained at 37±1 ^{0}C and bubbled with a mixture of 95% O_2 and 5% CO_2. An initial tension of 2 g was applied and the strips were allowed to equilibrate for 1 h before being challenged with KCl (124 mM),

which was repeated at the end of the experiment. Results were only accepted if both KCl responses varied in magnitude by less than 10%.

Initial experiments showed that UDP (a $P2Y_6$ receptor agonist) caused cavernosal relaxations. Subsequently, all tissues were precontracted with phenylephrine (PE) 10^{-4}M to quantify UDP-induced relaxations (10^{-3}M or 10^{-4}M, n=9 for each concentration). In some experiments, cibacron blue 3GA (a P2 receptor antagonist; 10^{-4}M, n=9) or L-NAME (a NO synthase inhibitor; 10^{-4}M, n=9) was added to the strips and left for 20 min prior to re-exposure to UDP 10^{-3}M. Control experiments were also performed examining the effect of distilled water on the UDP-induced relaxation using PE-precontracted strips.

PE, UDP, cibacron blue 3GA and KCl were supplied by Sigma Chemical Co. (Poole, UK).

Statistical Analysis:

Comparisons were made between UDP alone and following exposure to cibacron blue 3GA, L-NAME or distilled water using a one-way analysis of variance (ANOVA) followed by a *post hoc* test (Bonferroni's). A probability of $P<0.05$ was taken as significant. Results are presented as mean values +/- standard error of mean (SEM).

RT-PCR (controls)

Omitting the initial RNA during amplification acted as a control, with no band appearing on the gel (result not shown). Further control experiments that could have been performed include omitting the $P2Y_6$-specific primer during RNA amplification and repeating the experiment with a tissue known to contain the $P2Y_6$ receptor (positive control). RT-PCR was performed on CSM tissue from 4 patients (n=4).

Results

Immunohistochemistry

Subsequent tissue sections underwent one of three staining techniques: H&E staining identified the presence of muscle cells (Figure 4.1a), immunohistochemistry of the $P2Y_6$ receptor antibody showed positive staining on the muscle cells (Figure 4.1b), and immunohistochemistry following preabsorption with the $P2Y_6$ receptor antibody peptide showed reduced staining, the control (Figure 4.1c). This demonstrates the presence of $P2Y_6$ receptors on CSM

$P2Y_6$ receptor staining was seen on all 7 patients immunostained.

RT-PCR

RT-PCR demonstrated the presence of a band at 364 base pairs, which corresponds to the $P2Y_6$ receptor mRNA in cavernosal tissue (Figure 4.2). No other bands were seen during this preparation. The wide range DNA ladder is seen on the left side.

Pharmacology

UDP caused a significant and sustained relaxation of PE-precontracted tissue at both concentrations used (10^{-4}M, 4.9 +/- 0.8; 10^{-3}M, 15.7 +/- 2.8, both $p < 0.01$) when compared with controls (distilled water). The UDP-mediated relaxation (10^{-3}M) was significantly reduced by cibacron blue 3GA 10^{-4}M (8.2 +/- 1.5, $p < 0.05$). Whilst in contrast, L-NAME 10^{-4}M had no significant effect on UDP-mediated relaxations (10^{-3}M, 17.1 +/- 4.4) (Figure 4.3).

Ecto-enzymes (surface enzymes) may convert UDP to UTP (requiring the uptake of ATP), producing agonist actions of UTP on $P2Y_2$ and $P2Y_4$ receptors. Relaxations recorded may be a combination of UTP and UDP actions.

Discussion

Male erectile dysfunction affects 5% of men in their 40's, and increases in incidence with age. Causes include psychogenic, vasculogenic, neurological, endocrine (diabetes), traumatic and drug related. Strong evidence exists linking LUTS and ED[44]. $P2X_1$ receptor mediated detrusor muscle contraction is enhanced in LUTS in rabbits[260]. Purinergic signalling alterations affecting LUTS may also have effects on ED. Adenosine and ATP induce relaxation in CSM. Sensitivity is enhanced with diabetes and with increasing age[198,261]. This purinergic alteration could be a result of new nerve proliferation that release purines, upregulation of current purinergic receptors or enhanced purine receptor sensitivity that may occur during the diabetic and ageing changes of the CSM. Diabetes is also known to impair NO mediated CSM relaxation, leading to ED[262]. Purinergic signalling is known to be contributory in the proliferation of vascular smooth muscle and endothelial cells as well as cell death[103]. It is possible that the enhanced purinergic bioactivity in diabetes and ageing would lead to development or worsening of penile atherosclerosis in the long term. Therefore, although the purinergic enhancement might be compensatory initially, it could be detrimental to erectile function as time progresses in ageing and diabetic patients.

Using immunohistochemistry and RT-PCR techniques, the presence of mRNA for the $P2Y_6$ receptor and the receptor itself have been detected on CSM for the first time. Organ bath studies revealed that $P2Y_6$ receptor activation causes sustained relaxation of the CSM. Greater relaxation of the CSM was recorded with a more concentrated UDP solution. The significant reduction in relaxation in the presence of cibacron blue 3GA confirms a $P2Y_6$ receptor-mediated relaxation. This finding is in marked contrast to earlier studies on endothelium denuded rat mesenteric artery and human cerebral artery where vasoconstriction was reported[96,263]. This discrepancy implies that activation of the $P2Y_6$ receptor has a range of physiological functions with species and tissue variation.

P2Y receptor activation has been reported to induce endothelium-dependent relaxation of human CSM via NO production[205]. However we did not record a significant reduction in relaxation in the presence of the NO synthase inhibitor L-NAME. It is possible that there are two independent pathways involved in CSM relaxation: a $P2Y_6$ receptor-mediated pathway and a NO-mediated pathway. CSM stretching during tumescence may stimulate the release of ATP from endothelial cells which may lead to relaxation, as is seen in the bladder[264]. Studies showed that NO and ATP-sensitive potassium channels play a significant role in modulating human CSM relaxation, and therefore, erectile capacity[265,266]. Adenosine relaxes CSM via activation of the potassium ATP channels[261]. Both ATP and adenosine may therefore mediate relaxation either by NO production or via ATP-sensitive potassium channels. It must be remembered that ATP is hydrolysed by ectonucleotidases to adenosine. Both A_{2A} and A_{2B} have been demonstrated on CSM.

VIP is thought to play a role in the erectile process[267]. Chiang PH et al[196] demonstrated that the combination of adenosine and prostaglandin (PGE_1) is more effective than PGE_1 alone in promoting tumescence in man. This suggests that targeting purinergic pathway, possibly mediating the $P2Y_6$ receptor, may form a novel therapeutic option in the treatment of erectile dysfunction, in combination with other erectogens such as PDE_5 inhibitors and PGE_1. Modern drugs designed to increase arterial blood flow into the penis to promote tumescence may fail in practice. Reduced venous outflow is vital to the process. PGE_1 is able to induce a valid erection, but adenosine alone is unable to do so. Studies into this showed increasing concentrations of PGE_1 increased dorsal penile vein tone (reducing venous outflow) whilst adenosine showed no venous effect. However in NA precontracted venous strips, adenosine induced an almost complete relaxation (increasing venous outflow)[199]. Adenosine is thought to relax both arterial and venous vessels explaining why adenosine alone can not produce an erection.

Our findings reinforce the evidence that suggests involvement of the purinergic signalling system in the erectile process through the $P2Y_6$ receptor.

Figure 4.1: Immunohistochemisty of cavernosal smooth muscle. Three consecutive sections are shown; stained with H&E (a), immunofluorescent staining with a $P2Y_6$ receptor antibody (b) and immunofluorescent staining with a $P2Y_6$ receptor antibody following preabsorption with its peptide (control) (c).

Figure 4.1

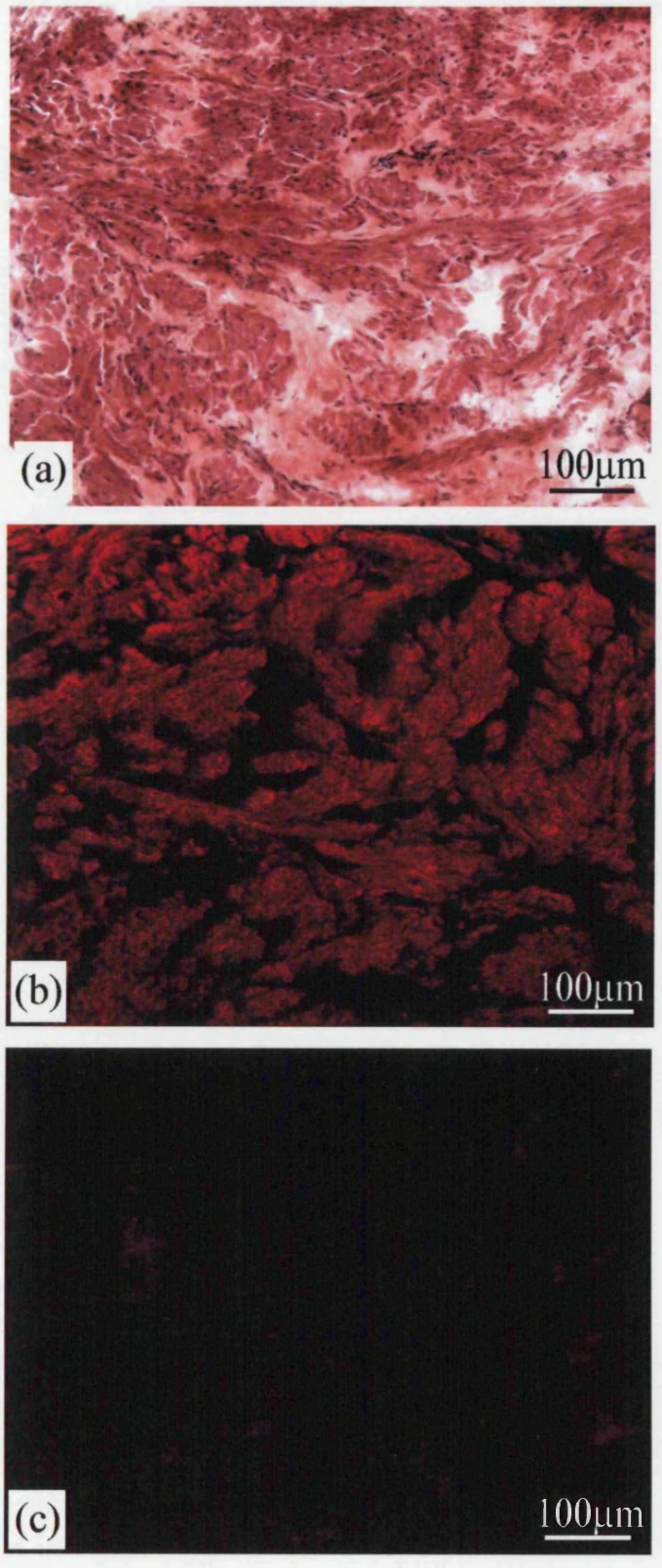


Figure 4.2: RT-PCR gel electrophoresis showing a DNA ladder (left side) and the presence of the 364 base pair mRNA of the $P2Y_6$ receptor in cavernosal muscle (right).

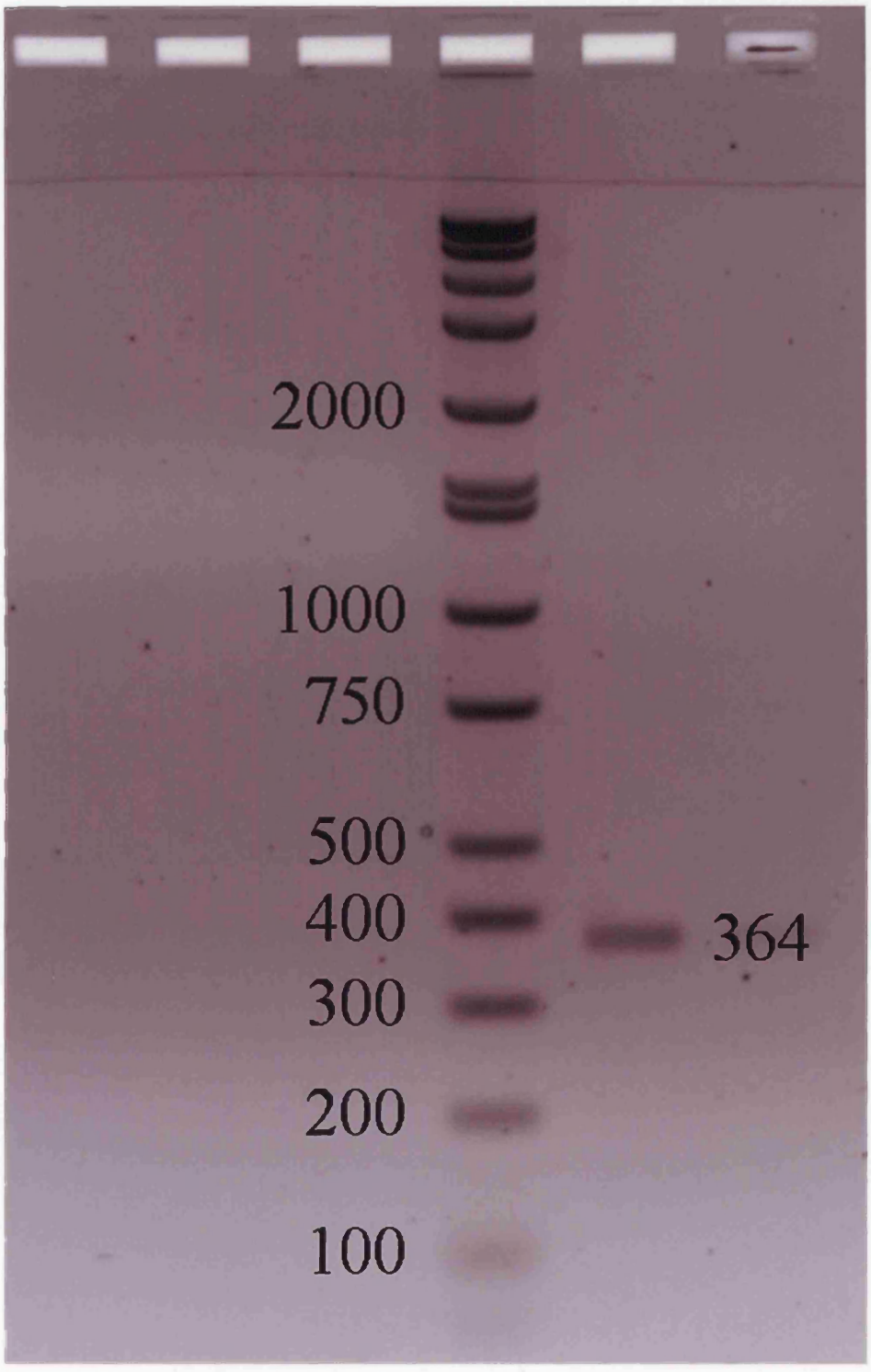
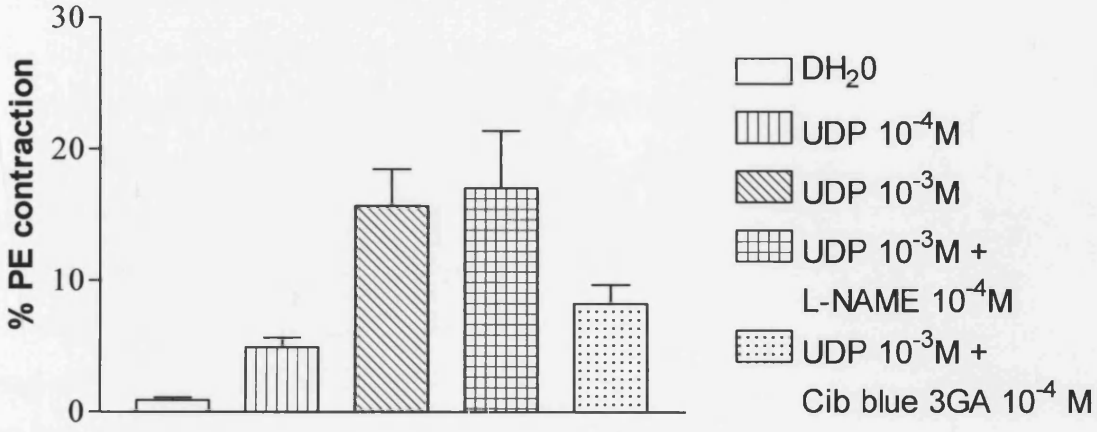


Figure 4.3: Bar chart representing organ bath pharmacology results. Cavernosal muscle relaxation is expressed as a percentage of the overall contraction induced by 10^{-4}M phenylephrine. Relaxations were measured to the following agents with standard error of the mean shown: DH_20 (distilled water), UDP (uridine 5'diphosphate) at 10^{-4} and 10^{-3} M concentrations, UDP 10^{-3}M and L-NAME 10^{-4}M (a nitric oxide synthase inhibitor), and UDP 10^{-3}M and Cib blue 3GA 10^{-4}M (Cibacron blue 3GA is a $P2Y_6$ antagonist).



Chapter 5

General Discussion

The role of purines in CVI has been explored in chapters 2 and 3 of this thesis. Purine receptor related changes have been identified between control and CVI tissues suggesting that purine-mediated actions are affected during CVI. This is the first time an association between purines and CVI has been demonstrated and opens up potential therapeutic pathways. Chapter 4 has identified relaxant properties of the $P2Y_6$ receptor on CSM. This is a new discovery and may help in our understanding of tumescence and subsequent clinical management of ED.

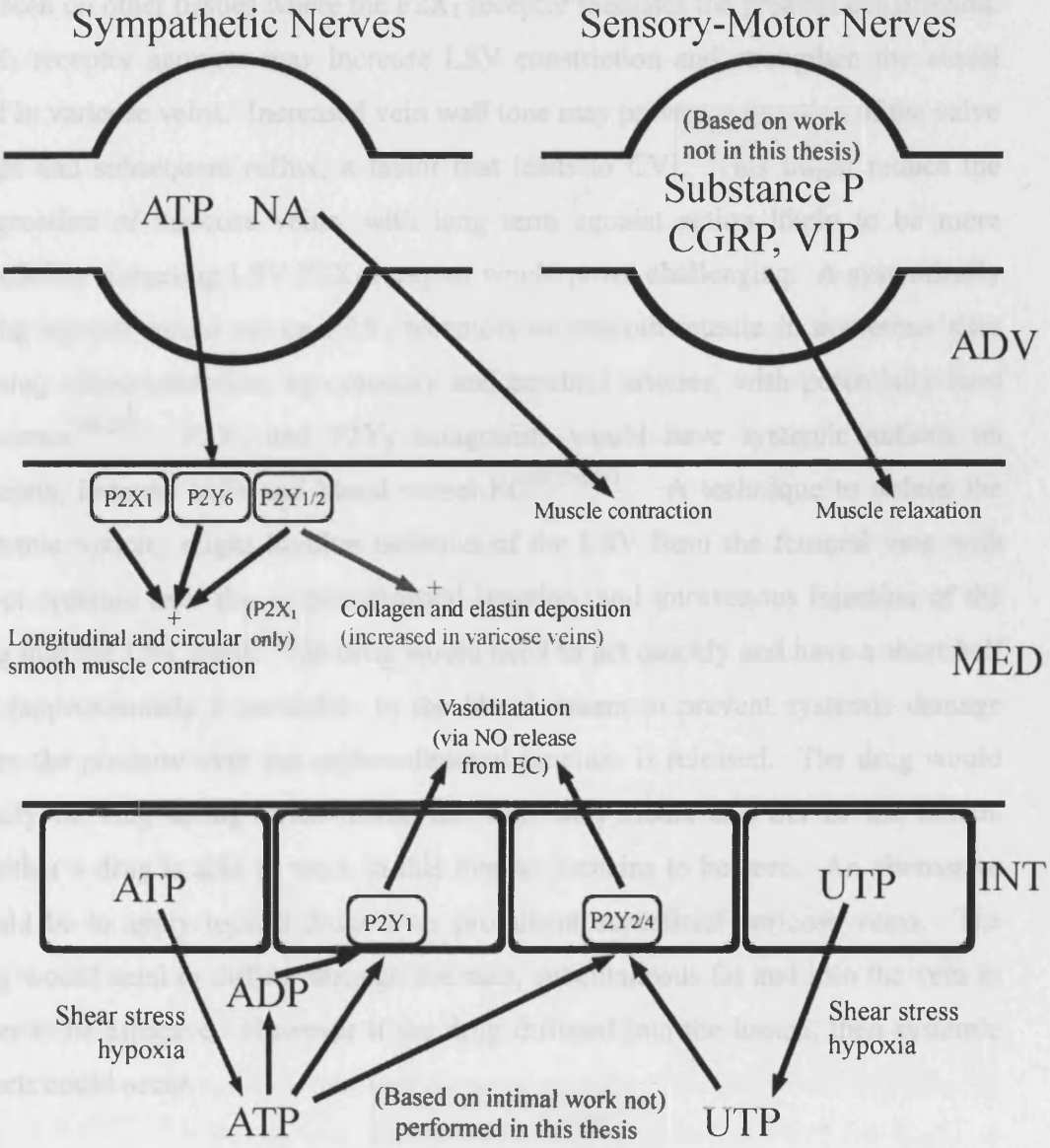
Purine receptors on varicose veins

Many studies exist on circular smooth muscle contraction of the LSV. This is the first study to contract longitudinal strips of LSV. Longitudinal fibres exist in both the intima and the adventitia of the LSV and I have aimed to contract these using the longitudinal axis of the vein, though it is impossible to separate them from the circular fibres. Circular muscle fibres that spiral along the long axis of the vein may account for contractions along the long axis. Both circular and longitudinal muscle contracted to NA and a $P2X_1$ receptor agonist, with reduced contractions seen in the varicose vein. Stimulation of $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors contracted longitudinal fibres only, with reduced contractions seen in varicose veins. Immunostaining of PX_1 receptor decreased and of $P2Y_1$ and $P2Y_2$ receptors increased in varicose veins, whilst that of $P2Y_4$ and $P2Y_6$ receptors remained the same. This summary of findings addresses the first part of the first hypothesis (purinoceptor changes occur between control and varicose LSV); in that purinoceptor variation does occur between the two tissues. The second part of the hypothesis (these changes are related to functional properties of the smooth muscle, either causal or consequential to varicose veins) refers to the EM findings. Here structural differences were identified along with synthetic phenotyped SMC in the varicose vein. The immunohistochemical findings of reduced $P2X_1$, increased $P2Y_1$ and $P2Y_2$ receptors in varicose tissue supports a purine receptor-mediated

phenotype change of the SMC and thus altered function. The first hypothesis has therefore been answered and the findings can be summarized in Fig 5.1

Whilst the findings describe a phenotype change in the varicose tissue, it is unclear whether this causes the varicose changes to occur, or whether this is a result of the varicose change and is a compensatory effect. It is still uncertain whether the aetiology of varicose veins is a defect within the muscle wall, such as an altered SMC phenotype, or due to refluxing valves. A refluxing valve would increase venous pressure and may stimulate structural changes.

Fig 5.1 Diagram representing the role of purinoceptors in the LSV based on findings in this research. ATP is released at the neuroeffector junction and acts on $P2X_1$, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors mediating vasoconstriction. In varicose veins, $P2Y_1$ and $P2Y_2$ receptor mediated actions include an increase in collagen and elastin deposition in the media. Shear stress stimulates ATP and UTP release from the endothelial cells which promotes vasodilatation via $P2Y_1$ and $P2Y_2$ receptors respectively.



Targeting purine receptors on the LSV may have a role in preventing varicose changes occurring. Competitive antagonists to $P2Y_1$ and $P2Y_2$ receptors may inhibit synthetically phenotyped SMC, reducing collagen deposition and inhibiting structural varicose changes occurring. As varicose changes occur over time, antagonistic action would need to either be long lasting or regularly administered. However $P2Y_1$ and $P2Y_2$ receptor antagonists would also reduce their contractile actions, as noted in the varicose tissue. The $P2X_1$ receptor mediated the greatest constriction out of the purinoceptors studied. Similar findings are seen on other tissues where the $P2X_1$ receptor mediates the greatest constriction. $P2X_1$ receptor agonists may increase LSV constriction and strengthen the vessel wall in varicose veins. Increased vein wall tone may prevent separation of the valve cusps and subsequent reflux, a factor that leads to CVI. This might reduce the progression of varicose veins, with long term agonist action likely to be more beneficial. Targeting LSV $P2X_1$ receptor would prove challenging. A systemically acting agonist would act on $P2X_1$ receptors on smooth muscle in numerous sites causing vasoconstriction, eg coronary and cerebral arteries, with potentially fatal outcomes[268,269]. $P2Y_1$ and $P2Y_2$ antagonists would have systemic actions on platelets, immune cells and blood vessel EC[90,270,271]. A technique to reduce the systemic toxicity might involve isolation of the LSV from the femoral vein with direct pressure over the saphenofemoral junction, and intravenous injection of the drug into the LSV itself. The drug would need to act quickly and have a short half life (approximately 3 seconds) in the blood stream to prevent systemic damage when the pressure over the saphenofemoral junction is released. The drug would ideally be long acting when inside the vein wall media and out of the lumen. Whether a drug is able to work in this manner remains to be seen. An alternative would be to apply topical drugs over prominent superficial varicose veins. The drug would need to diffuse through the skin, subcutaneous fat and into the vein in order to be effective. However if the drug diffused into the lumen, then systemic effects could occur.

The LSV has important uses in bypass surgery. Vein grafts have limited lifespans and their architecture changes when sited within the higher pressured arterial system. Intimal hyperplasia has been reported along with migration and proliferation of de-differentiated SMC[185]. ATP is released in large amounts from injured cells[103]. Atherosclerosis develops following EC injury; hence it is thought that ATP is released during the initial stages of atherosclerosis. ATP and adenosine have potent actions in SMC and EC growth, migration, proliferation and death[103]. Vascular EC are exposed to blood flow variation which plays a major role in the development of atherosclerosis. The shear stress that occurs during blood flow changes leads to ATP and UTP release from EC[88]. These purines may mediate the balance between proliferation and apoptosis during the development of atherosclerosis. $P2X_4$ and $P2Y_2$ receptors on EC are thought to mediate proliferation during atherosclerosis of the LSV when used as an arterial graft[92]. If $P2Y_1$ and $P2Y_2$ receptors are upregulated and $P2X_1$ receptor downregulated in the elevated pressure system seen in varicose veins and CVI, as our results suggest, then similar receptor upregulation may explain SMC proliferation seen in the higher pressure system during atherosclerosis. Taking our findings further, $P2Y_1$ and $P2Y_2$ antagonists may reduce the development of atherosclerosis. This would have huge clinical implications, as atherosclerosis is an underlying factor in many common conditions including myocardial infarctions, angina, cerebrovascular events and peripheral vascular disease. However it should be noted that no functioning EC where detected on the LSV tissue samples used in chapter 2 and hence were not studied. EC function is essential to atherosclerosis, where damage to their integrity initiates pathways leading to atherosclerosis.

Our findings excluded purine-mediated actions in EC, however interaction between EC and SMC does exist. In the saphenous vein, proven endothelium-mediated vasodilators include 5HT, ACh, Substance P, VIP and bradykinin[272-274]. ATP is thought to mediate vasodilatation via EC production of NO, endothelium-derived hyperpolarizing factor and prostanoids, via $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors, but this has not been demonstrated on the saphenous vein endothelium to date[61,61,89,89,275,276]. Purine mediated actions on saphenous vein EC are likely to

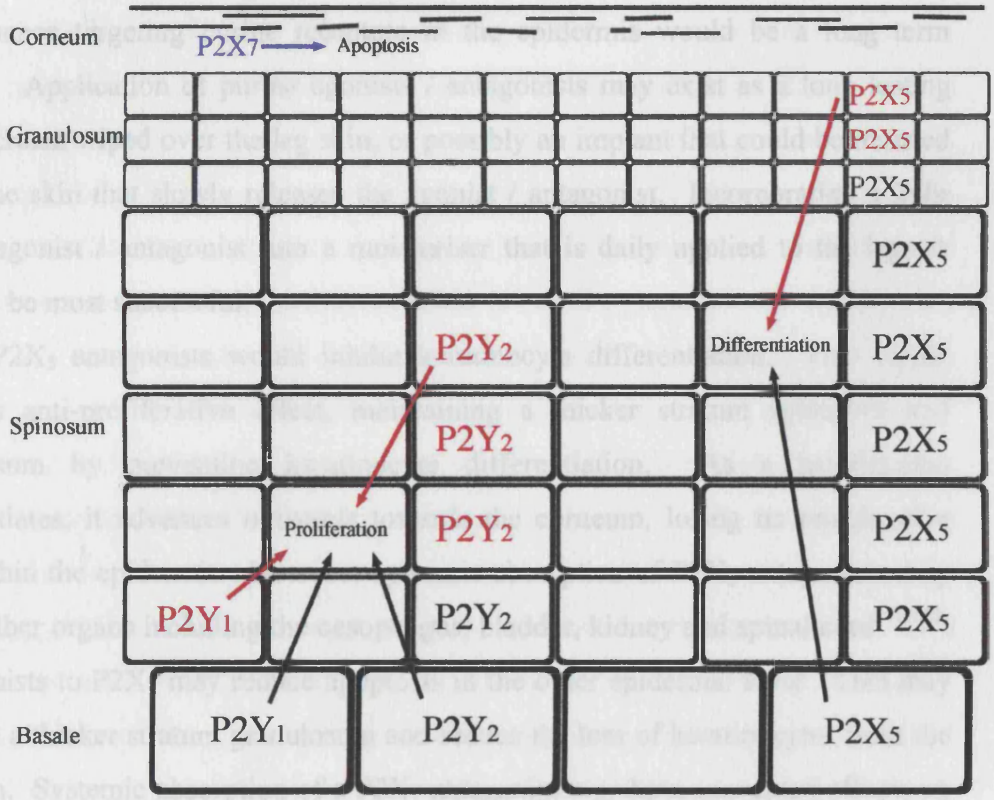
result in vasodilatation. Reducing vasodilatation increases vein wall tone and may prevent varicose changes occurring. Conversely a reduced vasodilatation would reduce the blood flow out of the leg possibly leading to a greater venous pressure and thus promoting CVI and varicose vein development. Whether EC function is affected in varicose veins is unknown. This is an area that has not been investigated in this study. Obtaining healthy intact endothelial cells has proved difficult. The vein is handled during its surgical preparation and this is likely to damage the delicate EC. Greater exposure of the vein, with a larger incision, to reduce its handling during surgery may be challenged by an ethical committee. Other sources of LSV could be from patients undergoing amputation for trauma or ischaemia, however proximal LSV would not be obtained. In these scenarios handling of the vein could be avoided during the amputation surgery. The LSV in an ischaemic leg would not be an ideal control as the venous blood would contain lactic acid and other potentially harmful chemicals that may directly damage the EC. Using animal (eg rat) LSV may be an alternative model. Creating a CVI environment may be obtained by narrowing the diameter of the proximal LSV with a ligature thus reducing blood flow through its lumen, elevating the pressure proximal to the ligature, and possibly resulting in valve reflux. However a CVI animal model may compensate the greater pressure in the LSV by shunting more blood into the deep veins. This model works on the theory that varicose veins develop from refluxing valves leading to elevated pressure and vein dilatation. If the main cause of varicose veins is with the SMC function, then this model will be ineffective.

Whilst varicose veins are common, their severity varies greatly. In some patients, varicose veins continue to develop and progress, leading to skin changes of CVI, whilst in others, varicose changes occur and remain stable with no deterioration. Whether its severity is due to the degree of elevated venous pressure, the function of the valves or due to changes occurring within the muscle wall itself is unclear. A combination of these factors may control the extent of the varicose changes.

Purine receptors on CVI epidermis

Many of the earlier studies of purines focused on their short term effects that occur in neurotransmission and secretion. Purine mediated trophic functions in the epidermis are a recent discovery[277]. This is the first study to date where immunostaining has demonstrated purine receptors in CVI epidermis and compared the findings to control epidermis. Chapter 3's findings of increased $P2Y_1$, $P2Y_2$ and $P2X_5$ receptor staining and reduced $P2X_7$ receptor staining in the CVI skin represents altered expression of these purine receptors. An alteration of their expression is likely to reflect changes in receptor activity and therefore receptor-mediated effects. To an extent the second hypothesis (purine-mediated effects on keratinocytes change in CVI skin) has been addressed, although the precise effect has not been demonstrated.

Fig 5.2 Diagram representing purine receptor changes occurring in CVI epidermis based on findings in this research. Receptor staining on control is shown in black. Increased intensity of receptor staining in CVI shown in red. Corneal staining of $P2X_7$ (blue) stains positive in control, but weakly stains in CVI skin.



The management of varicose veins aims to prevent the development of CVI. In the presence of venous ulcers, varicose vein surgery still has a role, alongside compression bandaging, skin grafts and numerous dressings including vacuum pump therapy[278]. Alteration of the purine mediated trophic effects in keratinocyte proliferation, differentiation and death may inhibit the development of CVI epidermal changes and promote venous ulcer healing. Potential advances in both ulcer prevention and ulcer healing may be obtained. CVI progresses over many years, hence targeting purine receptors in the epidermis would be a long term therapy. Application of purine agonists / antagonists may exist as a long lasting topical cream wiped over the leg skin, or possibly an implant that could be inserted under the skin that slowly releases the agonist / antagonist. Incorporating a 24hr lasting agonist / antagonist into a moisturiser that is daily applied to the legs is likely to be most successful.

$P2X_5$ antagonists would inhibit keratinocyte differentiation. This would have an anti-proliferative effect, maintaining a thicker stratum spinosum and granulosum by preventing keratinocyte differentiation. As a keratinocyte differentiates, it advances outwards towards the corneum, losing its proliferative role within the epidermis. However systemic absorption of $P2X_5$ antagonists may target other organs including the oesophagus, bladder, kidney and spinal cord[279-282]. Antagonists to $P2X_7$ may reduce apoptosis in the outer epidermal layer. This may result in a thicker stratum granulosum and reduce the loss of keratinocytes from the cornuem. Systemic absorption of a $P2X_7$ antagonist may have unwanted effects on the pancreas, lung and immune system[116,117,283]. $P2Y_1$ and $P2Y_2$ agonists would promote keratinocyte proliferation. In chapter 3 immunostaining of both receptors was increased suggesting that proliferation was already increased in CVI. Further agonist action on these receptors would promote epidermal thickening. The increased activity of both receptors in CVI is proposed to be a compensatory mechanism preventing epidermal loss. It should be noted that increased keratinocyte proliferation increases the risk of DNA mutations and has neoplastic potential. A side effect of $P2Y_1$ and $P2Y_2$ receptor agonists may be an increased risk of squamous and basal cell carcinomas.

Chapter 3 demonstrates the variation of immunostaining different purine receptors between control and CVI skin. Simultaneous staining of these sections with trophic markers, as described by Greig et al[190], would confirm the trophic roles of these receptors, rather than assuming they have identical functions seen in previous studies. Double labeling of $P2Y_1$ receptors with Ki-67 and $P2Y_2$ receptors with proliferating cell nuclear antigen (PCNA) would demonstrate that the increase in staining of these receptors on their respective epidermal layers coincided with increased cellular proliferation. Double staining of $P2X_5$ receptors with cytokeratin K10 or involucrin would strengthen our theory that the increase in its staining in CVI reflects increased early and late keratinocyte differentiation. In the same manner, a reduced $P2X_7$ receptor staining along with colocalised staining with caspase-3 or TdT-mediated dUTP nick end labeling strengthens the case for reduced apoptosis in the stratum corneum in CVI. A mismatch between receptor staining and trophic marker would suggest that variations in the purine staining intensity are due to new unidentified functions of the receptors.

Skin sections from classes CEAP 4a and 4b were used in the study. Comparing two different CEAP classes along with a control might reveal a trend in the keratinocyte function as the skin deteriorates with progression of CVI. Comparing classes 4a with 4b would be an appropriate comparison, as skin changes would be present and the epidermis would still be intact (no active ulceration present). However due to the limited sample size, comparisons between the CEAP classes were not made.

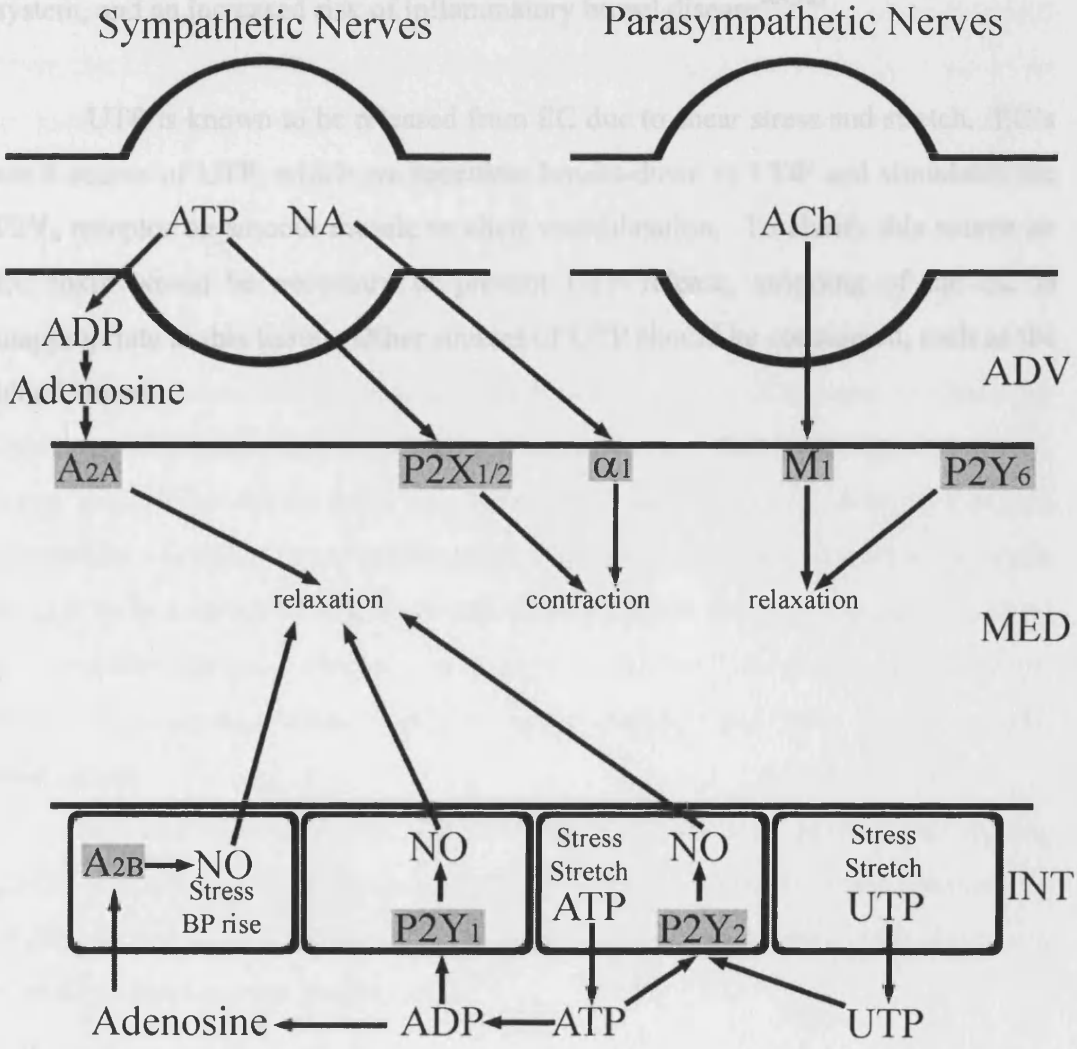
The findings in chapter 2 suggest an association between LSV SMC phenotype, purinoceptor expression and changes occurring in varicose veins. Chapter 3 describes an association between skin keratinocyte function, purinoceptor expression and changes occurring with CVI. These associations are observational only and are not definitive proof. Patient group differences and experimental limitations both weaken the strength of these associations. Further work is needed to fully validate these purinoceptor hypotheses.

Purine receptors on cavernosal smooth muscle

Immunohistochemistry and PT-PCR have demonstrated the presence of the $P2Y_6$ receptor in penile CSM, and organ pharmacology has shown its relaxant properties are NO independent. The hypothesis 'The $P2Y_6$ receptor plays a short term role in dilatations underlying penile erection' has been addressed as $P2Y_6$ has been shown to relax CSM and this is thought to induce tumescence.

The tumescence process may occur due to a positive feedback mechanism until a limiting factor is reached. Nerve stimulation may initiate the process with the release of ATP from sympathetic nerves and subsequent adenosine production, or with ACh, released from the parasympathetic nerves. However an increase in blood pressure or pulse rate may increase penile blood flow resulting in endothelial cell production of ATP, UTP and NO. NO production leads to relaxation. ATP and its breakdown products stimulate A_{2A} and A_{2B} receptors resulting in relaxation. Relaxation and a subsequent increase in arterial blood flow may promote further EC production of NO, ATP and UTP producing a positive feedback mechanism. The limiting factor in this process is unknown. An elevated arterial flow will compromise the venous outflow and eventually the penile pressure will prevent further arterial inflow. How the process is reversed in unknown. A drop in blood pressure may be the initiating factor, reducing EC production of NO and ATP. Increased NA production from sympathetic nerves or increased $P2X_1$ and $P2X_2$ CSM receptor activity is another possible mechanism as both lead to muscle contraction reducing arterial blood flow. $P2Y_6$ receptors in the media have been shown to mediate relaxation; however the source of its agonist is unknown.

Fig 5.3 Diagram representing purine receptor activity on cavernosal smooth muscle. Perivascular nerves in the adventitia release ATP and NA as cotransmitters from sympathetic nerves which result in CSM contraction mediated by $P2X_1$ and $P2X_2$ purinoceptors and α_1 receptors respectively. ATP is broken down to adenosine which stimulates A_{2A} receptors in the media resulting in muscle relaxation. ACh released from parasympathetic nerves stimulates M1 receptors resulting in relaxation. $P2Y_6$ receptor stimulation leads to relaxation. In EC, stress and stretch releases UTP and ATP which stimulate $P2Y_2$ receptors leading to NO mediated relaxation. In the lumen, ATP is broken down to ADP and adenosine which act on $P2Y_1$ and A_{2B} receptors respectively resulting in relaxation.



$P2Y_6$ agonists should mediate CSM relaxation and encourage tumescence. Excessive relaxation would be painful, impair venous outflow in the penis and result in structural damage, possibly preventing future erections. Impaired venous outflow may result in ischaemia and its consequences. Agonist effects would ideally last for approximately 30 mins, allowing time for intercourse and reducing the discomfort of prolonged tumescence. Agonists applied locally eg intracavernosal injections, may treat erectile dysfunction whether the cause is traumatic, diabetes, psychological or neurological. The benefit of a reduced venous outflow means there will be less release of the agonist into the systemic circulation and thus fewer complications. Systemic complications may include excess human nucleotide peptide induced IL-8 release and a subsequent overactive immune system, and an increased risk of inflammatory bowel disease[284,285].

UTP is known to be released from EC due to shear stress and stretch. EC's are a source of UTP, which we speculate breaks-down to UDP and stimulates the $P2Y_6$ receptor on smooth muscle to elicit vasodilatation. To clarify this source an EC toxin would be necessary to prevent UTP release, stripping of the EC is inappropriate in this tissue. Other sources of UTP should be considered, such as the blood stream.

Difficulties encountered

Stripping and harvesting the LSV inevitably involves instrument handling of the tissue. Obtaining skin biopsies from patients with CVI proved harder than anticipated. Patients were often reluctant to have an ellipse of skin excised as there was the possibility of a slightly greater scar. Although the operation is not performed for cosmetic reasons, an undeclared cosmetic improvement is often anticipated by the patient. Our biopsies were taken from the incision made below the knee. Samples were only taken where this occurred at sites of visible skin changes (CEAP 4a and 4b). Hence useful samples were only obtained when an incision was made over an appropriately affected skin area in consented patients. The skin incision was itself dependent on the pathway the stripper took as it passed down the LSV during the surgical procedure. This would often not be made in an affected skin area, or might be above or at knee level. These reasons explain the limited number of samples in the epidermis study. A larger patient number would make our results more convincing and enable us to compare different CEAP classes. This could be obtained by continuing the research for a longer period of time or by recruiting patients at other hospitals.

Using human tissue samples is complex as there will always be variability between individuals. Whilst known variables have been discussed (eg sex and age), many more exist which have not been mentioned including patients diet and occupation. Whether these variables are relevant is unknown. Varicose veins are thought to be increased in those who spend much of the day standing and immobile. It is possible that our varicose vein group consisted of 2 subdivisions of patients, those whose varicose veins are due to their occupation and those who have SMC weaknesses.

Control LSV and skin tissues were obtained from patients undergoing CABG surgery. Naturally these patients are on a long list of cardiac medications which patients with CVI were not taking. Interaction between these drugs and purine receptors can not be excluded.

A lack of visible varicose veins and a hand held doppler were used to exclude reflux and varicose veins in control patients. Veins that were slightly varicosed with early vein wall structural changes may not have been obvious to the naked eye. If no refluxing valves were detectable, then the vein would have been incorrectly labelled as a control vein.

Human penile tissue is a difficult tissue to obtain for research. With gender reassignment surgery on the increase, its availability is greater. When sufficient patient numbers are present to create a control and a disease group eg diabetes, then tumescence can be further evaluated in these diseases. At present many variables exist within the relatively small gender reassignment population.

Conclusion

For many years varicose veins and CVI have troubled patients and doctors. Despite new surgical techniques and conservative managements, they continue to be a financial burden on our underfunded National Health Service. For the first time the involvement of purines in the development of these conditions has been explored. The results show changes in purine receptor activity and further our understanding of these conditions. The identification of the $P2Y_6$ receptor in CSM provides more information into our understanding of the tumescence process. New targets for treatment have been highlighted through this research which should further encourage work into developing therapies to modify purine target activity.

Publications arising from this thesis

'Purinergic Signalling is altered in Varicose Veins and Reflects Changes in Purinoceptor Expression'.
Metcalfe MJ, Burnstock G, Baker DM
British Journal of Surgery 2005; 92 (4):503 (Abstract)

'Purinoceptor Expression on Keratinocytes Reflects their Function on the Epidermis during Chronic Venous Insufficiency'
Metcalfe MJ, Baker DM, Burnstock G
Archives of Dermatological Research 2006;298(6):301-307

'Alterations in Purinoceptor Expression in Human Long Saphenous Vein during Varicose Disease'
Metcalfe MJ; Baker DM; Turmaine M; Burnstock G
European Journal of Vascular and Endovascular Surgery – in press

'Purinergic P2Y6 Receptors Modulate Corpus Cavernosal Relaxation'.
David HW Lau, Matthew J Metcalfe, Daryll M Baker, Robert J Morgan, Faiz H Mumtaz, Dimitri P Mikhailidis, Cecil S Thompson.
Journal of Urology - submitted

Reference List

1. Guyton AC. *Textbook of Medical Physiology.*: 1991.

2. Olivencia JA. Pathophysiology of venous ulcers: surgical implications, review, and update. *Dermatol Surg* 1999; **25**: 880-5.

3. Lees TA, Redwood NFW. Chronic Venous Insufficiency and Lymphoedema. In: Beard JD, Gaines PA, eds. *Vascular and Endovascular Surgery.*: Saunders, 2001: 451-82.

4. Payne SP et al. Clinical significance of venous reflux detected by duplex scanning. *Br J Surg* 1994; **81**: 39-41.

5. Delis KT, Ibegbuna V, Nicolaides AN, Lauro A, Hafez H. Prevalence and distribution of incompetent perforating veins in chronic venous insufficiency. *J Vasc Surg* 1998; **28**: 815-25.

6. Malanin K, Haapanen A, Kolari PJ, Helander I, Havu VK. The peripheral resistance in arteries of legs is inversely proportional to the severity of chronic venous insufficiency. *Acta Derm Venereol* 1997; **77**: 22-5.

7. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. *J Biol Chem* 1999; **274**: 21491-4.

8. Coleridge Smith PD, Thomas P, Scurr JH, Dormandy JA. Causes of venous ulceration: a new hypothesis. *Br Med J (Clin Res Ed)* 1988; **296**: 1726-7.

9. Claudy AL, Mirshahi M, Soria C, Soria J. Detection of undegraded fibrin and tumor necrosis factor-alpha in venous leg ulcers. *J Am Acad Dermatol* 1991; **25**: 623-7.

10. Vanscheidt W, Laaff H, Weiss JM, Schopf E. Immunohistochemical investigation of dermal capillaries in chronic venous insufficiency. *Acta Derm Venereol* 1991; **71**: 17-9.

11. Leu HJ, Wenner A, Spycher MA, Brunner U. [Changes in the transendothelial permeability as the origin of edema in chronic venous insufficiency]. *Med Welt* 1980; **31**: 781-5.

12. Browse NL, Burnand KG. The cause of venous ulceration. *Lancet* 1982; **2**: 243-5.

13. Herrick SE et al. Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. *Am J Pathol* 1992; **141**: 1085-95.

14. Falanga V, Eaglstein WH. The "trap" hypothesis of venous ulceration. *Lancet* 1993; **341**: 1006-8.

15. Porter JM, Moneta GL. Reporting standards in venous disease: an update. International Consensus Committee on Chronic Venous Disease. *J Vasc Surg* 1995; **21**: 635-45.

16. Allegra C et al. The "C" of CEAP: suggested definitions and refinements: an International Union of Phlebology conference of experts. *J Vasc Surg* 2003; **37**: 129-31.

17. Jull A, Waters J, Arroll B. Pentoxifylline for treatment of venous leg ulcers: a systematic review. *Lancet* 2002; **359**: 1550-4.

18. Pascarella L, Schonbein GW, Bergan JJ. Microcirculation and venous ulcers: a review. *Ann Vasc Surg* 2005; **19**: 921-7.

19. Pierik EG, Wittens CH, van Urk H. Subfascial endoscopic ligation in the treatment of incompetent perforating veins. *Eur J Vasc Endovasc Surg* 1995; **9**: 38-41.

20. Eriksson I. Reconstructive surgery for deep vein valve incompetence in the lower limb. *Eur J Vasc Surg* 1990; **4**: 211-8.

21. PALMA EC, ESPERON R. Vein transplants and grafts in the surgical treatment of the postphlebitic syndrome. *J Cardiovasc Surg (Torino)* 1960; **1**: 94-107.

22. Bradbury A. Varicose Veins. In: Beard JD, Gaines PA, eds. *Vascular and Endovascular Surgery.*: Saunders, 2001: 483-514.

23. Rose SS, Ahmed A. Some thoughts on the aetiology of varicose veins. *J Cardiovasc Surg (Torino)* 1986; **27**: 534-43.

24. Lowell RC, Gloviczki P, Miller VM. In vitro evaluation of endothelial and smooth muscle function of primary varicose veins. *J Vasc Surg* 1992; **16**: 679-86.

25. Milroy CM, Scott DJ, Beard JD, Horrocks M, Bradfield JW. Histological appearances of the long saphenous vein. *J Pathol* 1989; **159**: 311-6.

26. Badier-Commander C et al. Smooth muscle cell modulation and cytokine overproduction in varicose veins. An in situ study. *J Pathol* 2001; **193**: 398-407.

27. Burnstock G. Local mechanisms of blood flow control by perivascular nerves and endothelium. *J Hypertens Suppl* 1990; **8**: S95-106.

28. Marin ML et al. Human greater saphenous vein: histologic and ultrastructural variation. *Cardiovasc Surg* 1994; **2**: 56-62.

29. Khan AA, Eid RA, Hamdi A. Structural changes in the tunica intima of varicose veins: a histopathological and ultrastructural study. *Pathology* 2000; **32**: 253-7.

30. Bourassa MG et al. Long-term fate of bypass grafts: the Coronary Artery Surgery Study (CASS) and Montreal Heart Institute experiences. *Circulation* 1985; **72**: V71-V78.

31. Sarjeant JM, Rabinovitch M. Understanding and treating vein graft atherosclerosis. *Cardiovasc Pathol* 2002; **11**: 263-71.

32. Kerr JB. *Atlas of Functional Histology.*: 1999.

33. Saenz dT, I, Goldstein I, Krane RJ. Local control of penile erection. Nerves, smooth muscle, and endothelium. *Urol Clin North Am* 1988; **15**: 9-15.

34. Levin RM, Hypolite JA, Broderick GA. Comparison of the pharmacological response of human corpus cavernosal tissue with the response of rabbit cavernosal tissue. *Gen Pharmacol* 1995; **26**: 1107-11.

35. Andersson KE, Holmquist F. Mechanisms for contraction and relaxation of human penile smooth muscles. *Int J Impot Res* 1990; **2**: 209-25.

36. Levin RM, Wein AJ. Adrenergic alpha receptors outnumber beta receptors in human penile corpus cavernosum. *Invest Urol* 1980; **18**: 225-6.

37. Hedlund P, Ny L, Alm P, Andersson KE. Cholinergic nerves in human corpus cavernosum and spongiosum contain nitric oxide synthase and heme oxygenase. *J Urol* 2000; **164**: 868-75.

38. Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N Engl J Med* 1992; **326**: 90-4.

39. Holmquist F, Andersson KE, Hedlund H. Actions of endothelin on isolated corpus cavernosum from rabbit and man. *Acta Physiol Scand* 1990; **139**: 113-22.

40. Feldman HA et al. Erectile dysfunction and coronary risk factors: prospective results from the Massachusetts male aging study. *Prev Med* 2000; **30**: 328-38.

41. Kirby M, Jackson G, Betteridge J, Friedli K. Is erectile dysfunction a marker for cardiovascular disease? *Int J Clin Pract* 2001; **55**: 614-8.

42. McMahon CN, Smith CJ, Shabsigh R. Treating erectile dysfunction when PDE5 inhibitors fail. *BMJ* 2006; **332**: 589-92.

43. Rosen R et al. Lower urinary tract symptoms and male sexual dysfunction: the multinational survey of the aging male (MSAM-7). *Eur Urol* 2003; **44**: 637-49.

44. McVary K. Lower urinary tract symptoms and sexual dysfunction: epidemiology and pathophysiology. *BJU Int* 2006; **97 Suppl 2**: 23-8.

45. Andersson KE. Erectile physiological and pathophysiological pathways involved in erectile dysfunction. *J Urol* 2003; **170**: S6-13.

46. Bloch W et al. Distribution of nitric oxide synthase implies a regulation of circulation, smooth muscle tone, and secretory function in the human prostate by nitric oxide. *Prostate* 1997; **33**: 1-8.

47. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet* 2005; **365**: 1415-28.

48. Persson K et al. Spinal and peripheral mechanisms contributing to hyperactive voiding in spontaneously hypertensive rats. *Am J Physiol* 1998; **275**: R1366-R1373.

49. McVary KT, Rademaker A, Lloyd GL, Gann P. Autonomic nervous system overactivity in men with lower urinary tract symptoms secondary to benign prostatic hyperplasia. *J Urol* 2005; **174**: 1327-433.

50. Bing W et al. Obstruction-induced changes in urinary bladder smooth muscle contractility: a role for Rho kinase. *Am J Physiol Renal Physiol* 2003; **285**: F990-F997.

51. Chang S et al. Increased corpus cavernosum smooth muscle tone associated with partial bladder outlet obstruction is mediated via Rho-kinase. *Am J Physiol Regul Integr Comp Physiol* 2005; **289**: R1124-R1130.

52. Azadzoi KM, Babayan RK, Kozlowski R, Siroky MB. Chronic ischemia increases prostatic smooth muscle contraction in the rabbit. *J Urol* 2003; **170**: 659-63.

53. Azadzoi KM, Tarcan T, Siroky MB, Krane RJ. Atherosclerosis-induced chronic ischemia causes bladder fibrosis and non-compliance in the rabbit. *J Urol* 1999; **161**: 1626-35.

54. Kozlowski R, Kershen RT, Siroky MB, Krane RJ, Azadzoi KM. Chronic ischemia alters prostate structure and reactivity in rabbits. *J Urol* 2001; **165**: 1019-26.

55. McVary KT. Erectile dysfunction and lower urinary tract symptoms secondary to BPH. *Eur Urol* 2005; **47**: 838-45.

56. Drury AN, Szent-Gyorgyi A. The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. *J Physiol* 1929; **68**: 213-37.

57. Buchthal F, Folkow B. Interaction between acetylcholine and adenosine triphosphate in normal, curarised and denervated muscle. *Acta Physiol Scand* 1948; 150-60.

58. HOLTON P. The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *J Physiol* 1959; **145**: 494-504.

59. Burnstock G. Purinergic nerves. *Pharmacol Rev* 1972; **24**: 509-81.

60. Ralevic V, Burnstock G. Involvement of purinergic signaling in cardiovascular diseases. *Drug News Perspect* 2003; **16**: 133-40.

61. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev* 1998; **50**: 413-92.

62. Burnstock G. A basis for distinguishing two types of purinergic receptor. In: Straub RW, Bolis L, eds. *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* New York: Raven Press, 1978: 107-18.

63. Burnstock G, Kennedy C. Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 1985; **16**: 433-40.

64. Fredholm BB et al. Nomenclature and classification of purinoceptors. *Pharmacol Rev* 1994; **46**: 143-56.

65. Schwiebert EM. Cellular mechanisms and physiology of nucleotide and nucleoside release from cells: current knowledge, novel assays to detect purinergic agonists, and future directions. *Curr Top Membr* 2003; 31-58.

66. Abbracchio MP et al. Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* 2003; **24**: 52-5.

67. North RA. Molecular physiology of P2X receptors. *Physiol Rev* 2002; **82**: 1013-67.

68. Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA. Allosteric control of gating and kinetics at P2X(4) receptor channels. *J Neurosci* 1999; **19**: 7289-99.

69. Lundy PM et al. Pharmacological differentiation of the P2X7 receptor and the maitotoxin-activated cationic channel. *Eur J Pharmacol* 2004; **487**: 17-28.

70. Bo X, Burnstock G. Distribution of [3H]alpha,beta-methylene ATP binding sites in rat brain and spinal cord. *Neuroreport* 1994; **5**: 1601-4.

71. Collo G et al. Cloning OF P2X5 and P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J Neurosci* 1996; **16**: 2495-507.

72. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* 1996; **272**: 735-8.

73. Burnstock G. Introduction: P2 receptors. *Curr Top Med Chem* 2004; **4**: 793-803.

74. Janssens R et al. Cloning and tissue distribution of the human P2Y1 receptor. *Biochem Biophys Res Commun* 1996; **221**: 588-93.

75. Webb TE, Simon J, Bateson AN, Barnard EA. Transient expression of the recombinant chick brain P2y1 purinoceptor and localization of the corresponding mRNA. *Cell Mol Biol (Noisy -le-grand)* 1994; **40**: 437-42.

76. Seye CI et al. Functional P2Y2 nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* 2002; **106**: 2720-6.

77. Robaye B et al. Loss of nucleotide regulation of epithelial chloride transport in the jejunum of P2Y4-null mice. *Mol Pharmacol* 2003; **63**: 777-83.

78. Anwar Z et al. Regulation of cyclic AMP by extracellular ATP in cultured brain capillary endothelial cells. *Br J Pharmacol* 1999; **128**: 465-71.

79. Communi D, Parmentier M, Boeynaems JM. Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem Biophys Res Commun* 1996; **222**: 303-8.

80. Hollopeter G et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; **409**: 202-7.

81. Unterberger U, Moskvina E, Scholze T, Freissmuth M, Boehm S. Inhibition of adenylyl cyclase by neuronal P2Y receptors. *Br J Pharmacol* 2002; **135**: 673-84.

82. Wihlborg AK et al. ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1810-5.

83. Fumagalli M et al. Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. *Biochem Pharmacol* 2004; **68**: 113-24.

84. Wang L, Jacobsen SE, Bengtsson A, Erlinge D. P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. *BMC Immunol* 2004; **5**: 16.

85. Spelta V, Jiang LH, Surprenant A, North RA. Kinetics of antagonist actions at rat P2X2/3 heteromeric receptors. *Br J Pharmacol* 2002; **135**: 1524-30.

86. Ralevic V, Burnstock G. Purinergic Receptors, Nitric Oxide, and Regional Blood Flow. In: Kadowitz PJ, McNamara DB, eds. *Nitric Oxide and the Regulation of the Peripheral Circulation.* 2000: 65-84.

87. Burnstock G. Vessel tone and remodeling. *Nat Med* 2006; **12**: 16-7.

88. Burnstock G. Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat* 1999; **194 (Pt 3)**: 335-42.

89. Ralevic V. Roles of purines and pyrimidines in endothelium. In: Abbracchio MP, Williams M, eds. *Purinergic and Pyrimidines in Endothelium.* Berlin: Springer, 2001: 101-20.

90. Wang L et al. P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 2002; **40**: 841-53.

91. Glass R, Loesch A, Bodin P, Burnstock G. P2X4 and P2X6 receptors associate with VE-cadherin in human endothelial cells. *Cell Mol Life Sci* 2002; **59**: 870-81.

92. Ray FR, Huang W, Slater M, Barden JA. Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selection of coronary artery grafts. *Atherosclerosis* 2002; **162**: 55-61.

93. Yamamoto K et al. Impaired flow-dependent control of vascular tone and remodeling in P2X4-deficient mice. *Nat Med* 2006; **12**: 133-7.

94. Guns PJ et al. Pharmacological characterization of nucleotide P2Y receptors on endothelial cells of the mouse aorta. *Br J Pharmacol* 2005; **146**: 288-95.

95. Bodin P, Burnstock G. Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J Cardiovasc Pharmacol* 2001; **38**: 900-8.

96. Malmsjo M et al. The stable pyrimidines UDPbetaS and UTPgammaS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery. *Br J Pharmacol* 2000; **131**: 51-6.

97. Corr L, Burnstock G. Analysis of P2-purinoceptor subtypes on the smooth muscle and endothelium of rabbit coronary artery. *J Cardiovasc Pharmacol* 1994; **23**: 709-15.

98. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; **362**: 801-9.

99. Sjolund M, Hedin U, Sejersen T, Heldin CH, Thyberg J. Arterial smooth muscle cells express platelet-derived growth factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. *J Cell Biol* 1988; **106**: 403-13.

100. Erlinge D, Hou M, Webb TE, Barnard EA, Moller S. Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by quantitative RT-PCR. *Biochem Biophys Res Commun* 1998; **248**: 864-70.

101. Hou M, Moller S, Edvinsson L, Erlinge D. MAPKK-dependent growth factor-induced upregulation of P2Y2 receptors in vascular smooth muscle cells. *Biochem Biophys Res Commun* 1999; **258**: 648-52.

102. Hou M et al. UDP acts as a growth factor for vascular smooth muscle cells by activation of P2Y(6) receptors. *Am J Physiol Heart Circ Physiol* 2002; **282**: H784-H792.

103. Burnstock G. Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* 2002; **22**: 364-73.

104. Di Virgilio F, Solini A. P2 receptors: new potential players in atherosclerosis. *Br J Pharmacol* 2002; **135**: 831-42.

105. Cristalli G et al. 2-Aralkynyl and 2-heteroalkynyl derivatives of adenosine-5'-N-ethyluronamide as selective A2a adenosine receptor agonists. *J Med Chem* 1995; **38**: 1462-72.

106. Gurden MF et al. Functional characterization of three adenosine receptor types. *Br J Pharmacol* 1993; **109**: 693-8.

107. Huttemann E, Ukena D, Lenschow V, Schwabe U. Ra adenosine receptors in human platelets. Characterization by 5'-N-ethylcarboxamido[3H]adenosine binding in relation to adenylate cyclase activity. *Naunyn Schmiedebergs Arch Pharmacol* 1984; **325**: 226-33.

108. Monopoli A et al. Pharmacology of the new selective A2a adenosine receptor agonist 2-hexynyl-5'-N-ethylcarboxamidoadenosine. *Arzneimittelforschung* 1994; **44**: 1296-304.

109. Mellion BT et al. Evidence for the inhibitory role of guanosine 3', 5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981; **57**: 946-55.

110. Zimmermann H, Braun N. Extracellular metabolism of nucleotides in the nervous system. *J Auton Pharmacol* 1996; **16**: 397-400.

111. Di Virgilio F et al. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 2001; **97**: 587-600.

112. Burnstock G. Overview of P2 Receptors: Possible Functions in Immune Cells. *Drug Development Research* 2001; 53-9.

113. Wihlborg AK et al. Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor. *Br J Pharmacol* 2003; **138**: 1451-8.

114. Di Virgilio F, Falzoni S, Mutini C, Sanz JM, Chiozzi P. Purinergic P2X7 Receptor: A Pivotal Role in Inflammation and Immunomodulation. *Drug Development Research* 1998; **45**: 207-13.

115. Williams M, Jarvis MF. Purinergic and pyrimidinergic receptors as potential drug targets. *Biochem Pharmacol* 2000; **59**: 1173-85.

116. Baraldi PG, Di Virgilio F, Romagnoli R. Agonists and antagonists acting at P2X7 receptor. *Curr Top Med Chem* 2004; **4**: 1707-17.

117. Lemaire I, Leduc N. Purinergic P2X7 receptor function in lung alveolar macrophages: Pharmacologic characterization and bidirectional regulation by Th1 and Th2 cytokines. *Drug Dev Res* 2004; **59**: 118-27.

118. Feng C, Mery AG, Beller EM, Favot C, Boyce JA. Adenine nucleotides inhibit cytokine generation by human mast cells through a Gs-coupled receptor. *J Immunol* 2004; **173**: 7539-47.

119. Stober CB et al. ATP-mediated killing of Mycobacterium bovis bacille Calmette-Guerin within human macrophages is calcium dependent and associated with the acidification of mycobacteria-containing phagosomes. *J Immunol* 2001; **166**: 6276-86.

120. Luttikhuizen DT, Harmsen MC, de Leij LF, van Luyn MJ. Expression of P2 receptors at sites of chronic inflammation. *Cell Tissue Res* 2004; **317**: 289-98.

121. Duhant X et al. Extracellular adenine nucleotides inhibit the activation of human CD4+ T lymphocytes. *J Immunol* 2002; **169**: 15-21.

122. Schnurr M et al. ATP gradients inhibit the migratory capacity of specific human dendritic cell types: implications for P2Y11 receptor signaling. *Blood* 2003; **102**: 613-20.

123. Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev* 2006; **58**: 58-86.

124. Gibbons GH. Autocrine-paracrine factors and vascular remodeling in hypertension. *Curr Opin Nephrol Hypertens* 1993; **2**: 291-8.

125. Schachter M. Endothelium and smooth muscle: trophic interactions and potential for therapeutic intervention. *J Hum Hypertens* 1990; **4 Suppl 4**: 17-20.

126. Greig AV, Linge C, Cambrey A, Burnstock G. Purinergic receptors are part of a signaling system for keratinocyte proliferation, differentiation, and apoptosis in human fetal epidermis. *J Invest Dermatol* 2003; **121**: 1145-9.

127. Erlinge D. Extracellular ATP: a growth factor for vascular smooth muscle cells. *Gen Pharmacol* 1998; **31**: 1-8.

128. Van Daele P, Van Coevorden A, Roger PP, Boeynaems JM. Effects of adenine nucleotides on the proliferation of aortic endothelial cells. *Circ Res* 1992; **70**: 82-90.

129. Wang DJ, Huang NN, Heppel LA. Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. *J Cell Physiol* 1992; **153**: 221-33.

130. Burnstock G. Purinergic System. In: Offermanns S, Rosenthal W, eds. *Encyclopedic Reference of Molecular Pharmacology.*: Springer, 2003: 784-90.

131. Dale HH. Pharmacology and nerve endings. *Proceedings of the Royal Society of Medicine* 1935; **28**: 319-32.

132. Burnstock G. Do some nerve cells release more than one transmitter? *Neuroscience* 1976; **1**: 239-48.

133. Burnstock G. Cotransmission. *Curr Opin Pharmacol* 2004; **4**: 47-52.

134. Burnstock G. The Autonomic Neuroeffector Junction. In: Robertson D, Low P, Burnstock G, Biaggioni I, eds. *Primer on the Autonomic Nervous System.* San Diego: Elsevier Academic Press, 2004: 29-33.

135. Moore LK, Burt JM. Gap junction function in vascular smooth muscle: influence of serotonin. *Am J Physiol* 1995; **269**: H1481-H1489.

136. Su C, Bevan JA, Burnstock G. [3H]adenosine triphosphate: release during stimulation of enteric nerves. *Science* 1971; **173**: 336-8.

137. Langer SZ, Pinto JE. Possible involvement of a transmitter different from norepinephrine in the residual responses to nerve stimulation of the cat nictitating membrane after pretreatment with reserpine. *J Pharmacol Exp Ther* 1976; **196**: 697-713.

138. Fedan JS, Hogaboom GK, O'Donnell JP, Colby J, Westfall DP. Contribution by purines to the neurogenic response of the vas deferens of the guinea pig. *Eur J Pharmacol* 1981; **69**: 41-53.

139. Sneddon P, Westfall DP. Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *J Physiol* 1984; **347**: 561-80.

140. Sneddon P, Burnstock G. ATP as a co-transmitter in rat tail artery. *Eur J Pharmacol* 1984; **106**: 149-52.

141. Burnstock G, Warland JJ. A pharmacological study of the rabbit saphenous artery in vitro: a vessel with a large purinergic contractile response to sympathetic nerve stimulation. *Br J Pharmacol* 1987; **90**: 111-20.

142. Katsuragi T, Su C. Augmentation by theophylline of [3H]purine release from vascular adrenergic nerves: evidence for presynaptic autoinhibition. *J Pharmacol Exp Ther* 1982; **220**: 152-6.

143. Ishikawa S. Actions of ATP and alpha, beta-methylene ATP on neuromuscular transmission and smooth muscle membrane of the rabbit and guinea-pig mesenteric arteries. *Br J Pharmacol* 1985; **86**: 777-87.

144. Muramatsu I, Kigoshi S. Purinergic and non-purinergic innervation in the cerebral arteries of the dog. *Br J Pharmacol* 1987; **92**: 901-8.

145. Kennedy C, Saville VL, Burnstock G. The contributions of noradrenaline and ATP to the responses of the rabbit central ear artery to sympathetic nerve stimulation depend on the parameters of stimulation. *Eur J Pharmacol* 1986; **122**: 291-300.

146. Burnstock G, Ralevic V. New insights into the local regulation of blood flow by perivascular nerves and endothelium. *Br J Plast Surg* 1994; **47**: 527-43.

147. Lundberg JM. Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical and functional studies. *Acta Physiol Scand Suppl* 1981; **496**: 1-57.

148. Fujii K. Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J Physiol* 1988; **404**: 39-52.

149. MacKenzie I, Burnstock G, Dolly JO. The effects of purified botulinum neurotoxin type A on cholinergic, adrenergic and non-adrenergic, atropine-resistant autonomic neuromuscular transmission. *Neuroscience* 1982; **7**: 997-1006.

150. Sneddon P. Electrophysiology of autonomic neuromuscular transmission involving ATP. *J Auton Nerv Syst* 2000; **81**: 218-24.

151. Gibbins IL et al. Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci Lett* 1985; **57**: 125-30.

152. Gulbenkian S, Merighi A, Wharton J, Varndell IM, Polak JM. Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. *J Neurocytol* 1986; **15**: 535-42.

153. Manzini S et al. Neurochemical evidence of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) release from capsaicin-sensitive nerves in rat mesenteric arteries and veins. *Gen Pharmacol* 1991; **22**: 275-8.

154. Gibbins IL, Furness JB, Costa M. Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystokinin and dynorphin in neurons of the dorsal root ganglia of the guinea-pig. *Cell Tissue Res* 1987; **248**: 417-37.

155. Burnstock G. Autonomic neuroeffector junctions--reflex vasodilatation of the skin. *J Invest Dermatol* 1977; **69**: 47-57.

156. Maggi CA, Meli A. The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen Pharmacol* 1988; **19**: 1-43.

157. Furness JB, Morris JL, Gibbins IL, Costa M. Chemical coding of neurons and plurichemical transmission. *Annu Rev Pharmacol Toxicol* 1989; **29**: 289-306.

158. Hassall CJ, Burnstock G. Neuropeptide Y-like immunoreactivity in cultured intrinsic neurones of the heart. *Neurosci Lett* 1984; **52**: 111-5.

159. Hassall CJ, Burnstock G. Intrinsic neurones and associated cells of the guinea-pig heart in culture. *Brain Res* 1986; **364**: 102-13.

160. Huang MH, Sylven C, Pelleg A, Smith FM, Armour JA. Modulation of in situ canine intrinsic cardiac neuronal activity by locally applied adenosine, ATP, or analogues. *Am J Physiol* 1993; **265**: R914-R922.

161. Neel DS, Parsons RL. Catecholamine, serotonin, and substance P-like peptide containing intrinsic neurons in the mudpuppy parasympathetic cardiac ganglion. *J Neurosci* 1986; **6**: 1970-5.

162. Bradley E, Law A, Bell D, Johnson CD. Effects of varying impulse number on cotransmitter contributions to sympathetic vasoconstriction in rat tail artery. *Am J Physiol Heart Circ Physiol* 2003; **284**: H2007-H2014.

163. Ventura S, Dewalagama RK, Lau LC. Adenosine 5'-triphosphate (ATP) is an excitatory cotransmitter with noradrenaline to the smooth muscle of the rat prostate gland. *Br J Pharmacol* 2003; **138**: 1277-84.

164. Lundberg JM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol Rev* 1996; **48**: 113-78.

165. Langer SZ, Shepperson NB. Prejunctional modulation of noradrenaline release by alpha 2-adrenoceptors: physiological and pharmacological implications in the cardiovascular system. *J Cardiovasc Pharmacol* 1982; **4 Suppl 1**: S35-S40.

166. Merighi A. Costorage and coexistence of neuropeptides in the mammalian CNS. *Prog Neurobiol* 2002; **66**: 161-90.

167. Burnstock G. The fifth Heymans memorial lecture-Ghent, February 17, 1990. Co-transmission. *Arch Int Pharmacodyn Ther* 1990; **304**: 7-33.

168. Khakh BS, Zhou X, Sydes J, Galligan JJ, Lester HA. State-dependent cross-inhibition between transmitter-gated cation channels. *Nature* 2000; **406**: 405-10.

169. Yang B, Slonimsky JD, Birren SJ. A rapid switch in sympathetic neurotransmitter release properties mediated by the p75 receptor. *Nat Neurosci* 2002; **5**: 539-45.

170. Andersson KE. Emptying against outflow obstruction--pharmacological aspects. *Scand J Urol Nephrol Suppl* 1997; **184**: 77-84.

171. Andersson KE, Hedlund P. Pharmacologic perspective on the physiology of the lower urinary tract. *Urology* 2002; **60**: 13-20.

172. Crowe R et al. Vasoactive intestinal polypeptide-like immunoreactive nerves in diabetic penis. A comparison between streptozotocin-treated rats and man. *Diabetes* 1983; **32**: 1075-7.

173. Belai A et al. Enteric nerves in diabetic rats: increase in vasoactive intestinal polypeptide but not substance P. *Gastroenterology* 1985; **89**: 967-76.

174. Bishop AE, Polak JM, Bryant MG, Bloom SR, Hamilton S. Abnormalities of vasoactive intestinal polypeptide-containing nerves in Crohn's disease. *Gastroenterology* 1980; **79**: 853-60.

175. Burnstock G. Autonomic neuromuscular junctions: current developments and future directions. *J Anat* 1986; **146**: 1-30.

176. Ralevic V, Burnstock G. Interactions Between Perivascular Nerves and Endothelial Cells in Control of Local Vascular Tone. In: Bennett T, Gardiner S, eds. *Nervous Control of Blood Vessels.*: Harwood Academic Publishers, 1996: 135-75.

177. Burnstock G. Integration of factors controlling vascular tone. Overview. *Anesthesiology* 1993; **79**: 1368-80.

178. Young B, Heath JW. *Wheater's Functional Histology.*: Churchill Livingstone, 2000.

179. Racchi H et al. Adenosine 5'-triphosphate and neuropeptide Y are co-transmitters in conjunction with noradrenaline in the human saphenous vein. *Br J Pharmacol* 1999; **126**: 1175-85.

180. Rump LC, von K, I. A study of ATP as a sympathetic cotransmitter in human saphenous vein. *Br J Pharmacol* 1994; **111**: 65-72.

181. Borna C et al. Contractions in human coronary bypass vessels stimulated by extracellular nucleotides. *Ann Thorac Surg* 2003; **76**: 50-7.

182. Conant AR, Fisher MJ, McLennan AG, Simpson AW. Diadenosine polyphosphates are largely ineffective as agonists at natively expressed P2Y(1) and P2Y(2) receptors on cultured human saphenous vein endothelial cells. *J Vasc Res* 2000; **37**: 548-55.

183. Cario-Toumaniantz C, Loirand G, Ladoux A, Pacaud P. P2X7 receptor activation-induced contraction and lysis in human saphenous vein smooth muscle. *Circ Res* 1998; **83**: 196-203.

184. Ziganshin AU et al. Varicose disease affects the P2 receptor-mediated responses of human greater saphenous vein. *Vascular Pharmacology* 2005.

185. Yamada T et al. Immunohistochemical and ultrastructural examination of smooth muscle cells in aortocoronary saphenous vein grafts. *Angiology* 1997; **48**: 381-90.

186. Cox JL, Chiasson DA, Gotlieb AI. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences between veins and arteries. *Prog Cardiovasc Dis* 1991; **34**: 45-68.

187. Newby AC, George SJ. Proliferation, migration, matrix turnover, and death of smooth muscle cells in native coronary and vein graft atherosclerosis. *Curr Opin Cardiol* 1996; **11**: 574-82.

188. Davies MG, Ramkumar V, Hagen PO. Adenosine responses in experimental vein bypass grafts. *J Vasc Surg* 1998; **28**: 929-38.

189. Denda M, Inoue K, Fuziwara S, Denda S. P2X purinergic receptor antagonist accelerates skin barrier repair and prevents epidermal hyperplasia induced by skin barrier disruption. *J Invest Dermatol* 2002; **119**: 1034-40.

190. Greig AV, Linge C, Terenghi G, McGrouther DA, Burnstock G. Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes. *J Invest Dermatol* 2003; **120**: 1007-15.

191. Groschel-Stewart U, Bardini M, Robson T, Burnstock G. Localisation of P2X5 and P2X7 receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell Tissue Res* 1999; **296**: 599-605.

192. Inoue K et al. Characterization of multiple P2X receptors in cultured normal human epidermal keratinocytes. *J Invest Dermatol* 2005; **124**: 756-63.

193. Burrell HE, Bowler WB, Gallagher JA, Sharpe GR. Human keratinocytes express multiple P2Y-receptors: evidence for functional P2Y1, P2Y2, and P2Y4 receptors. *J Invest Dermatol* 2003; **120**: 440-7.

194. Yoshida H, Kobayashi D, Ohkubo S, Nakahata N. ATP stimulates interleukin-6 production via P2Y receptors in human HaCaT keratinocytes. *Eur J Pharmacol* 2006.

195. Tong YC, Broderick G, Hypolite J, Levin RM. Correlations of purinergic, cholinergic and adrenergic functions in rabbit corporal cavernosal tissue. *Pharmacology* 1992; **45**: 241-9.

196. Chiang PH et al. Adenosine modulation of neurotransmission in penile erection. *Br J Clin Pharmacol* 1994; **38**: 357-62.

197. Mantelli L, Amerini S, Ledda F, Forti G, Maggi M. The potent relaxant effect of adenosine in rabbit corpora cavernosa is nitric oxide independent and mediated by A2 receptors. *J Androl* 1995; **16**: 312-7.

198. Ragazzi E, Chinellato A, Italiano G, Pagano F, Calabro A. Characterization of in vitro relaxant mechanisms in erectile tissue from rabbits of different ages. *Urol Res* 1996; **24**: 317-22.

199. Filippi S et al. Functional adenosine receptors in human corpora cavernosa. *Int J Androl* 2000; **23**: 210-7.

200. Noto T, Inoue H, Mochida H, Kikkawa K. Role of adenosine and P2 receptors in the penile tumescence in anesthetized dogs. *Eur J Pharmacol* 2001; **425**: 51-5.

201. Filippi S, Amerini S, Maggi M, Natali A, Ledda F. Studies on the mechanisms involved in the ATP-induced relaxation in human and rabbit corpus cavernosum. *J Urol* 1999; **161**: 326-31.

202. Burnstock G. Potential therapeutic targets in the rapidly expanding field of purinergic signalling. *Clin Med* 2002; **2**: 45-53.

203. Wu HY, Broderick GA, Suh JK, Hypolite JA, Levin RM. Effects of purines on rabbit corpus cavernosum contractile activity. *Int J Impot Res* 1993; **5**: 161-7.

204. Shalev M, Staerman F, Allain H, Lobel B, Saiag B. Stimulation of P2y purinoceptors induces, via nitric oxide production, endothelium-dependent relaxation of human isolated corpus cavernosum. *J Urol* 1999; **161**: 955-9.

205. Staerman F, Shalev M, Legrand A, Lobel B, Saiag B. P2y and P2x purinoceptors are respectively implicated in endothelium- dependent relaxation and endothelium independent contraction in human corpus cavernosum. *Adv Exp Med Biol* 2000; **486**: 189-95.

206. Obara K, Lepor H, Walden PD. Localization of P2Y1 purinoceptor transcripts in the rat penis and urinary bladder. *J Urol* 1998; **160**: 587-91.

207. Pinna C et al. Purine- and pyrimidine-induced responses and P2Y receptor characterization in the hamster proximal urethra. *Br J Pharmacol* 2005; **144**: 510-8.

208. Erlinge D, You J, Wahlestedt C, Edvinsson L. Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol* 1995; **289**: 135-49.

209. Dixon CJ et al. Regulation of epidermal homeostasis through P2Y2 receptors. *Br J Pharmacol* 1999; **127**: 1680-6.

210. Lee HY, Bardini M, Burnstock G. P2X receptor immunoreactivity in the male genital organs of the rat. *Cell Tissue Res* 2000; **300**: 321-30.

211. Wali MA, Eid RA. Smooth muscle changes in varicose veins: an ultrastructural study. *J Smooth Muscle Res* 2001; **37**: 123-35.

212. Bogdanov YD, Wildman SS, Clements MP, King BF, Burnstock G. Molecular cloning and characterization of rat P2Y4 nucleotide receptor. *Br J Pharmacol* 1998; **124**: 428-30.

213. Ciardullo AV et al. High endogenous estradiol is associated with increased venous distensibility and clinical evidence of varicose veins in menopausal women. *J Vasc Surg* 2000; **32**: 544-9.

214. Mashiah A et al. Estrogen and progesterone receptors in normal and varicose saphenous veins. *Cardiovasc Surg* 1999; **7**: 327-31.

215. Haynes JM, Pennefather JN, Sikorski B. Purinoceptor-mediated contractility of the perfused uterine vasculature of the guinea-pig: influence of oestradiol and pregnancy. *Clin Exp Pharmacol Physiol* 2003; **30**: 329-35.

216. Uvelius B, Arner A, Johansson B. Structural and mechanical alterations in hypertrophic venous smooth muscle. *Acta Physiol Scand* 1981; **112**: 463-71.

217. Arnould T, Michiels C, Janssens D, Berna N, Remacle J. Effect of Ginkor Fort on hypoxia-induced neutrophil adherence to human saphenous vein endothelium. *J Cardiovasc Pharmacol* 1998; **31**: 456-63.

218. Janssens D et al. Increase in circulating endothelial cells in patients with primary chronic venous insufficiency: protective effect of Ginkor Fort in a randomized double-blind, placebo-controlled clinical trial. *J Cardiovasc Pharmacol* 1999; **33**: 7-11.

219. Kohler TR, Kirkman TR, Kraiss LW, Zierler BK, Clowes AW. Increased blood flow inhibits neointimal hyperplasia in endothelialized vascular grafts. *Circ Res* 1991; **69**: 1557-65.

220. Yamamura S, Okadome K, Onohara T, Komori K, Sugimachi K. Blood flow and kinetics of smooth muscle cell proliferation in canine autogenous vein grafts: in vivo BrdU incorporation. *J Surg Res* 1994; **56**: 155-61.

221. Lee KM, Tsai KY, Wang N, Ingber DE. Extracellular matrix and pulmonary hypertension: control of vascular smooth muscle cell contractility. *Am J Physiol* 1998; **274**: H76-H82.

222. Travers JP et al. Assessment of wall structure and composition of varicose veins with reference to collagen, elastin and smooth muscle content. *Eur J Vasc Endovasc Surg* 1996; **11**: 230-7.

223. Bujan J et al. Expression of elastic components in healthy and varicose veins. *World J Surg* 2003; **27**: 901-5.

224. Rizzi A et al. Effects of vasoactive agents in healthy and diseased human saphenous veins. *J Vasc Surg* 1998; **28**: 855-61.

225. Kawasaki K, Iino T, Hasegawa H, Miyazawa I, Hosoda S. The function of intimal longitudinal smooth muscles of the human coronary artery. *Experientia* 1986; **42**: 1222-4.

226. Kennedy C, Burnstock G. Evidence for two types of P2-purinoceptor in longitudinal muscle of the rabbit portal vein. *Eur J Pharmacol* 1985; **111**: 49-56.

227. Reilly WM, Burnstock G. The effect of ATP analogues on the spontaneous electrical and mechanical activity of rat portal vein longitudinal muscle. *Eur J Pharmacol* 1987; **138**: 319-25.

228. Todd ME. Changes in size and shape of smooth muscle cells from the portal vein of spontaneously hypertensive rats: an ultrastructural analysis. *Arch Histol Cytol* 1992; **55 Suppl**: 95-104.

229. Todd ME. Hypertensive structural changes in blood vessels: do endothelial cells hold the key? *Can J Physiol Pharmacol* 1992; **70**: 536-51.

230. Ralevic V, Burnstock G. Roles of P2-purinoceptors in the cardiovascular system. *Circulation* 1991; **84**: 1-14.

231. Thyberg J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int Rev Cytol* 1996; **169**: 183-265.

232. Harper S, Webb TE, Charlton SJ, Ng LL, Boarder MR. Evidence that P2Y4 nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. *Br J Pharmacol* 1998; **124**: 703-10.

233. Schuller-Petrovic S et al. Imbalance between the endothelial cell-derived contracting factors prostacyclin and angiotensin II and nitric oxide/cyclic GMP in human primary varicosis. *Br J Pharmacol* 1997; **122**: 772-8.

234. Dubey RK, Gillespie DG, Jackson EK. Adenosine inhibits collagen and total protein synthesis in vascular smooth muscle cells. *Hypertension* 1999; **33**: 190-4.

235. Burnstock G. Noradrenaline and ATP as cotransmitters in sympathetic nerves. *Neurochem Int* 1990; **17**: 357-68.

236. Saharay M, Shields DA, Porter JB, Scurr JH, Coleridge Smith PD. Leukocyte activity in the microcirculation of the leg in patients with chronic venous disease. *J Vasc Surg* 1997; **26**: 265-73.

237. Junger M, Steins A, Hahn M, Hafner HM. Microcirculatory dysfunction in chronic venous insufficiency (CVI). *Microcirculation* 2000; **7**: S3-12.

238. Coleridge Smith PD. Deleterious effects of white cells in the course of skin damage in CVI. *Int Angiol* 2002; **21**: 26-32.

239. Weyl A et al. Expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin and their ligands VLA-4 and LFA-1 in chronic venous leg ulcers. *J Am Acad Dermatol* 1996; **34**: 418-23.

240. Korthuis RJ, Unthank JL. Experimental models to investigate inflammatory processes in chronic venous insufficiency. *Microcirculation* 2000; **7**: S13-S22.

241. Peschen M et al. Changes of cytokeratin expression in the epidermis with chronic venous insufficiency. *Vasa* 1997; **26**: 76-80.

242. Slater M, Scolyer RA, Gidley-Baird A, Thompson JF, Barden JA. Increased expression of apoptotic markers in melanoma. *Melanoma Res* 2003; **13**: 137-45.

243. Galkowska H, Olszewsk WL, Wojewodzka U, Mijal J, Filipiuk E. Expression of apoptosis- and cell cycle-related proteins in epidermis of venous leg and diabetic foot ulcers. *Surgery* 2003; **134**: 213-20.

244. Labasi JM et al. Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol* 2002; **168**: 6436-45.

245. Brough D et al. Ca2+ stores and Ca2+ entry differentially contribute to the release of IL-1 beta and IL-1 alpha from murine macrophages. *J Immunol* 2003; **170**: 3029-36.

246. Baricordi OR et al. An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes. *Blood* 1996; **87**: 682-90.

247. Di Virgilio F. The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. *Immunol Today* 1995; **16**: 524-8.

248. Okamoto H, Mizuno K, Horio T. Monocyte-derived multinucleated giant cells and sarcoidosis. *J Dermatol Sci* 2003; **31**: 119-28.

249. Ferrari D et al. Mouse microglial cells express a plasma membrane pore gated by extracellular ATP. *J Immunol* 1996; **156**: 1531-9.

250. Zheng LM, Zychlinsky A, Liu CC, Ojcius DM, Young JD. Extracellular ATP as a trigger for apoptosis or programmed cell death. *J Cell Biol* 1991; **112**: 279-88.

251. Saharay M et al. Endothelial activation in patients with chronic venous disease. *Eur J Vasc Endovasc Surg* 1998; **15**: 342-9.

252. Namazi MR. Cetirizine and allopurinol as novel weapons against cellular autoimmune disorders. *Int Immunopharmacol* 2004; **4**: 349-53.

253. Huttunen M et al. Inhibition of keratinocyte growth in cell culture and whole skin culture by mast cell mediators. *Exp Dermatol* 2001; **10**: 184-92.

254. Lee YH, Lee SJ, Seo MH, Kim CJ, Sim SS. ATP-induced histamine release is in part related to phospholipase A2-mediated arachidonic acid metabolism in rat peritoneal mast cells. *Arch Pharm Res* 2001; **24**: 552-6.

255. Pappas PJ et al. Dermal tissue fibrosis in patients with chronic venous insufficiency is associated with increased transforming growth factor-beta1 gene expression and protein production. *J Vasc Surg* 1999; **30**: 1129-45.

256. Saito S et al. Role of matrix metalloproteinases 1, 2, and 9 and tissue inhibitor of matrix metalloproteinase-1 in chronic venous insufficiency. *J Vasc Surg* 2001; **34**: 930-8.

257. Herouy Y et al. Lipodermatosclerosis is characterized by elevated expression and activation of matrix metalloproteinases: implications for venous ulcer formation. *J Invest Dermatol* 1998; **111**: 822-7.

258. Wang CM, Chang YY, Sun SH. Activation of P2X7 purinoceptor-stimulated TGF-beta 1 mRNA expression involves PKC/MAPK signalling pathway in a rat brain-derived type-2 astrocyte cell line, RBA-2. *Cell Signal* 2003; **15**: 1129-37.

259. Montesinos MC et al. Wound healing is accelerated by agonists of adenosine A2 (G alpha s-linked) receptors. *J Exp Med* 1997; **186**: 1615-20.

260. Calvert RC et al. Alterations in cholinergic and purinergic signaling in a model of the obstructed bladder. *J Urol* 2001; **166**: 1530-3.

261. Gur S, Ozturk B. Altered relaxant responses to adenosine and adenosine 5'-triphosphate in the corpus cavernosum from men and rats with diabetes. *Pharmacology* 2000; **60**: 105-12.

262. Thompson CS et al. The effect of sildenafil on corpus cavernosal smooth muscle relaxation and cyclic GMP formation in the diabetic rabbit. *Eur J Pharmacol* 2001; **425**: 57-64.

263. Malmsjo M, Hou M, Pendergast W, Erlinge D, Edvinsson L. Potent P2Y6 receptor mediated contractions in human cerebral arteries. *BMC Pharmacol* 2003; **3**: 4.

264. Sun Y, Chai TC. Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. *Am J Physiol Cell Physiol* 2006; **290**: C27-C34.

265. Burnett AL. Novel nitric oxide signaling mechanisms regulate the erectile response. *Int J Impot Res* 2004; **16 Suppl 1**: S15-S19.

266. Lee SW, Wang HZ, Christ GJ. Characterization of ATP-sensitive potassium channels in human corporal smooth muscle cells. *Int J Impot Res* 1999; **11**: 179-88.

267. Adaikan PG, Kottegoda SR, Ratnam SS. Is vasoactive intestinal polypeptide the principal transmitter involved in human penile erection? *J Urol* 1986; **135**: 638-40.

268. Bo X, Karoon P, Nori SL, Bardini M, Burnstock G. P2X purinoceptors in postmortem human cerebral arteries. *J Cardiovasc Pharmacol* 1998; **31**: 794-9.

269. Lewis CJ, Evans RJ. P2X receptor immunoreactivity in different arteries from the femoral, pulmonary, cerebral, coronary and renal circulations. *J Vasc Res* 2001; **38**: 332-40.

270. Jin J, Dasari VR, Sistare FD, Kunapuli SP. Distribution of P2Y receptor subtypes on haematopoietic cells. *Br J Pharmacol* 1998; **123**: 789-94.

271. Vial C, Hechler B, Leon C, Cazenave JP, Gachet C. Presence of P2X1 purinoceptors in human platelets and megakaryoblastic cell lines. *Thromb Haemost* 1997; **78**: 1500-4.

272. Chester AH et al. Structural, biochemical and functional effects of distending pressure in the human saphenous vein: implications for bypass grafting. *Coron Artery Dis* 1998; **9**: 143-51.

273. Fabi F, Argiolas L, Chiavarelli M, del Basso P. Nitric oxide-dependent and -independent modulation of sympathetic vasoconstriction in the human saphenous vein. *Eur J Pharmacol* 1996; **309**: 41-50.

274. Luu TN, Chester AH, O'Neil GS, Tadjkarimi S, Yacoub MH. Effects of vasoactive neuropeptides on human saphenous vein. *Br Heart J* 1992; **67**: 474-7.

275. Malmsjo M, Edvinsson L, Erlinge D. P2X receptors counteract the vasodilatory effects of endothelium derived hyperpolarising factor. *Eur J Pharmacol* 2000; **390**: 173-80.

276. Motte S, Communi D, Pirotton S, Boeynaems JM. Involvement of multiple receptors in the actions of extracellular ATP: the example of vascular endothelial cells. *Int J Biochem Cell Biol* 1995; **27**: 1-7.

277. Abbracchio MP, Burnstock G. Purinergic signalling: pathophysiological roles. *Jpn J Pharmacol* 1998; **78**: 113-45.

278. Halter G, Kapfer X, Liewald F, Bischoff M. Vacuum-sealed mesh graft transplantation in chronic cutaneous ulcers of the lower leg. *Vasa* 2003; **32**: 155-8.

279. Groschel-Stewart U, Bardini M, Robson T, Burnstock G. Localisation of P2X5 and P2X7 receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell Tissue Res* 1999; **296**: 599-605.

280. Le KT, Boue-Grabot E, Archambault V, Seguela P. Functional and biochemical evidence for heteromeric ATP-gated channels composed of P2X1 and P2X5 subunits. *J Biol Chem* 1999; **274**: 15415-9.

281. Lee HY, Bardini M, Burnstock G. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* 2000; **163**: 2002-7.

282. Turner CM, Vonend O, Chan C, Burnstock G, Unwin RJ. The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: an immunohistological study. *Cells Tissues Organs* 2003; **175**: 105-17.

283. Amstrup J, Novak I. P2X7 receptor activates extracellular signal-regulated kinases ERK1 and ERK2 independently of Ca2+ influx. *Biochem J* 2003; **374**: 51-61.

284. Khine AA et al. Human neutrophil peptides induce interleukin-8 production through the P2Y6 signaling pathway. *Blood* 2006; **107**: 2936-42.

285. Somers GR, Hammet FM, Trute L, Southey MC, Venter DJ. Expression of the P2Y6 purinergic receptor in human T cells infiltrating inflammatory bowel disease. *Lab Invest* 1998; **78**: 1375-83.

Patient Demographics for Functional Pharmacology (Chapter 2)

Tables show patient code (identification), pharmacology experiment performed and medications taken in patients.

Circular muscle pharmacology in control patients

Patient	NA	ATP	α,β-meATP	KCl	Aspirin	β Blocker	Statin	Angiotensin converting enzyme inhibitor	Calcium channel blocker	Nitrate
1	X	X	X			X	X			
2	X	X	X			X	X			
3	X	X	X			X	X	X	X	X
4	X	X	X			X	X			X
5	X	X	X	X	X	X	X	X		
6	X	X	X	X	X	X	X	X	X	
7	X	X	X	X	X	X	X	X		
8				X	X	X	X		X	
9				X	X	X	X		X	
10				X	X	X				X
11				X			X	X		X
	N=7	N=7	N=7	N=7	N=6	N=10	N=10	N=5	N=4	N=4

Circular muscle pharmacology in varicose patients

Patient	NA	ATP	α,β-meATP	KCl	β blocker
1	X	X	X		
2	X	X	X		
3	X	X	X		
4	X	X	X		
5	X	X	X	X	X
6	X	X	X	X	
7	X	X	X	X	
8	X	X	X	X	
9	X	X	X	X	
10	X	X	X	X	
11	X			X	
12	X			X	
13	X			X	
14				X	
15				X	
16				X	
17				X	
18				X	
	N=13	N=10	N=10	N=14	N=1

Longitudinal muscle pharmacology in control patients

Patient	NA	ATP	α,β-meATP	2-MeSADP	KCl	UDP	UTP	EFS	Aspirin	β Blocker	Statin	Angiotension Converting Enzyme Inhibitor	Calcium Channel Blocker	Nitrate
1	X	X	X							X	X			
2	X	X	X						X		X		X	X
3	X	X	X						X		X		X	X
4	X	X	X		X				X	X	X	X		
5	X	X	X		X				X	X				X
6	X	X	X		X				X	X				
7	X	X	X		X				X	X		X		X
8		X			X				X		X	X		
9		X			X				X	X	X			X
10		X			X				X	X	X			X
11		X			X				X		X			
12		X			X				X	X		X		X
13		X			X						X	X	X	
14							X		X					
15							X		X	X	X	X		
16							X		X		X	X	X	
17							X		X	X		X		X
18				X					X	X		X		X
19				X					X					
20				X					X	X	X	X		
21				X					X		X			
22				X						X		X		
23								X	X				X	X
24								X	X		X	X		
25								X	X					
26								X		X	X			X
27						X			X	X	X	X		
28						X			X	X	X	X		
29						X			X		X	X		
30						X			X		X			
31						X					X			X
	N=7	N=13	N=7	N=5	N=10	N=5	N=4	N=4	N=26	N=16	N=20	N=15	N=5	N=12

Longitudinal muscle pharmacology in varicose patients

Patient	NA	ATP	α,β-meATP	KCl	2-MeSADP	UDP	UTP	Aspirin
1	X	X	X					
2	X	X	X					
3	X	X	X					
4	X	X	X	X				
5	X	X	X	X				
6	X	X	X	X				
7	X	X	X	X				
8	X	X	X	X				
9	X	X	X	X				
10	X	X	X	X				
11		X		X				
12		X		X				
13		X		X				X
14				X				
15				X				
16				X				
17				X				
18				X				
19				X				
20					X			
21					X			
22					X			
23					X			
24						X		
25						X		
26						X		
27							X	
28							X	
29							X	
30							X	
31							X	
	N=10	N=13	N=10	N=16	N=4	N=3	N=5	N=1