Glucocorticoid metabolism and the vascular response to injury

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Abstract

Atherosclerotic and restenotic lesions develop in the blood vessel wall as a result of chronic inflammatory and uncontrolled proliferative responses to injury. Glucocorticoids are steroid hormones which exert both anti-inflammatory and anti-proliferative actions in the body, and can interact directly with the vascular wall. The effect of these hormones is controlled, in part, by the tissue-specific pre-receptor generation of active steroid by the enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1). Whilst administration of exogenous glucocorticoids can inhibit restenosis, it is not known whether the availability of endogenous glucocorticoids influences the vascular response to injury. We hypothesised that endogenous glucocorticoids will directly inhibit vascular lesion formation, and that this effect will be modulated by 11β-HSD1.

In order to study the influence of glucocorticoids on vascular lesion formation, a model of intra-luminal, wire-induced vascular injury in the mouse femoral artery was developed. This model caused extensive stretching of the arterial wall and denudation of the endothelium, followed by the time-dependent formation of smooth muscle-rich neointimal lesions.

The effects of exogenous glucocorticoids on lesion development were assessed using different methods of administration. Systemic administration of dexamethasone by sub-cutaneous injection (1mg/kg/day, 21 days) reduced the size of smooth muscle-rich lesions after injury, but also promoted the formation of large thrombotic lesions. These occluded the lumen, leading to a similar reduction in luminal diameter to that seen in vehicle-treated controls. In contrast, local application of cortisol at the vessel wall via sustained release from an implanted pellet (21 days), significantly reduced neointimal lesion growth when compared to contra-lateral vehicle controls, without the development of thrombotic lesions. The influence of endogenous glucocorticoid activity on neointimal proliferation in the femoral artery was assessed using administration of a glucocorticoid receptor antagonist (RU38486, via implanted pellet for 21 days) or via selective pharmacological inhibition of 11β–HSD1 (60mg/kg/day via oral gavage, 14 days) and the use of mice with transgenic disruption of 11β–HSD1. Neither glucocorticoid receptor antagonism nor

11β–HSD1 inhibition or deletion significantly altered neointimal lesion development after injury.

These results indicate that exogenous glucocorticoids do inhibit neointimal proliferation in this model but their effectiveness depends upon whether they are administered systemically or locally at the vessel wall. Furthermore, it was demonstrated that metabolism of endogenous glucocorticoids within the artery wall does not significantly regulate neointimal proliferation in response to injury. Therefore, whereas the manipulation of endogenous glucocorticoid generation in the vessel wall may not represent a novel therapeutic target, the application of glucocorticoids locally at this site may be beneficial in the treatment of arterial remodelling.

Declaration

I declare that I have written this thesis and that the data presented represent my own work, with the exceptions listed below:

Ms Eileen Miller performed immunohistochemical staining for Mac-2, and Ms Margaret Ross performed immunohistochemical staining for proliferating cell nuclear antigen.

Ms Jill Harrison carried out measurement of plasma cortisol levels by radioimmunoassay.

Study of arterial function 14 days after wire-induced injury was performed by Mr Nick Kirkby.

Femoral artery injury operations were carried out jointly with Dr Patrick Hadoke, who also carried out independent analysis of arterial sections from animals treated with systemic dexamethasone.

I declare that this work has not been submitted for any other degree.

Linsay Joanne Macdonald

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List of abbreviations

A	11-dehydrocorticosterone
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of Variance
ANP	Atrial natriuretic peptide
ApoA-1	Apolipoprotein A-1
ApoE	Apolipoprotein E
AVP	Arginine vasopressin
B	Corticosterone
bFGF	Basic fibroblast growth factor
BrdU	5-bromo-2deoxyuridine
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft
C/EBP	CAAT/ enhancer-binding protein
CHD	Coronary heart disease
CPM	Counts per minute
CRB	Corticosteroid-binding protein
CREB	cAMP responsive element-binding protein
CRH	Corticotrophin releasing hormone
CRP	C-reactive protein
CVD	Cardiovascular disease
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EEL	External elastic lamina
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
F	Cortisol
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive element
HB-EGF	Heparin-binding epidermal growth factor

HCl	Hydrochloric acid
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic pituitary adrenal
HPLC	High performance liquid chromatography
11β-HSD	11β-hydroxysteroid dehydrogenase
ICAM-1	Intercellular cell adhesion molecule-1
IEL	Internal elastic lamina
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
KPSS	High potassium physiological salt solution
LDL	Low density lipoprotein
LPS	Lipopolysachharide
MCP-1	Macrophage chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NA-K	10^{-5}M noradrenaline in KPSS
NFκB	Nuclear factor κB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reactions
PDGF	Platelet derived growth factor
PET	Positron emission tomography
PSS	Physiological salt solution
RNA	Ribonucleic acid
SAME	Syndrome of apparent mineralocorticoid excess
S.E.M	Standard error of the mean
SPA	Scintillation proximity assay
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TNF	Tumour necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor

List of publications and presentations

Reviews

Hadoke PW, Macdonald L, Logie JJ, Small GR, Dover AR & Walker BR. (2006) Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function. *Cell Mol.Life Sci.* 63: 565-578.

Original research

Dover AR, Hadoke PW, Macdonald LJ, Miller E, Newby DE & Walker BR. (2007) Intravascular glucocorticoid metabolism during inflammation and injury in mice. *Endocrinology* 148: 166-172.

Presented abstracts

Macdonald LJ, Walker BR & Hadoke PW. (2006) Dexamethasone inhibits neointimal proliferation after wire-induced injury of the mouse femoral artery. Poster presented at Scottish Cardiovascular Forum, Strathclyde University, Glasgow.

Macdonald LJ, Walker BR & Hadoke PW. (2006) Dexamethasone alters the composition of neointimal lesions in mouse femoral artery. *Microcirculation* 13(6): 511-34. Poster presented at meeting of British Microcirculation Society, Dundee University.

Macdonald LJ, Dover AR, Miller E, Walker BR & Hadoke PW. (2006) Intra-vascular glucocorticoid metabolism by 11β-hydroxysteroid dehydrogenase type 1 following arterial injury. *Hypertension* 48(4): 756-85. Poster presented at meeting of European Council for Cardiovascular Research, Nice, France.

Macdonald LJ, Dover AR, Miller E, Walker BR & Hadoke PW. (2007) Effect of injury and inflammation on vascular 11β-hydorxysteroid dehydrogenase type 1 activity. Poster presented at Scottish Cardiovascular Forum, Queen's University, Belfast.

Macdonald LJ, Walker BR & Hadoke PW. (2007) Local glucocorticoid administration decreases luminal narrowing following injury in the mouse femoral artery. Selected oral presentation at International Networking for Young Scientists workshop, Bratislava, Slovakia.

Macdonald LJ, Walker BR & Hadoke PW. (2007) Local, but not systemic, glucocorticoid administration reduces luminal narrowing following injury in the mouse femoral artery. Poster presented at meeting of American Endocrine Society, Toronto, Canada.

Chapter 1

Introduction

Cardiovascular disease is the single biggest cause of death and disability in developed countries. Central to its pathogenesis is the development of arterial lesions, a process thought to occur in response to chronic injury of the vascular wall. Since localised inflammation and cellular proliferation are key features of this vascular response to injury, pharmacological manipulations of inflammation and proliferation have been identified as potential therapeutic approaches to modulating lesion development. Glucocorticoid hormones have widely-recognised anti-inflammatory and anti-proliferative properties which appear to make them ideal candidates for inhibition of vascular lesion development. However, although administration of glucocorticoids to experimental animals does inhibit the growth of vascular lesions in some models, systemic excess or increased tissue sensitivity to these hormones in humans is associated with *increased* cardiovascular risk (e.g. central obesity, insulin resistance, dyslipidemia and hypertension). Recent demonstrations that glucocorticoid activity is regulated by pre-receptor metabolism in target tissues, and indications that modulation of this pathway can reduce cardiovascular risk, make it increasingly important to clarify the mechanisms through which glucocorticoids regulate vascular remodelling in response to injury. This will improve our understanding of both the role of endogenous glucocorticoids in regulating cardiovascular risk and lesion pathogenesis, and the therapeutic potential of manipulating glucocorticoid activity for the treatment of cardiovascular disease. This introduction will consider the mechanisms that contribute to arterial remodelling in response to injury, the physiological role of glucocorticoids and the evidence that glucocorticoid activity regulates development of vascular lesions.

1.1 The vascular response to injury

Vascular lesion development in response to injury is implicated in a variety of conditions (including atherosclerosis, restenosis and chronic graft rejection) and its clinical significance is indisputable. Diseases of the heart and circulatory system (cardiovascular disease or CVD) are the principle cause of death in the developed world, including the United States, Europe and much of Asia (Ross 1999). In the United Kingdom CVD, leading to myocardial infarction, heart failure, stroke and peripheral vascular disease, is responsible for 36% of all deaths each year (www.heartstats.org). About half of these deaths are caused by coronary heart

disease (CHD), a condition which is itself the most common cause of death in the U.K., affecting almost 2.6 million people in this country (www.heartstats.org). Although mortality from CHD is currently falling, death rates in the U.K. are still amongst the highest in Western Europe, whilst morbidity from this condition is rising (www.heartstats.org). Furthermore, the rapid increase in the incidence of cardiovascular risk factors, such as obesity and diabetes, threatens to reverse the current decrease in CHD-related mortality. Consequently, CVD continues to command a high cost in terms of human suffering and economic burden. It is essential to improve current understanding of the factors that contribute to the development of CVD and identify new therapeutic options for treatment of this condition. CHD is caused by the formation of lipid-laden atherosclerotic lesions in the coronary circulation. In order to understand the vascular remodelling that occurs during atherogenesis, it is necessary to consider the structure of the healthy arterial wall and the functional role of its component layers.

1.1.1 The structure of the normal artery wall

The healthy arterial wall comprises three distinct layers (Figure 1.1a).

1.1.1.1 Tunica intima

The innermost layer of the arterial wall, which lines the vascular lumen, predominantly consists of a monolayer of endothelial cells seated on a basement membrane, and is separated from the tunica media by the internal elastic lamina (IEL). In some arteries, the intima can be more extensive, containing focal accumulations of modified vascular smooth muscle cells (VSMCs) (Stary 1990). Intimal thickening occurs during normal development and aging (Velican & Velican 1976). The endothelial cells are crucial to the regulation of vascular tone, as they can release a variety of vasodilators (e.g. nitric oxide (NO), prostacyclin (Furchgott & Zawadzki 1980; Ignarro *et al.* 1987; Palmer *et al.* 1987)) and vasoconstrictors (e.g. endothelin-1, thromboxane). In addition, the endothelium acts as a barrier between the blood and pro-thrombotic components of the vascular wall, and is an important modulator of inflammation and new blood vessel growth (Levick 2003).

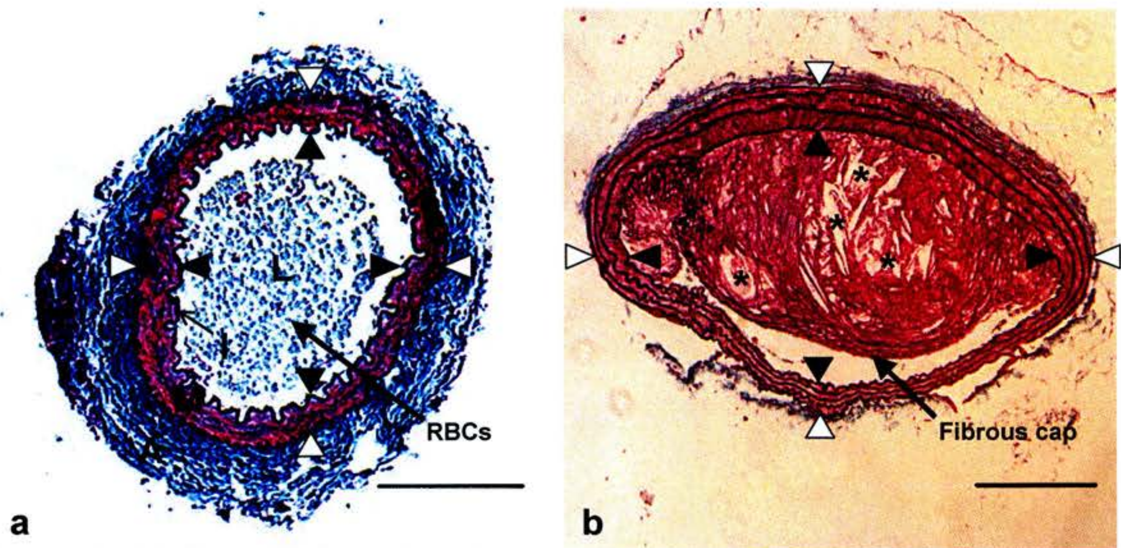


Figure 1.1: Structure of the artery wall in health and disease

(a) Transverse section of a normal mouse femoral artery. The tunica adventitia (A) lies outside of the external elastic lamina (EEL, marked by white arrow heads), while the tunica media (M) lies between the EEL and internal elastic lamina (IEL, marked by filled arrow heads). The tunica intima (I) is difficult to see in this artery, since it consists of only a monolayer of endothelial cells lining the surface of the lumen (L). Red blood cells (RBCs) are present in the lumen of the artery.

(b) Transverse section of the brachiocephalic artery from an Apolipoprotein E deficient mouse, which is susceptible to the development of atherosclerosis when fed a high fat diet. In this high fat-fed animal, an atherosclerotic lesion blocks the lumen of the artery. The lesion contains fibrous material and sites of lipid incorporation (*), and the luminal surface is covered by a fibrous cap. Medial remodelling is also evident in this artery.

Scale bar = 100 µm. Both sections are stained with the United States trichrome histological stain (elastin, purple; collagen, blue/green; smooth muscle, pink).

1.1.1.2 Tunica media

The middle layer of the vessel wall consists of VSMCs and supporting extracellular matrix (ECM). Medial VSMCs respond to stimulation by blood-borne signals, factors released by the endothelium and neurotransmitters released by nerve endings to regulate luminal diameter. In this way they regulate vascular tone and, hence, blood flow and pressure. The ECM consists of concentric sheets of elastin that enable the vessel to expand and recoil, in order to accommodate blood ejected from the heart and convert pulsatile flow in arteries close to the heart into a continuous flow through more distal arteries (Levick 2003). The ECM also contains collagen, which is important in the maintenance of vessel shape and integrity, and prevents over-expansion of the artery wall (Levick 2003).

1.1.1.3 Tunica adventitia

The outermost layer of the vessel wall is separated from the media by the external elastic lamina (EEL). It consists of connective tissue, VSMCs, fibroblasts and macrophages. The adventitia can also contain nerve cells and small blood vessels (vasa vasorum), which supply the vessel wall with nutrients (Levick 2003). This layer gives the blood vessel stability and strength, and connects it to surrounding tissues. In addition to its supportive role, the adventitia can also regulate vascular structure and function. For example, NO can be released from the adventitia via the action of inducible nitric oxide synthase (iNOS) (Muller *et al.* 2000) and neuronal nitric oxide synthase (nNOS) (Pluta 2006). In addition, perivascular adventitial fat can influence vascular function through the release of an (as yet unidentified) adventitial-derived relaxing factor (Dubrovska *et al.* 2004) and vasoactive adipocytokines (a mechanism that may contribute to the relationship between visceral obesity and vascular disease (Yudkin *et al.* 2005)). The adventitia may also influence vascular remodelling, as it has been proposed that proliferation and migration of adventitial fibroblasts contributes to the development of atherosclerotic lesions (Xu *et al.* 2007).

1.1.2 Atherosclerosis

As mentioned above, atherosclerosis is the most clinically significant manifestation of vascular lesion development. Atherosclerosis is primarily a disease of the large and medium-sized elastic and muscular arteries, and is characterised by the

accumulation of lipids and fibrous material within the arterial wall (Figure 1.1b). Development of atherosclerotic lesions (or plaques) usually occurs silently, over several decades, and throughout the vascular network. When symptoms do occur they are predominantly due to interruption of the blood supply, with their precise nature dependent upon the site of lesion development. For example, arterial occlusion in the coronary circulation results in loss of blood supply to the myocardium, which can produce angina or myocardial infarction, the hallmarks of CHD. The mechanisms underlying interruption of blood flow in atherosclerotic arteries can include gradual occlusion of the lumen by an expanding lesion, and spasm of the arterial wall. However, the current opinion is that rupture of unstable plaques, leading to acute and potentially occlusive thrombosis, is the most clinically significant mechanism of vascular occlusion associated with atherosclerosis (Weissberg 2000).

Atherosclerotic plaques are classically described on the basis of pathological studies (Stary 1989; Wissler 1992; Badimon *et al.* 1993) and are usually divided into three main types of lesion (Ross & Glomset 1976a; Ross & Glomset 1976b): the fatty streak, the fibrous plaque and the complicated lesion. They are predominantly intimal in nature (Wissler 1992), although their development is often accompanied by medial remodelling or atrophy (Davies & Woolf 1993).

1.1.2.1 The fatty streak

The fatty streak is commonly observed in early life, and can be found in the aorta of virtually every child from the age of 10 years, regardless of race, sex or environment (Stary 1983). It is characterised by its yellow colour, due to lipid deposition in the artery wall, and often occurs at vascular branch points and sites of pre-existing 'cushions' of intimal VSMCs (Ross & Glomset 1976a). Although originally described as focal accumulations of lipid-containing smooth muscle cells and macrophages (Ross & Glomset 1976a), the fatty streak has more recently been described as an inflammatory lesion (Ross 1999), consisting primarily of macrophages and T lymphocytes (Stary *et al.* 1994). These lesions cause little or no obstruction, produce no clinical symptoms, and are only detected at post mortem.

1.1.2.2 The fibrous plaque

The fibrous plaque is characteristic of progressing atherosclerosis. It consists principally of a lipid-laden core, often containing some necrotic material, covered by a fibrous cap of VSMCs, collagen fibres and proteoglycans (Badimon *et al.* 1993). The development of fibrous plaques in anatomical locations associated with fatty streaks in earlier life (Leary 1951; Robertson *et al.* 1963), and the suggestion that fibrous plaques are preceded by an intermediate (or fibrofatty) lesion (Wissler 1992), prompted the proposal that fatty streaks are the precursors of fibrous plaques (McGill 1984). More recently, however, it has been proposed that the process of plaque development is dynamic, with lesions in a constant, but clinically silent, flux between formation and regression (Weissberg 2000).

1.1.2.3 The complicated lesion

The complicated lesion distinguishes advanced atherosclerosis and can be thought of as a fibrous plaque that has undergone necrosis, calcification, rupture and focal thrombosis (Wissler 1992). As described above, the clinical consequences of atherosclerosis have been attributed to progressive occlusion of the vascular lumen which, in the coronary circulation, can cause chronic stable angina. However, lesion development does not always lead to vascular occlusion, as atherosclerotic arteries can undergo outward remodelling, or compensatory enlargement, to accommodate lesion growth (Figure 1.2) (Glagov *et al.* 1987). During this process, which involves the degradation and deposition of ECM molecules (Ward *et al.* 2000), an increase in cross-sectional area within the EEL preserves luminal patency as the lesion expands; this continues until later stages of the disease. Consequently, large lesions can be asymptomatic, and are not visible using imaging techniques such as angiography, which are based on measurement of the lumen.

It is now widely accepted that plaque rupture and consequent thrombosis (Weissberg 2000) are the more clinically significant mechanisms of vascular occlusion. In this model, plaque stability is not determined primarily by size or degree of luminal obstruction, but by composition. Collagen provides most of the biomechanical strength of the fibrous cap, and plaques with a thick cap are more able to resist local mechanical stresses created by blood flow and pressure (Libby 2000).

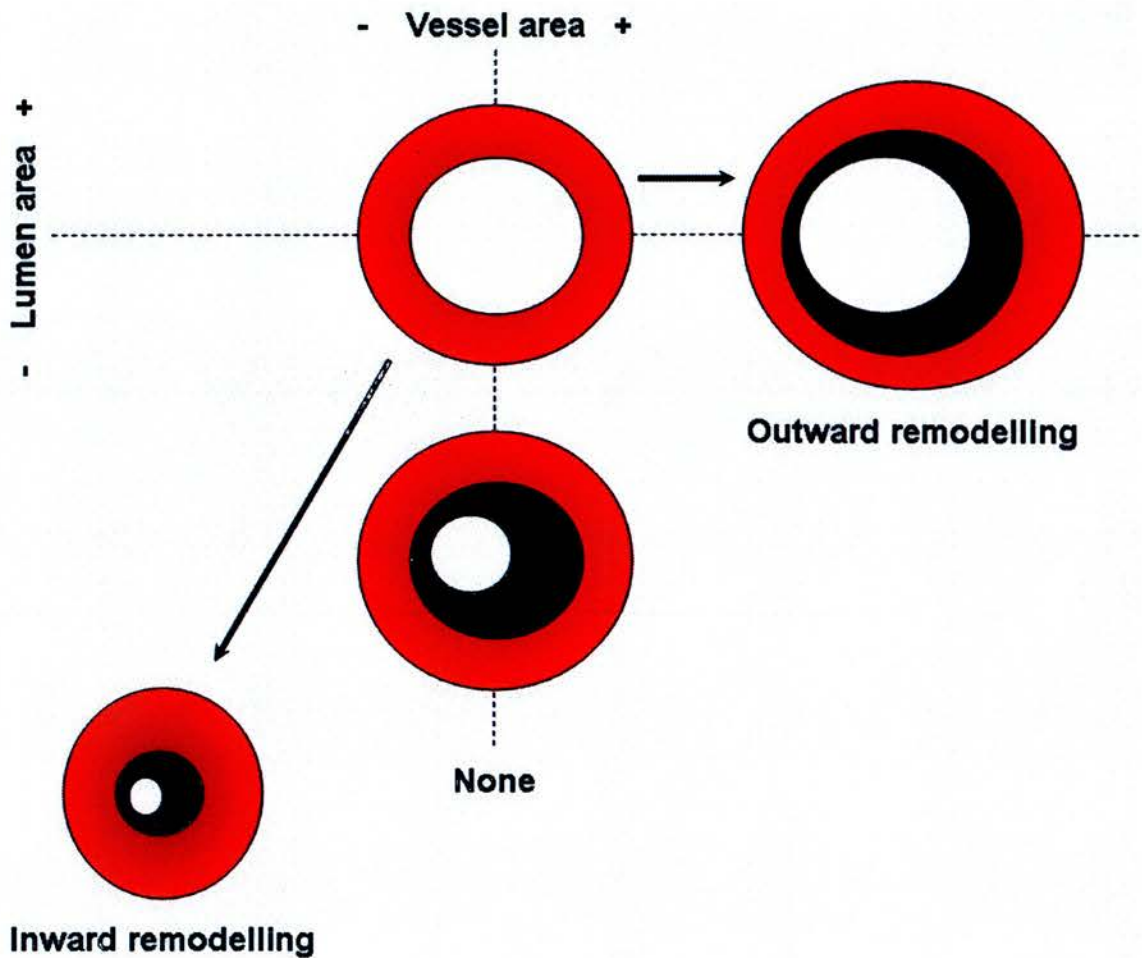


Figure 1.2: Artery remodelling during vascular lesion growth

During the growth of atherosclerotic lesions (indicated by black area), an increase in vessel area via outward remodelling can preserve luminal patency. If no artery remodelling occurs during vascular lesion growth, luminal area is decreased. During vascular healing after plaque rupture, or restenosis in response to injury caused by coronary revascularisation interventions, inward remodelling of the vessel wall may occur. This causes a reduction in vessel area which, along with vascular lesion growth, decreases luminal area. Adapted from (Ward *et al.* 2000).

However, a high content of inflammatory cells and a lack of VSMCs, particularly at the edges (or shoulder regions) of a plaque, can contribute to instability (Lendon *et al.* 1991; van der Wal *et al.* 1994). Therefore, the likelihood of rupture is greatest in lesions with a large lipid pool and a thin fibrous cap, containing more inflammatory cells than VSMCs and collagen (Libby 2000). Over the last ten years, these observations have led to the new paradigm which proposes that 'vulnerable' lesions provide the greatest risk of morbidity and mortality from atherosclerosis (Weissberg 2000). Therefore, the development of interventions which increase lesion stability represents a current area of investigation for the treatment of atherosclerosis. For example, the therapeutic benefit of treating existing atherosclerosis with statins is thought to be due, at least in part, to their ability to increase plaque stability (Libby & Aikawa 2003).

1.1.2.4 Pathogenesis of atherosclerosis: the response to injury hypothesis

The slow and silent development of atherosclerosis has made it difficult to study; consequently, its pathogenesis is not completely understood. The most widely accepted hypothesis proposes that lesion development is the result of an excessive wound-healing response to injury in the blood vessel wall. This hypothesis developed from the work of Von Rokitansky and Virchow in the mid 19th century, was refined and compared with alternative hypotheses by Ross & Glomset in 1976 (Ross & Glomset 1976a; Ross & Glomset 1976b), and has subsequently been extensively updated and revised (Ross 1986; Ross 1993; Ross 1999).

Initiation of vascular injury. The response-to-injury hypothesis proposes that atherosclerosis begins as the result of a wound-healing response that has spiralled out of control. This process is influenced by genetic pre-disposition to the condition, and occurs in response to injury induced by local formation of harmful compounds after exposure to risk factors for atherosclerosis (which include cigarette smoking, diabetes mellitus, hyperlipidemia and hypertension). Initially, damage to the endothelium stimulates an inflammatory response, and the incorporation of lipid into the vascular wall, leading to fatty streak formation.

The initiating injury (the precise nature of which remains unclear) induces endothelial dysfunction, including an increase in endothelial cell adhesiveness and

permeability, allowing plasma constituents to pass into the sub-endothelial space. Increased adhesiveness is mediated by expression of surface-bound cell adhesion molecules such as P-selectin (Johnson *et al.* 1997) and vascular cell adhesion molecule-1 (VCAM-1) (Li *et al.* 1993). Monocytes and lymphocytes adhere to the endothelium and migrate into the sub-endothelial space in response to chemoattractant chemokines such as macrophage chemoattractant protein-1 (MCP-1) (Gu *et al.* 1998). Increased transport of lipids across the endothelium also occurs, during which lipid oxidation can occur. Monocytes within the intima mature into macrophages, under the influence of MCP-1 and macrophage colony-stimulating factor (M-CSF) (Smith *et al.* 1995). Macrophages accumulate oxidized lipid via uptake through scavenger receptors to become foam cells (Brown & Goldstein 1983), which are the hallmark of the fatty streak. Activated macrophages and T cells express a variety of cytokines (such as interferon γ (IFNγ), tumour necrosis factor α and β (TNFα/ β), and interleukin-1 (IL-1)) that recruit and activate further leukocytes, and stimulate endothelial cells and VSMCs within the vessel wall to produce additional inflammatory mediators (Libby 2000). These processes lead to the reversible formation of a fatty streak, containing lipid and leukocytes. However, with continued exposure to risk factors for atherosclerosis, this inflammatory response can not effectively interrupt and repair chronic injury to the vessel wall, and it continues indefinitely.

Fibrous plaque development. Lesion progression involves both propagation of the initial inflammatory response, and additional, excessive wound-healing responses in the arterial wall. Continued injury and inflammation produces a cascade of proliferative and chemotactic factors that promote activation of VSMCs, and their migration to, and proliferation in, the intima. This results in the development of a fibrous plaque with a smooth muscle cell-rich cap. VSMCs within the intimal lesion change phenotype from a 'contractile' state into a 'synthetic' state and secrete ECM components, which contribute to cap formation (Chamley-Campbell & Campbell 1981). The predisposition for lesions to develop at sites with pre-existing 'cushions' of intimal VSMCs makes it difficult to determine the origin of the VSMCs that form the fibrous cap (Ross & Glomset 1976a). Indeed, it is not clear whether these

VSMCs originate predominantly in the intima or the media, or, alternatively, are derived from cells circulating in the blood (Dzau *et al.* 2002).

Proliferation and migration of VSMCs is stimulated by a variety of growth factors (including platelet derived growth factor (PDGF) (Ferns *et al.* 1991), basic fibroblast growth factor (bFGF) (Lindner & Reidy 1991), insulin-like growth factor (IGF) (Bornfeldt *et al.* 1992; Bornfeldt *et al.* 1994), epidermal growth factor (EGF) (Mitsumata *et al.* 1994) and angiotensin II (Geisterfer *et al.* 1988; Berk *et al.* 1989)) released by vascular and inflammatory cells within the lesion. Increased expression of cytokines, such as TNFα and IL-1, by leukocytes also contributes to the induction of VSMC proliferation (Ikeda *et al.* 1990; Fukumoto *et al.* 1997; Selzman *et al.* 1999).

The original hypothesis that VSMC proliferation was a key initiating event during atherogenesis (Ross & Glomset 1976a; Ross & Glomset 1976b) has given way to the belief that it represents an important reparative response to inflammation. VSMC migration and proliferation at the site of injury, with subsequent release of ECM proteins such as collagen and elastin, is central to the production of a fibrous cap (Newby & Zaltsman 2000). This cap separates the highly thrombogenic lipid core of the plaque from the blood, and also confers structural stability to the lesion, and therefore prevents plaque rupture and focal thrombosis.

Progression to complicated lesion. Further expansion and development of the fibrous plaque will produce a complicated lesion with characteristic necrosis, calcification, thinning of the fibrous cap and protrusion into the lumen (Wissler 1992). This may be accompanied by loss of overlying endothelial cells, thrombosis on the lesion surface, fissuring and plaque rupture.

Continuation of the inflammatory response, which increases the numbers of macrophages and T lymphocytes in the lesion, exacerbates the release of hydrolytic enzymes, chemokines, cytokines, and growth factors (Ross 1999). This causes further damage and, eventually, focal necrosis. For example, inflammatory cytokines such as IFNγ can induce macrophage apoptosis, contributing to the development of a necrotic core (Inagaki *et al.* 2002). The lesion increases in size and becomes structurally modified, with the fibrous cap covering a core of lipid and necrotic

tissue. Generation of this characteristic advanced, complicated lesion is often accompanied by loss of outward remodelling (Figure 1.2) of the vessel wall (Glagov *et al.* 1987). As a result, the lesion protrudes into the vascular lumen and restricts blood flow. These advanced lesions may be subject to silent, non-occlusive episodes of plaque rupture and thrombosis (Weissberg 2000), resulting in platelet adhesion and activation, PDGF release and thrombin formation. PDGF and thrombin act to cause further VSMC recruitment, proliferation and ECM synthesis (Fingerle *et al.* 1989; Ferns *et al.* 1991; Kanthou *et al.* 1995; Molloy *et al.* 1996), and thus stimulate the formation of a new fibrous cap over the thrombus. In this way, atherosclerotic plaques can grow in size due to repeated episodes of rupture and repair (Weissberg 2000).

As discussed above, many clinical consequences of atherosclerosis are the result of plaque rupture and occlusive thrombosis (Libby 1995). Thrombosis occurs when plaque contents come into contact with circulating clotting factors. Tissue factor is the main mediator of thrombosis in atheroma. It is produced by macrophages in response to stimulation by T cell-derived CD40 (Mach *et al.* 1997), and also by endothelial cells (Bevilacqua *et al.* 1984) and VSMCs (Schecter *et al.* 1997) exposed to inflammatory cytokines such as IL-1 and TNFα, highlighting a link between inflammation and thrombosis in atherosclerosis (Libby & Simon 2001). Resultant clots can produce sudden occlusion of the vascular lumen which, in the coronary arteries, leads to myocardial infarction.

The susceptibility of plaque shoulder regions to rupture is increased when inflammatory cell content is high in these areas (Davies 1996). Inflammatory cells can decrease plaque stability in several ways: (i) inflammatory cytokines can increase the expression of matrix metalloproteinase (MMP) enzymes, which degrade collagen in the cap (Galis *et al.* 1994a), and activated macrophages within the lesion can produce these enzymes (Galis *et al.* 1994b), (ii) T cells can provoke activation of macrophages, and MMP production, by cell-cell contact through CD40 ligand (Mach *et al.* 1997), (iii) inflammatory cytokines such as IFNγ inhibit VSMC proliferation and collagen synthesis (Amento *et al.* 1991), and (iv) the inflammatory cytokines IFNγ, IL-1β and TNFα all induce apoptosis in VSMCs (Geng *et al.* 1996), whilst activated macrophages can induce VSMC apoptosis by direct cell-cell contact (Boyle

et al. 2001). In this way inflammation plays an active role in mediating plaque stability.

1.1.2.5 Current treatments for atherosclerosis

Treatment options for atherosclerosis can be broadly divided into primary prevention, secondary prevention and treatment of symptoms. Lifestyle change (including weight loss, dietary modification, stopping smoking and increased exercise) is an important aspect of primary prevention. In patients with established disease (or those with an increased risk of developing atheroma), these strategies can be augmented by pharmacological treatments (for example, to improve plasma lipid profiles or reduce blood pressure). Medical interventions (including vasodilators such as sublingual glyceryl trinitrate) are also used for the treatment of clinical complications of CHD, such as stable and unstable angina. Thrombolytic and anti-platelet drugs are used to treat myocardial infarction, in an attempt to open the blocked coronary artery.

Surgical intervention is often necessary in patients with severe angina or obstruction of the coronary circulation. Essentially, this involves bypassing the occluded area using a coronary artery bypass graft (CABG) formed from the great saphenous vein or the internal mammary artery (Favaloro 1968). Alternatively, an internal thoracic artery can be anastomosed with the blocked coronary artery. A less invasive alternative to CABG is percutaneous coronary intervention (PCI), based on the technique of percutaneous transluminal balloon angioplasty originally introduced by Andreas Gruntzig (Gruntzig *et al.* 1979). This procedure has grown in importance, and has been developed by the introduction of laser catheters, atherectomy catheters and stents. Its aim is to widen the arterial lumen and improve blood flow to tissue downstream of the atherosclerotic lesion, via the insertion of an intra-luminal device. This is achieved by compression or removal of the plaque, and can also involve stretching of the artery wall (Gruntzig *et al.* 1979). PCI was greatly improved by the introduction of coronary stents, which act as a scaffold to hold the artery open, and has become an increasingly important interventional approach to arterial occlusion. Indeed, in 2005 over 70,000 PCI procedures were carried out in the U.K. alone (www.heartstats.org). The rise in the use of PCI has occurred despite the procedure being limited by some significant acute and chronic complications. The most

important of these is chronic restenosis, which involves inward remodelling of the arterial wall (Figure 1.2) and the growth of fibroproliferative neointimal lesions in response to vascular injury, and so leads to re-occlusion of the arterial lumen. Interestingly, the neointimal development involved in restenosis has provided a useful model for investigating the mechanisms of arterial lesion formation in response to vascular injury (discussed below in section 1.1.4).

1.1.3 Restenosis

Initial success using balloon angioplasty for the revascularisation of atherosclerotic arteries has been limited by three major complications (Serruys *et al.* 1988): (i) acute dissection of the artery wall (requiring emergency CABG), (ii) acute re-occlusion due to elastic recoil, and (iii) chronic restenosis, which generally occurs in 30–50% of patients within 6 months of the initial procedure (Serruys *et al.* 1988; Bourassa *et al.* 1989). The introduction of intra-coronary stents has been highly effective in reducing these complications, by decreasing the need for emergency CABG (Al *et al.* 2000) and reducing early elastic recoil and chronic inward remodelling in nearly all patients (Fischman *et al.* 1994; Hoffmann *et al.* 1996). In-stent restenosis, however, remains a significant problem, affecting approximately 20% of patients (Welt & Rogers 2002). This is perhaps not surprising, since stents have been shown to induce increased proliferation of VSMCs (Edelman & Rogers 1996) and the creation of a more intense and prolonged inflammatory state (Welt & Rogers 2002). Therefore, stents can actually cause an increase in neointimal lesion formation.

Restenosis is the re-occlusion of an artery following PCI for the treatment of atherosclerosis. It involves the rapid development of fibroproliferative neointimal lesions in response to the acute injury caused by revascularisation. Since PCI techniques can produce severe damage to the vessel wall (including endothelial denudation, transient stretching, and medial fracture (Edelman & Rogers 1996)) the inflammatory and proliferative response to injury is more rapid than in atherosclerosis. The use of stents can cause a more vigorous neointimal response as this induces deep, focal trauma to the media at sites where the struts penetrate into the vessel wall (Edelman & Rogers 1996). Indeed, the extent of this injury predicts subsequent neointimal lesion formation (Schwartz *et al.* 1992b). In addition, the stent also produces a permanent mechanical stretch on the artery wall, and remains as

foreign material in the vessel, triggering an inflammatory response (Welt & Rogers 2002). Understanding the pathogenesis of the neointimal lesion formation that underpins restenosis is important for the development of new anti-restenotic treatments. However, neointimal proliferation also provides a rapid model for investigating the mechanisms regulating the vascular response to injury.

1.1.3.1 Pathogenesis of neointimal lesion formation

The formation of neointimal lesions after revascularisation of a plaque-bearing artery during restenosis has a similar mechanism to the neointimal proliferation which is induced in response to experimental mechanical injury of a previously normal artery. In addition, both processes incorporate components of the chronic wound-healing response to the less severe injury that is central to atherosclerosis. Neointimal proliferation comprises a complex series of events involving cells of the artery wall and blood (VSMCs, endothelial cells, leukocytes and platelets). In a simplified model (Lee *et al.* 1993), neointimal lesions are thought to form in three phases (Figure 1.3). The first, acute phase involves the interaction of platelets, thrombin and leukocytes with the site of injury, the subsequent release of a range of biologically active signals, and the resulting activation of VSMCs. A second, intermediate phase follows, characterised by proliferation of VSMCs and their migration to the intima. A final, chronic phase involves phenotypic modulation of intimal VSMCs, which produce large amounts of ECM molecules, contributing to the formation of a fibroproliferative neointimal lesion.

Endothelial damage and medial stretch injury expose the underlying ECM of the vessel wall, which induces the adhesion of platelets. Activation of tissue factor and thrombin generation (Fager 1995) causes further platelet activation and the release of PDGF, which acts as a potent chemoattractant for VSMCs during lesion development (Fingerle *et al.* 1989; Ferns *et al.* 1991). In addition to PDGF, various other growth factors up-regulated after vascular injury have been implicated in inducing VSMC proliferation, including: bFGF (released directly from injured VSMCs) (Lindner & Reidy 1991; Olson *et al.* 1992), IGF-1 (Cercek *et al.* 1990; Bornfeldt *et al.* 1992), thrombin (Kanthou *et al.* 1995; Molloy *et al.* 1996),

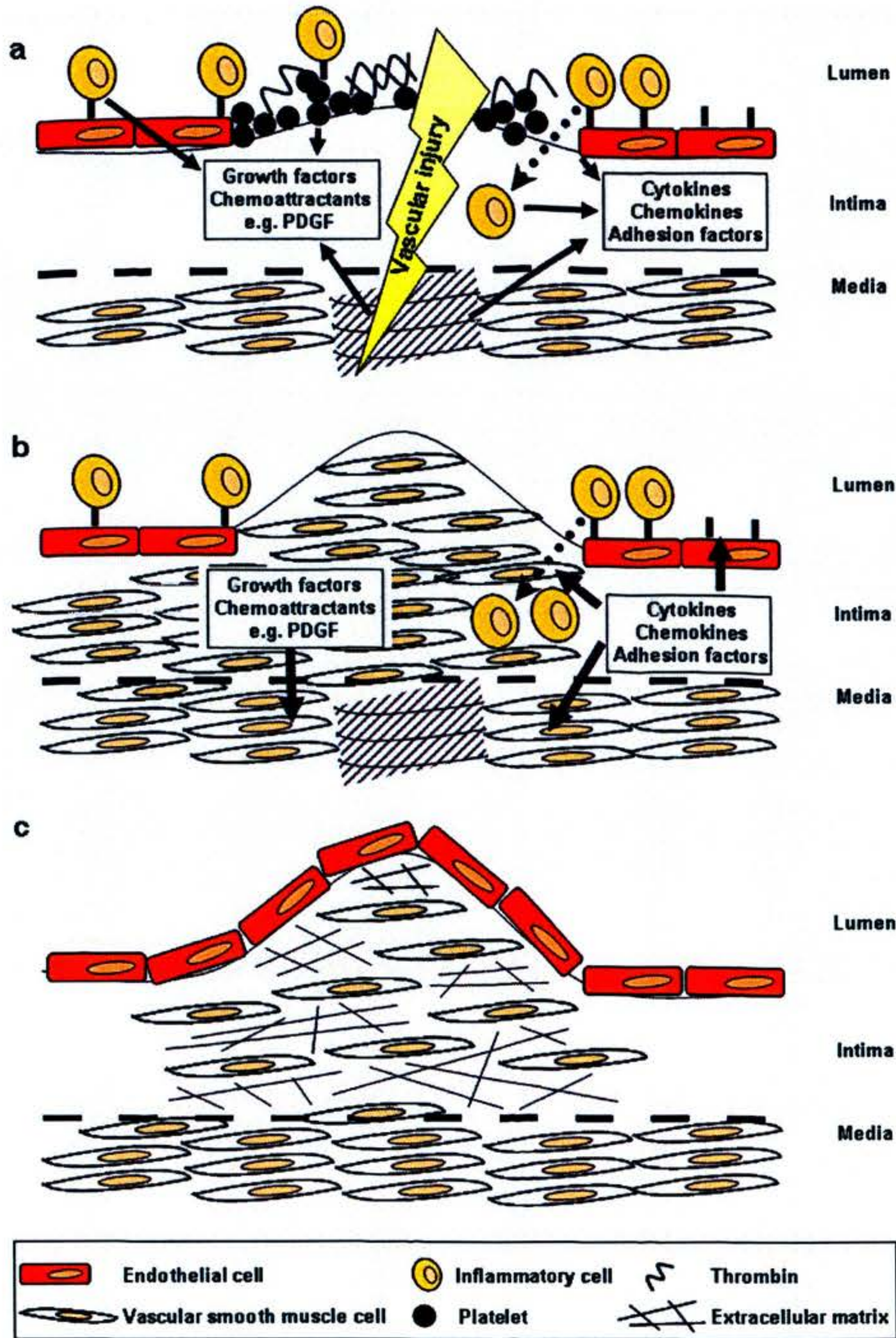


Figure 1.3: Neointimal lesion formation in response to vascular injury

Acute vascular injury causes endothelial denudation and damage to the underlying media (hatched area). Lesion formation is initiated by interaction of platelets, thrombin and inflammatory cells with the site of injury, and the release of various biologically active signals (a). These factors stimulate the proliferation and migration of VSMCs in the neointima, and further activation of inflammation (b). Finally, VSMCs in the intima secrete extracellular matrix molecules to produce a stable fibrous lesion (c). Re-endothelialisation may also occur.

endothelin-1 (Weissberg *et al.* 1990) and angiotensin II (Powell *et al.* 1989; Daemen *et al.* 1991). Whilst thrombin generated in response to injury can act as a mitogen for VSMCs, the development of platelet-rich mural thrombi over stent struts is also thought to act as a 'scaffold', into which inflammatory cells and VSMCs migrate (Edelman & Rogers 1996). In addition to growth factors, other mediators involved in ECM remodelling, such as heperanases and proteases, are activated after vascular injury. For example, MMPs are up-regulated after balloon angioplasty (Bendeck *et al.* 1994; Zempo *et al.* 1994; Southgate *et al.* 1996), and contribute to VSMC migration by degrading ECM molecules (Jackson & Reidy 1992).

The recruitment of leukocytes induced by endothelial denudation or medial damage, and subsequent activation of inflammatory mechanisms, plays a key role in neointimal lesion development (Wainwright *et al.* 2001; Welt & Rogers 2002; Toutouzas *et al.* 2004). Studies using animal models show that leukocyte infiltration into the vessel wall occurs rapidly following injury, with neutrophils observed within 6-24 hours (Cole *et al.* 1987; Welt *et al.* 2000), and T lymphocytes (Tanaka *et al.* 1993) and monocytes (Miller *et al.* 1996) appearing after 2-14 days. Inflammatory cell recruitment and infiltration is mediated by: (i) interactions with surface molecules on adherent platelets (P-selectin, Mac-1) at the site of injury (Diacovo *et al.* 1996), (ii) release of chemokines such as MCP-1 (Rollins 1996) and IL-8 (Webb *et al.* 1993) from VSMCs, endothelial cells and leukocytes at the site of injury, and (iii) up-regulation of endothelial adhesion molecules (Tanaka *et al.* 1993; Kennedy *et al.* 2000a). The central role of this inflammatory response in subsequent lesion development is illustrated by the demonstration that leukocyte depletion virtually abolishes neointimal formation following balloon angioplasty in rabbits (Miller *et al.* 2001).

Clinical evidence also supports a critical role of inflammation in neointimal lesion formation during restenosis. Leukocytes are activated in patients within 24-48 hours of angioplasty (De Servi & *et al.* 1990; Steg *et al.* 1993), with marked increases in the plasma concentrations of leukocyte- and endothelium-derived soluble adhesion molecules evident (Inoue *et al.* 1996; Neumann *et al.* 1996; Mickelson *et al.* 1996; Serrano, Jr. *et al.* 1997; Inoue *et al.* 2003). Furthermore, elevated plasma levels of cytokines and chemokines (Pietersma *et al.* 1995; Hojo *et al.* 2000; Suzuki *et al.*

2000; Cipollone *et al.* 2001) and C-reactive protein (CRP) (Gaspardone *et al.* 1998; Gottsauner-Wolf *et al.* 2000; Almagor *et al.* 2003) predict a high risk of restenosis. Circulating monocyte counts after stenting show a positive correlation with neointimal lesion area 6 months after PCI (Fukuda *et al.* 2004). Interestingly, in patients with a genetic polymorphism resulting in increased production of the natural antagonist of IL-1 (IL-1ra), there is a lower risk of restenosis (Kastrati *et al.* 2000). Expression of platelet and leukocyte surface adhesion molecules are increased by coronary stent implantation when compared with balloon angioplasty (Inoue *et al.* 2000), which may explain the increased neointimal lesion formation seen after stenting. In pathological studies, markers of inflammation in arterial tissue correlate strongly with restenosis after angioplasty or stenting (Moreno *et al.* 1996; Farb *et al.* 2002). In addition to inflammatory responses triggered by PCI, the inflammatory status of the underlying atherosclerotic plaque being treated must also be taken into account. Indeed, correlations between pre-interventional inflammatory activation and post-interventional restenosis or adverse cardiac events have been found in several sub-groups of patients (Toutouzas *et al.* 2004).

There are several mechanisms by which activated inflammatory cells may contribute to neointimal lesion formation. These cells release a wide range of vasoactive substances, including cytokines, chemokines, growth factors, proteolytic enzymes and oxygen-derived free radicals, all of which can contribute to the vascular response to injury. The expression of the cytokines TNFα (Tanaka *et al.* 1996) and IL-1β (Wang *et al.* 2000) is increased after balloon angioplasty in animal models; these cytokines induce smooth muscle cell proliferation *in vitro* (Ikeda *et al.* 1990; Selzman *et al.* 1999) and neointimal lesion growth in normal porcine coronary arteries (Fukumoto *et al.* 1997). Furthermore, the anti-inflammatory cytokine IL-10 attenuated macrophage infiltration and VSMC proliferation after balloon injury or stenting in rabbits (Feldman *et al.* 2000). Cytokine release also causes the further activation of leukocytes, and an increase in the adhesiveness of endothelial cells, promoting leukocyte adherence and migration into the intima (Furie & McHugh 1989; Moser *et al.* 1989; Miller *et al.* 2005).

1.1.4 Models for studying the vascular response to injury

The development of atherosclerosis without symptoms and over several decades makes investigation of lesion generation in humans extremely difficult (reviewed in (Toutouzas *et al.* 2004)). Information on lesion pathology obtained from post-mortem and atherectomy specimens provides only a 'snapshot' in development. Vascular imaging techniques (e.g. angiography, intra-vascular ultrasound, magnetic resonance imaging (MRI), positron emission tomography (PET)) can provide information on luminal narrowing and vascular lesion size, but give little or no information on the molecular, biochemical and cellular events involved in lesion formation. However, current advances in techniques such as MRI may allow a more detailed cellular and molecular characterisation of atherosclerosis in the near future (Canet-Soulas & Letourneur 2007). Vascular imaging can confirm the success or failure of anti-atherosclerotic and anti-restenotic treatments, but provides no information on the processes involved in the outcome (Toutouzas *et al.* 2004). In addition, many imaging techniques are invasive and so would be impractical and unethical to repeat frequently in longitudinal studies. Longitudinal studies are also hampered by the extended duration of lesion development, and the fact that the insidious development of atherosclerosis makes it difficult to identify a suitable patient population. As a consequence, clinical studies require large groups of patients and a long time-scale, making them labour intensive and expensive. They can also be difficult to control (due to environmental and genetic differences between individuals) and, if data are acquired from patients selected for coronary revascularisation, a bias towards high-risk subjects can be introduced (Toutouzas *et al.* 2004).

The practical obstacles to clinical investigations into the pathogenesis of atherosclerosis and restenosis have resulted in much of our knowledge of these processes being gained from animal models of vascular lesion formation. The advantage of animal models is that lesion development can be induced more rapidly than in humans, and conditions can be controlled more closely than in clinical studies (Smith & Breslow 1997). However, it is important to acknowledge that no animal model provides a perfect reproduction of vascular lesion development in humans. Indeed, specific features of different animal models will determine their suitability

for a particular investigation, and need to be considered carefully when extrapolating findings to the condition in humans.

1.1.4.1 Choice of species

Species used in experimental models of vascular lesion formation can be broadly divided into large animals, such as the pig, dog and non-human primate, and small animals, including the rabbit, rat and mouse (Mehta *et al.* 1996; Narayanaswamy *et al.* 2000). The main advantages of large animals are their relative physiological similarity to humans and the ability to assess clinically relevant arteries (i.e. the coronary circulation). Practical factors that limit the use of these species include lower availability compared with small animals, higher purchase and maintenance costs and greater difficulties associated with husbandry and handling. In addition, lesion development may be slower than in small animals and, significantly, there may be increased ethical concerns (particularly with the use of primates). Small animals are increasingly favoured as they are readily available, cost relatively little to purchase and house, are easy to handle and their small size reduces the amount of drug needed for intervention studies. The rapid growth of lesions allows a quick assessment of therapeutic agents, and it is possible to include large numbers in each group for more powerful statistical analysis. Practical disadvantages include the small size of arteries studied, the need to assess lesion formation in arteries that are not as relevant to the clinical situation (e.g. femoral, iliac), and poor replication of the condition in humans. However, perhaps the most significant advantage of using small animals, in particular mice, for investigations of lesion growth stems from developments in gene targeting techniques. Over the last decade, this has resulted in the generation of an array of transgenic and knock-out mice (Allayee *et al.* 2003). Transgenic techniques have been used to generate mice that are prone to the development of atherosclerosis, such as the low density lipoprotein (LDL) receptor and Apolipoprotein E (ApoE) knockout mice (Breslow 1993). Furthermore, these techniques make it possible to assess lesion development in animals with transgenic deletion of key factors involved in the vascular response to injury. This allows the molecular mechanisms involved in arterial lesion formation to be investigated at a genetic level.

1.1.4.2 Choice of disease model

Animal models of vascular lesion development have been generated which replicate features of atherosclerosis, induce neointimal proliferation in previously normal arteries, or attempt to model neointimal lesion development during restenosis.

Classically, atherogenesis has been induced in pigs, primates and rabbits by prolonged feeding with a high cholesterol diet (although some animals, such as the Watanabe heritable hyperlipidemic rabbit, develop spontaneous lesions (Watanabe 1980)). However, the lesions that form in rabbits rarely advance beyond the fatty streak stage (Prior *et al.* 1961), and so only replicate the early stages of atherosclerosis. In addition, rats and mice do not even develop these early lesions of atherosclerosis when fed a high-fat diet (Vesselinovitch & Wissler 1968), as a result of differences in lipid handling compared with humans (Paigen *et al.* 1994). Despite this, the mouse has become an increasingly popular model of atherosclerosis, following the introduction of single gene deletion strains which can develop hypercholesterolemia and atherosclerotic lesions. The two most widely-used murine models of atherosclerosis are the ApoE deficient (Piedrahita et al. 1992) and LDL receptor knockout (Ishibashi *et al.* 1993) mice. The former spontaneously develop atherosclerotic lesions that can be exacerbated by feeding a high fat 'western' diet (Paigen *et al.* 1987). ApoE serves as a ligand for LDL receptor-mediated removal of chylomicrons and very low density lipoproteins (VLDL) from the blood. Consequently, mice lacking ApoE develop hypercholesterolemia and atherosclerotic lesions (Plump et al. 1992; Zhang et al. 1992) which are morphologically similar to human lesions and occur at similar sites in the vasculature (Nakashima et al. 1994; Reddick et al. 1994; VanderLaan et al. 2004). Similarly, LDL receptor knockout mice are hypercholesterolemic (Ishibashi *et al.* 1993) and develop lipid-laden lesions with a necrotic core when fed a high-fat diet (Ishibashi *et al.* 1994). These two strains are now the basis of many investigations into the mechanisms of atherosclerotic lesion formation.

Neointimal proliferation can be induced in animal models by acute injury to the artery wall, and this approach has been used in a variety of different species (Mehta *et al.* 1996; Narayanaswamy *et al.* 2000; Xu 2004; Wang *et al.* 2006). These models mimic the injury caused to the blood vessel wall by angioplasty and stenting, in the

absence of a pre-existing atherosclerotic lesion, and induce the rapid development of a fibroproliferative lesion. Consequently, they can be used as a tool to study specific biological factors, such as the role of inflammation in the vascular response to injury and the mechanisms of VSMC proliferation and migration during lesion formation. These models have also been used extensively to assess the therapeutic potential of anti-restenotic interventions.

Attempts have been made to improve study of the pathogenesis and treatment of restenosis in mice by combining models of atherosclerosis and acute injury (Leidenfrost *et al.* 2003; Weingartner *et al.* 2005), in which injury is performed in arteries with existing lesions. This is important as it is likely that the arterial response to injury differs in diseased vessels (Carmeliet *et al.* 1998). Inevitably, however, these models are technically more demanding and their validity is doubtful given that the initial atherosclerotic lesion is likely to be a fatty streak (Lardenoye *et al.* 2000; Ivan *et al.* 2002), rather then the advanced lesion that would be subjected to PCI in a patient. This combination of atherogenesis and mechanical injury has also been used in rabbits to produce lesions that more closely resemble human plaques (Abela *et al.* 1995; Skinner *et al.* 1995; Aikawa *et al.* 1998). However, this advantage is offset by the complexity of the model (high fat feeding combined with repeated intra-luminal injury) and the time required for lesions to develop (9-12 months).

1.1.4.3 Induction of acute injury to the arterial wall

Animal models of acute vascular injury use a variety of techniques to damage the wall and induce neointimal proliferation in several different arteries. These were originally developed in large animals (primarily pigs and dogs), and subsequently applied to smaller species (rabbits and rats). More recently, they have been refined for use in mice. This has occurred despite the anatomical and physiological differences between mice and humans, and the structural differences of the mouse arterial wall (which is thin, with only 2-3 layers of VSMCs, few intimal VSMCs and a narrow lumen (Carmeliet *et al.* 1998)). The four most commonly used techniques in the mouse (Table 1.1) are: (i) intra-luminal mechanical injury, (ii) arterial ligation, (iii) electrical injury, and (iv) cuff placement. Each model induces either intra-vascular or perivascular injury, which results in the generation of an inflammatory response followed by neointimal proliferation.

	Mechanical injury	Ligation	Electrical injury	Cuff placement
Endothelial removal	Yes	No	Yes	No
Medial injury	Yes	No	Yes	No
Blood flow	Not changed	Changed	Not changed	Not changed
Thrombotic involvement	Some	Possible	High	None
Inflammatory response	Medium	Low	Extensive	Low
Difficulty of surgery	Hard	Low	Low	Medium
Examples of genes studied	P-selectin, VEGF, ApoE, ApoA-1	MMP-2 and -9, eNOS	PAI-1	IL-1ra, VLDLR

Table 1.1: Comparing four different models of vascular injury in the mouse

The most commonly used models of arterial injury in the mouse are intra-luminal mechanical injury (Lindner *et al.* 1993; Sata *et al.* 2000), ligation of the carotid artery (Kumar & Lindner 1997), electrical injury across the wall of the femoral artery (Carmeliet *et al.* 1997c) and cuff placement around the femoral artery (Moroi *et al.* 1998). Components of the injury induced and surgical difficulty for each model are compared. Examples of genes with a role in the response to injury studied using each model are also given. P-selectin (Smyth *et al.* 2001), VEGF: vascular endothelial growth factor (Hutter *et al.* 2004), ApoE: apolipoprotein E (De *et al.* 1997b; Weingartner *et al.* 2005), ApoA-1: apolipoprotein A-1 (De *et al.* 1997b), MMP: matrix metalloproteinase (Kuzuya *et al.* 2003; Galis *et al.* 2002), eNOS: endothelial nitric oxide synthase (Kawashima *et al.* 2001), PAI-1: plasminogen activator inhibitor-1 (Carmeliet *et al.* 1997b), IL-1ra: interleukin-1 receptor antagonist (Isoda *et al.* 2003), VLDLR: very low density lipoprotein receptor (Tacken *et al.* 2002). Adapted from (Xu 2004; Wang *et al.* 2006).

Intra-luminal mechanical injury. This method was originally introduced in the mouse by Lindner *et al*, using a flexible guidewire inserted into the common carotid artery (Lindner *et al.* 1993). Subsequently, it was applied to the femoral artery (Roque *et al.* 2000; Sata *et al.* 2000) whilst more recently, a method of balloon injury to the mouse aorta has been developed (Petrov *et al.* 2005). Intra-luminal injury causes endothelial denudation, and stretches the artery wall inducing disruption of the IEL and damage to VSMCs in the media. Early responses to injury include the adherence of platelets and neutrophils to the artery surface (Lindner *et al.* 1993; Roque *et al.* 2000; Petrov *et al.* 2005), and medial cell apoptosis (Sata *et al.* 2000; Petrov *et al.* 2005). Neointimal lesions are first evident from 7-8 days after injury (Lindner *et al.* 1993; Sata *et al.* 2000), and continue to grow in size until around 28 days, after which time they stabilise (Sata *et al.* 2000). These lesions are VSMC-rich (Lindner *et al.* 1993; Sata *et al.* 2000; Petrov *et al.* 2005), and become re-endothelialised from about 3 weeks after injury (Lindner *et al.* 1993; Sata *et al.* 2000).

In models of intra-luminal vascular injury, it is important that the artery targeted is: (i) relatively accessible, (ii) free from branches, (iii) large enough in diameter to introduce an intra-vascular device, and (iv) long enough to provide a sufficient area for lesion growth. The mouse carotid artery produces only a relatively small neointimal proliferative response to intra-luminal injury, with lesions around 2-3 cells thick (Lindner *et al.* 1993). In addition, these lesions are less reproducible and have a higher inter-animal variability than reported for models of femoral artery injury (Sata *et al.* 2000). This may be due in part to the challenging surgery involved in cannulating the carotid artery, which also increases the risk of complications such as stroke. In contrast, femoral arteries produce large, occlusive neointimal lesions in response to injury, with less adventitial remodelling than is seen in the carotid artery (Lindner *et al.* 1993; Sata *et al.* 2000). Aortic angioplasty in the mouse has two main advantages over other models; it involves the use of a balloon catheter as in the clinical setting, and allows the recovery of sufficient quantities of intact ribonucleic acid (RNA) from injured vessels for molecular analysis (Petrov *et al.* 2005). However, unlike the carotid and femoral arteries, this model does not permit the use of a contra-lateral artery as a control.

Artery ligation. Kumar and Lindner developed a ligation model using the mouse carotid artery (Kumar & Lindner 1997). Ligation of the common carotid artery, at its bifurcation into the internal and external carotid arteries, is thought to induce remodelling in response to altered shear stress on the vessel wall (Kohler *et al.* 1991; Geary *et al.* 1994). Large, concentric VSMC-rich neointimal lesions develop 2-4 weeks after ligation (Kumar & Lindner 1997) without the extensive endothelial cell removal, mechanical trauma and thrombosis produced by intra-luminal injury. Early platelet and leukocyte infiltration, and medial VSMC death are followed by luminal narrowing, caused in part by neointimal lesion growth and in part by inward remodelling of the artery wall (Kumar & Lindner 1997). The main advantage of this model is that the surgery required is relatively simple, and gives good reproducibility and less variation in lesion development than occurs after wire injury of the carotid artery. The pathogenesis of lesion formation remains unclear but it is possible that changes in blood flow alter the expression of adhesion molecules and chemokines by endothelial cells and leukocytes, leading to the observed influx of inflammatory cells and the activation of an inflammatory response (Carmeliet *et al.* 1998).

Electrical injury. Passing an electrical current through the wall of the femoral artery produces severe damage, killing many cells across the vessel wall, including endothelial cells, VSMCs and adventitial fibroblasts (Carmeliet *et al.* 1997c). Early platelet- and fibrin-rich thrombosis, and removal of necrotic material by leukocyte infiltration, is followed by the proliferation and migration of VSMCs from regions of the vessel wall bordering the site of injury. This results in the formation of a re-endothelialised neointimal lesion 2 weeks after injury. Adventitial and outward vascular remodelling also take place, which prevents luminal narrowing despite neointimal growth. The main advantage of this procedure is that it avoids the technical challenge of inserting intra-vascular devices.

Cuff placement. Models of cuff placement used in the rat and rabbit were adapted for use in mice (Moroi *et al.* 1998). The femoral artery is isolated and loosely sheathed with polyethylene tubing, which does not restrict blood flow. A neointimal lesion is evident after 14 days, comprising VSMCs, with minimal thrombosis, cell death or endothelial cell denudation. The mechanisms leading to lesion formation are incompletely understood but studies in rabbits have indicated that it may result from

endothelial cell activation, distortion of the artery, vasospasm, increased blood flow velocity, blockage of lymphatic drainage, damage to adventitial nerves and vasa vasorum (causing hypoxia of the media) or toxicity of the cuff (Kockx *et al.* 1992). It has been suggested that leukocytes play a central role in activation of the VSMC response (Carmeliet *et al.* 1998). This model can be extended by using drug-eluting cuffs, to allow local drug administration during lesion formation (Pires *et al.* 2005).

Other models. Acute vascular injury in the mouse has also been induced via endothelial denudation by air-drying the carotid artery (Simon *et al.* 2000), and endothelial denudation and occlusive thrombus generation by injection of rose bengal and irradiation of the femoral artery (Kikuchi *et al.* 1998). In addition, several mouse models of vein grafting have been introduced (Xu 2004), which allow the study of mechanisms of neointimal hyperplasia after CABG.

1.1.4.4 Advantages and disadvantages of animal models of vascular lesion formation

The main advantages of larger animal models are their susceptibility to experimental atherosclerosis, their physiological similarity to humans, accessibility of a clinically relevant artery, and a vascular remodelling process which is similar to that seen in humans. For example, pigs (Buchwald *et al.* 1992), and some non-human primates (Strong *et al.* 1994; Fincham *et al.* 1998), develop atherosclerosis in response to dietary manipulation; however dogs do not (Lafont & Faxon 1998). (The similarity of canine and human coronary artery morphology has led to dogs being used primarily for testing interventional cardiology devices (Roubin *et al.* 1987; Schatz *et al.* 1987)). In addition, the response to vascular injury and neointimal lesion composition in pigs (Schwartz *et al.* 1990), dogs (Lafont & Faxon 1998) and primates (Geary *et al.* 1996) is also very similar to that seen in humans.

The clinical relevance of large animal models is demonstrated by their ability to predict the effect of therapeutic interventions in humans. For example, the success of both probucol (Setsuda *et al.* 1993; Watanabe *et al.* 1996) and irradiation (Teirstein *et al.* 1997; Condado *et al.* 1997) in clinical trials was consistent with reduced neointimal growth in porcine models (Schneider *et al.* 1993; Waksman *et al.* 1995). However, this consistency with clinical studies is not always seen, with the

anti-proliferative action of low molecular weight heparin observed in pigs (Buchwald *et al.* 1992), not recapitulated in baboons (Geary *et al.* 1995) or humans (Faxon *et al.* 1994). This type of discrepancy is probably due to physiological differences between species. In pigs, for example, the haemostatic system differs from that in humans (Johnson *et al.* 1999), whilst in primates it is more similar to humans (Hanson 1991).

The major drawback of small animal models is that they have failed to predict the effectiveness of a number of pharmacological interventions in clinical trials (reviewed in (Johnson *et al.* 1999)), having produced both false positive and false negative results. For example, although angiotensin converting enzyme (ACE) inhibitors reduced neointimal lesion formation in rats (Powell *et al.* 1989) and rabbits (Shibutani *et al.* 1994), clinical studies found no effect on restenosis in humans (Faxon 1995). Several possibilities have been suggested to explain this shortcoming, including differences in: (i) species, (ii) size of artery used, (ii) structure of artery used (elastic vs. muscular), (iv) the protocol of drug administration and, (iv) the method used to assess outcome (Johnson *et al.* 1999). Alternatively, the poor predictive ability of small animal models may be attributable to their failure to replicate the pathophysiology of neointimal growth during restenosis in humans. For example, in the well-characterised model of balloon injury to the rat carotid artery (Clowes *et al.* 1983) the response to injury is limited to VSMC proliferation and migration, with no involvement of thrombosis and the absence of an underlying atherosclerotic lesion (Mehta *et al.* 1996). Similarly, although hyperlipidemic rabbits develop atherosclerotic lesions (Block *et al.* 1980) these are mainly composed of foam cells, and lack other features (e.g. fibromuscular caps) of human plaques (Prior *et al.* 1961). Indeed, the neointimal lesions that develop after balloon injury in hyperlipidemic rabbits tend to be lipid-rich with a scarcity of VSMCs (Johnson *et al.* 1999).

Thus, large animal models, such as the atherosclerotic pig, produce the best representation of neointimal formation during restenosis in humans and, therefore, are valuable for pre-clinical validation of potential therapeutic strategies (Johnson *et al.* 1999). Small animal models, in contrast, are useful in the experimental study of specific components involved in the vascular response to injury (such as mechanisms

of inflammation or VSMC proliferation); however, results must be interpreted with caution when extrapolating to the human conditions of restenosis and atherosclerosis.

1.1.4.5 Mechanistic insights from murine models of neointimal lesion development

The powerful combination of genetic manipulation and arterial injury in the mouse has provided important insights into the cellular and molecular mechanisms of neointimal proliferation (Table 1.1). A role for inflammation, including activation of adhesion molecules, has been confirmed with the demonstration that lesion size is increased by deletion of IL-1ra (Isoda *et al.* 2003), but reduced in the absence of P-selectin (Smyth *et al.* 2001). Similarly, NO synthesis has been shown to regulate lesion formation; eNOS deletion is associated with increased lesion size (Moroi *et al.* 1998) whilst over-expression of this enzyme results in smaller lesions (Kawashima *et al.* 2001). Other processes that have been addressed include the action of MMPs (with deletion of MMP-2 (Kuzuya *et al.* 2003) or MMP-9 (Galis *et al.* 2002) causing a reduction in lesion size), growth factors (over-expression of vascular endothelial growth factor (VEGF) protects against neointimal formation (Hutter *et al.* 2004)) and the fibrinolytic system (showing that urokinase and plasminogen activator inhibitor-1 modulate lesion development (Carmeliet *et al.* 1997a; Carmeliet *et al.* 1997b)). Mouse models have also been used to address the suggestion that circulating progenitor cells contribute to lesion formation (Hibbert *et al.* 2003). This work has shown that the origin of neointimal cells is dependent upon the initial injury model (Tanaka *et al.* 2003). After wire-induced injury a significant number of neointimal and medial cells are derived from the bone marrow; fewer cells of this lineage are observed after ligation and bone marrow-derived cells are seldom observed in the arterial wall after cuff placement.

The role of plasma lipids in neointimal lesion formation has been addressed, for example by genetic deletion of the VLDL receptor (Tacken *et al.* 2002), which increased neointimal proliferation, and by gene transfer of apolipoprotein A-1 (ApoA-1), which increased high density lipoprotein (HDL) and attenuated lesion development (De *et al.* 1997b). An alternative approach has been to perform arterial injury in atherosclerosis prone (ApoE knockout) mice, which develop larger lesions

than controls (De *et al.* 1997b; Weingartner *et al.* 2005). Therefore, these studies indicate that dyslipidemia exacerbates lesion development following arterial injury.

1.1.5 Targeting inflammation in the treatment of vascular lesions

Various approaches for the treatment of vascular lesion development have been attempted, such as targeting VSMC proliferation, platelet adhesion and activation, and the generation of thrombin. However, the volume of evidence showing that chronic vascular injury produces a cascade of inflammatory and proliferative signals has led to a general acceptance over the last twenty years that vascular disease is an inflammatory condition (Ross 1999; Wainwright *et al.* 2001; Libby *et al.* 2002). Given this central role for inflammation, it seems logical that anti-inflammatory interventions would be effective inhibitors of atherogenesis and neointimal lesion development.

Inhibition of leukocyte adhesion and platelet interaction after vascular injury has been successful in animals. Indeed, attenuation of intercellular adhesion molecule-1 (ICAM-1) (Yasukawa *et al.* 1997), Mac-1 (Rogers *et al.* 1998) and P-selectin (Hayashi *et al.* 2000) binding with monoclonal antibodies reduces neointimal formation in a range of animal models. Furthermore, the response to injury is inhibited in ICAM-1 (Zou *et al.* 2000) and Mac-1 (Simon *et al.* 2000) deficient mice. This work is consistent with many clinical trials that have focused on the prevention of restenosis using anti-inflammatory approaches. Due to their pleiotropic mode of action, statins exert well-described anti-inflammatory effects in the vasculature (Forrester & Libby 2007), and have been administered systemically for the treatment of restenosis. Improvements in both clinical and angiographic outcomes have been observed after statin treatment (Mulder *et al.* 2000; Walter *et al.* 2000; Walter *et al.* 2001a), even in patients with normal cholesterol levels (Serruys *et al.* 2002). Several agents have also been administered locally at the vessel wall. For example, coating stents with agents such as the immunosuppressant drug sirolimus (Suzuki *et al.* 2001; Morice *et al.* 2002) and the mitotic inhibitor paclitaxel (Park *et al.* 2003; Stone *et al.* 2004), which also has anti-inflammatory properties, has been found to reduce neointimal inflammation and lesion formation. Since the inflammatory response to injury is prolonged after stenting, the use of stents as a platform for the sustained delivery of anti-inflammatory drugs is very attractive. Therefore, although some

previous trials have found a lack of efficacy when investigating the effect of potential anti-inflammatory agents on restenosis (Ellis *et al.* 1989), a better understanding of both the biological response to injury and the pharmacokinetics and pharmacodynamics of certain agents has resulted in more recent clinical successes with agents such as sirolimus and paclitaxel.

The potential benefit of targeting local inflammation for prevention of vascular lesion formation suggests that glucocorticoids may provide a therapeutic option, as these hormones have well-described anti-inflammatory effects. In addition, glucocorticoids are anti-proliferative, which could also contribute to reduced lesion development. Furthermore, since glucocorticoids are endogenous hormones, their activity, metabolism and availability in the vessel wall may be an important influence during the vascular response to injury. For these reasons, the current investigation focuses on the effect of glucocorticoids on the vascular response to injury, and so a detailed review of the biology of these hormones is appropriate.

1.2 Glucocorticoids

Glucocorticoids (cortisol (F) in man and corticosterone (B) in rodents) are steroid hormones synthesised from cholesterol in the adrenal cortex, and released from the adrenal gland into the systemic circulation.

1.2.1 Physiological actions of glucocorticoids

Glucocorticoids exert wide-ranging effects in the body, and regulate a number of important metabolic and homeostatic processes. They influence the cardiovascular, immune and central nervous systems, and have effects during growth and development. The most important physiological role of glucocorticoids is thought to be during the stress response (Munck *et al.* 1984), during which increased levels of hormone alter metabolism to increase blood glucose levels, thus preparing the body for the 'fight or flight' response. The main metabolic effects of glucocorticoids in organs such as the liver, adipose tissue, and muscle are to increase the conversion of stored energy (in glycogen, triglycerides and protein) into fuel (glucose, free fatty acids and amino acids) for mitochondrial oxidation; thereby opposing the effects of insulin (Dallman *et al.* 1993). They also induce the differentiation of pre-adipocyte

stromal cells into mature adipocytes (Hauner *et al.* 1987), promoting the accumulation of lipid. The physiological roles of these hormones have been clarified by clinical observations of the symptoms of adrenal disease. Glucocorticoid excess (e.g. in Cushing's syndrome) is characterised by central obesity, insulin resistance, hyperglycemia and hypertension. In contrast, glucocorticoid insufficiency (e.g. in Addison's disease) causes weight loss, loss of appetite, hypoglycemia and postural hypotension.

1.2.1.1 Anti-inflammatory and immunosuppressive actions

The anti-inflammatory and immunosuppressive effects of glucocorticoids are well described, and account for their most common therapeutic applications in the treatment of inflammation, autoimmune disease and the prevention of rejection of transplanted organs.

Endogenous glucocorticoids can regulate both the adaptive and innate immune systems, including the first line of defence: inflammatory responses. The anti-inflammatory actions of glucocorticoids are due to interactions with blood vessels, inflammatory cells and mediators of inflammatory responses (Barnes & Adcock 1993). By altering the transcription of several genes, they decrease expression of adhesion factors, chemokines, cytokines and their receptors, and increase the expression of anti-inflammatory mediators such as IL-10 and IL-1ra (Barnes 1998). Therefore, glucocorticoids alter the recruitment of immune cells such as neutrophils, granulocytes and monocytes to the site of inflammation, cause a decrease in leukocyte activation and proliferation, and thus suppress the initial inflammatory response. Furthermore, these hormones modify granulocyte apoptosis (Meagher *et al.* 1996), promote the phagocytosis of apoptotic leukocytes (Liu *et al.* 1999), and regulate the maturation of monocytes into highly phagocytic macrophages (Giles *et al.* 2001), promoting the resolution of inflammation (Heasman *et al.* 2003).

It should be noted that glucocorticoids are not exclusively anti-inflammatory agents, and that at physiological levels they may both suppress and stimulate inflammatory responses (Yeager *et al.* 2004). A model has been proposed where physiological levels of glucocorticoids exert permissive effects on inflammation at low levels, and stimulatory actions as concentrations increase during normal diurnal variation. At

high stress levels, or following pharmacological administration, glucocorticoids inhibit inflammation (Sapolsky *et al.* 2000). Therefore, the physiological actions of glucocorticoids are best described as immunomodulatory, rather than immunosuppressive.

1.2.1.2 Effects on the Cardiovascular System

Observations in patients with adrenal disease have indicated that normal physiological levels of glucocorticoids are needed to maintain normal blood pressure and fluid volume. These hormones increase blood pressure and fluid volume in a homeostatic way, in response to stimuli such as septic shock. However, the exact mechanisms responsible for regulation of these factors by glucocorticoids are not clear, since the effects of these hormones on the cardiovascular system are wide ranging, with cardiac, renal and vascular actions documented. Glucocorticoids can regulate cardiac output by influencing contractility and work performance of the heart. They also have an important role in the regulation of fluid and electrolyte balance, and increase renal sodium retention and plasma volume expansion by the kidney. This may be mediated via direct influences on sodium handling (Montrella-Waybill *et al.* 1991), or in-directly via the regulation of angiotensinogen production by the liver, arginine vasopressin (AVP) production by the hypothalamus (Raff 1987) and atrial natriuretic peptide (ANP) production by cardiac myocytes (Shields *et al.* 1988). All of these peptide hormones can influence salt and water handling in the kidney. Glucocorticoids also have direct effects on the tone of the vessel wall, modulating its sensitivity to vasoconstrictors and the production of vasodilators (reviewed in (Ullian 1999)). This possible contribution to blood pressure regulation, and a detailed examination of the effects of glucocorticoids on the blood vessel wall, is discussed later (section 1.3). Increases in circulating glucocorticoid levels above physiological concentrations can have adverse effects on the cardiovascular system (see section 1.2.6).

1.2.2 Regulation of glucocorticoid action

Due to their varied physiological functions throughout the body, the actions of glucocorticoid hormones are tightly regulated by several mechanisms.

1.2.2.1 The hypothalamic-pituitary-adrenal axis

Glucocorticoids are released from the adrenal cortex when required, under the control of the hypothalamic-pituitary-adrenal (HPA) axis. Release of corticotrophin-releasing hormone (CRH) (Vale *et al.* 1981) from the hypothalamus causes the stimulation of CRH receptors in the anterior pituitary, and the secretion of adrenocorticotrophic hormone (ACTH) into the systemic circulation (Horrocks *et al.* 1990). AVP can also stimulate ACTH release (Salata *et al.* 1988), and there is a synergistic interaction between CRH and AVP (Rivier & Vale 1983). ACTH acts on adrenal cells to stimulate steroid hormone biosynthesis, catalysed by members of the cytochrome P450 oxidative enzyme family, resulting in glucocorticoid secretion. It does so by regulating the first, rate-limiting step in the steroid biosynthetic pathway: the conversion of cholesterol to pregnenolone.

There is a basal release of glucocorticoids which shows a normal diurnal variation, with concentrations at a peak at the beginning of the active period. Stimulation of glucocorticoid release can also be produced by physiological stimuli such as stress, injury or infection, which increase the secretion of CRH. Glucocorticoids provide a negative feedback loop to regulate their own release and maintain physiological plasma levels (de Kloet 1991). They do so by exerting a negative effect on the HPA axis at the level of both the hypothalamus and the pituitary.

1.2.2.2 Regulation of circulating hormone concentrations

Glucocorticoids are lipophilic, which enables them to pass through cell membranes. However, circulating glucocorticoids are predominantly bound to corticosteroid-binding globulin (CBG) and albumin, and only the small proportion of free hormone in the plasma can enter cells. In this way, glucocorticoid-binding proteins act to regulate the bioavailability of glucocorticoids in the plasma.

1.2.2.3 Receptor-mediated glucocorticoid action

Glucocorticoids can bind to and activate the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), which are both members of the nuclear hormone receptor super family of ligand-activated transcription factors (Parker 1993). However, glucocorticoids exert most of their physiological effects through GR. In the absence of hormone, GR are present in the cytosol, in association with a complex

of heat shock proteins which maintain the receptor in an inactive state. Upon ligand binding, GR dissociates from these proteins, forms homodimers and translocates to the nucleus. GR homodimers can then bind specific deoxyribonucleic acid (DNA) sequences, called glucocorticoid-responsive elements (GRE) in target genes to initiate their transcription. In addition, GR is able to repress the expression of specific genes by several mechanisms, and can also modulate the expression of target genes via cross-talk with other transcription factors, including AP1, cAMP responsive element- binding protein (CREB) and nuclear factor κB (NFκB) (Auphan *et al.* 1995).

GR are ubiquitously expressed but expression levels vary between and within tissues (Reul & de Kloet 1985; Reul *et al.* 1989), contributing to tissue-specific sensitivity to glucocorticoids. GR levels are programmed perinatally, by environmental factors such as neonatal stress (de Kloet *et al.* 1990) or prenatal glucocorticoid exposure (Nyirenda *et al.* 1998). This process is regulated in a tissue-specific manner by the existence of alternative promoters/ exons 1 in the GR gene, which generate several GR messenger RNA (mRNA) variants (McCormick *et al.* 2000). In the adult, autoregulation of expression around its predetermined level by glucocorticoids allows short-term control of GR levels (Rosewicz *et al.* 1988); this does not involve differential mRNAs (Freeman *et al.* 2004).

There is increasing evidence that glucocorticoids can exert non-genomic actions; for example the rapid activation of eNOS, which is blocked by GR antagonism but not by inhibition of transcription (Hafezi-Moghadam *et al.* 2002; Limbourg *et al.* 2002). These effects are thought to be mediated by membrane-bound GR (Bartholome *et al.* 2004), although the associated intracellular signalling pathways have not been determined, or by cytosolic GR (Croxtall *et al.* 2002) in association with molecular chaperones acting as signalling components. However, it remains uncertain whether non-genomic receptor-mediated responses to glucocorticoids have any physiological relevance (Tasker *et al.* 2006).

1.2.3 Glucocorticoid metabolism

Hormone metabolism provides additional control of glucocorticoid activity. The peripheral metabolism of glucocorticoids results in conversion of active steroid into inactive metabolites, and provides a means of clearing these hormones from the circulation. Metabolism of glucocorticoids (Figure 1.4) occurs mainly in the liver and involves reduction, oxidation, hydroxylation and conjugation reactions; the resultant metabolites are then excreted by the kidney. Inter-conversion of corticosterone (B) with its inactive 11-keto metabolite 11-dehydrocorticosterone (A) is catalyzed by the two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 2); the subsequent metabolism of corticosterone and 11-dehydrocoticosterone then follow similar paths, leading eventually to their excretion.

1.2.3.1 The 11β-hydroxysteroid dehydrogenase enzymes

The 11β-HSDs are microsomal enzymes of the short-chain alcohol dehydrogenase super family (Stewart & Krozowski 1999). The inter-conversion of active glucocorticoids with their inert 11-keto forms by 11β-HSD was first described over fifty years ago (Amelung *et al.* 1953), but its significance has only become apparent more recently.

The importance of 11β-HSD activity was demonstrated in patients with the rare, congenital 'syndrome of apparent mineralocorticoid excess' (SAME), which occurs as a result of mutations in the 11β-HSD2 gene (Stewart *et al.* 1996). The symptoms of excessive MR activation (severe hypertension associated with sodium retention, low plasma renin and hypoalkemia) in SAME, despite low circulating concentrations of its natural ligand aldosterone, were shown to be due to a congenital deficiency of 11β-HSD. This results in impaired inactivation of cortisol to cortisone (Ulick *et al.* 1979; Shackleton *et al.* 1985; Monder *et al.* 1986) and inappropriate binding of glucocorticoids to MR in the distal nephron, confirming the link between 11β-HSD activity and apparent mineralocorticoid excess (Edwards *et al.* 1988; Funder *et al.* 1988). Inactivation of cortisol by 11β-HSD activity also explained why MR, which have similar affinities for aldosterone and cortisol (Funder *et al.* 1988), preferentially bind aldosterone despite the presence of glucocorticoids at 100 fold higher

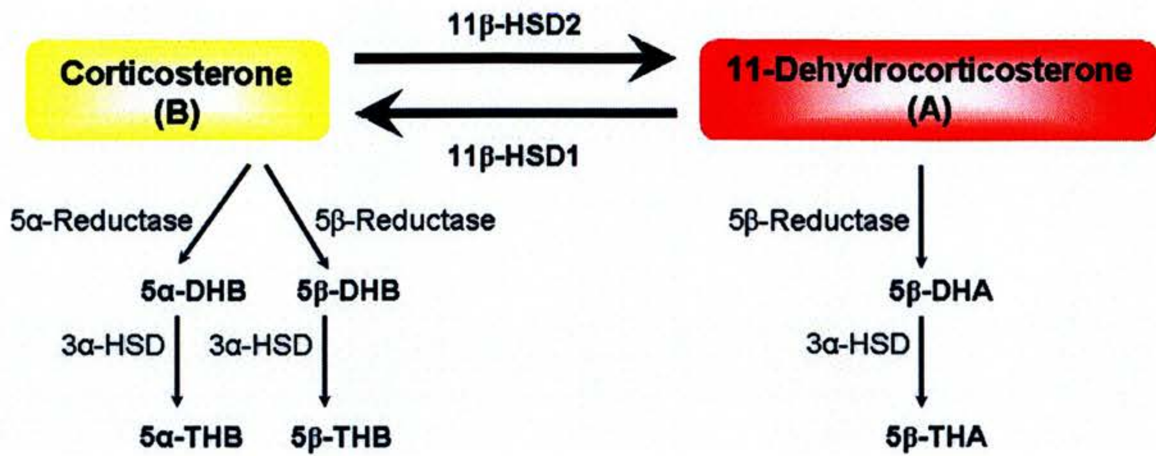


Figure 1.4: Metabolism of corticosterone

This pathway involves the inter-conversion of active (B) and inactive (A) hormone by the isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). Reduction by 5α- or 5β-reductase produces dihydro metabolites (5α/5β-DHB, 5β-DHA) that are then rapidly converted to tetrahydro metabolites (5α/5β-THB, 5β-THA) by 3α-hydroxysteroid dehydrogenase (3α-HSD). These metabolites are then conjugated and excreted from the body.

circulating concentrations (Sheppard & Funder 1987). Thus, it was proposed that the physiological role of 11β-HSD was to protect MR from inappropriate activation by glucocorticoids, by pre-receptor conversion of cortisol to cortisone.

Subsequent studies identified two isozymes of 11β-HSD. The first isozyme to be cloned (Agarwal *et al.* 1989) and purified from liver had a Km in the μM range for both cortisol and corticosterone (Lakshmi & Monder 1988), and did not co-localise with MR in the kidney (Edwards *et al.* 1988; Rundle *et al.* 1989; Stewart *et al.* 1991). This tissue distribution, and the fact that MR have a Kd in the nM range for glucocorticoids, indicated that this hepatic enzyme could not be responsible for the protection of MR from cortisol in the distal nephron of the kidney. A second isozyme of 11β-HSD, with nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase activity and a Km for cortisol and corticosterone in the nM range, was later identified in the aldosterone target cells of the kidney (Naray-Fejes-Toth *et al.* 1991; Mercer & Krozowski 1992; Rusvai & Naray-Fejes-Toth 1993; Stewart *et al.* 1994). Cloning of this 11β-HSD type 2 isozyme confirmed limited tissue distribution consistent with a role in protection of MR (Agarwal *et al.* 1994; Albiston *et al.* 1994). The first enzyme cloned from rat liver became known as 11β-HSD1. The physiology of both isozymes has subsequently been explored in greater detail, particularly since the generation of mice with selective transgenic disruption of each isozyme (Kotelevtsev *et al.* 1997; Kotelevtsev *et al.* 1999).

Physiology of 11β-HSD1. 11β-HSD1 is a low affinity nicotinamide adenine dinucleotide phosphate (NADP)-dependent enzyme. It has a wide expression pattern, being found in many glucocorticoid target tissues including the liver, lung, adipose tissue, brain, vascular smooth muscle, skeletal muscle, anterior pituitary, gonads and adrenal cortex (Stewart & Krozowski 1999; Tomlinson *et al.* 2004). 11β-HSD1 is under complex regulatory control. Factors that alter its synthesis and activity *in vivo* or *in vitro* include stress, sex steroids, growth hormone, inflammatory cytokines, peroxisome proliferator activated receptor agonists and glucocorticoids themselves (Stewart & Krozowski 1999; Tomlinson *et al.* 2004). The CAAT/ enhancer-binding protein (C/EBP) family of transcription factors represent one major mechanism by which the expression of 11β-HSD1 is regulated in the liver (Williams *et al.* 2000). In addition, its expression may be altered in a tissue-specific manner via the existence

of at least three alternative promoters in the 11β-HSD1 gene with differential expression patterns (Moisan *et al.* 1992; Bruley *et al.* 2006).

Original indications suggested that 11β-HSD1 was bi-directional, with both dehydrogenase and reductase activities observed *in vitro* (Monder & Lakshmi 1989). However, dehydrogenase activity of 11β–HSD1 is now attributed to release of enzyme from damaged or dying cells in culture, and dissociation from hexose-6-phosphate dehydrogenase, which maintains NADP concentrations needed for reductase activity (Bujalska *et al.* 2005; Hewitt *et al.* 2005). In intact cells or organs *in vitro* and *in vivo* (including liver (Jamieson *et al.* 1995; Jamieson *et al.* 2000), adipose tissue (Bujalska *et al.* 1997), neurones (Rajan *et al.* 1996) and vascular smooth muscle (Brem *et al.* 1995)), 11β-HSD1 acts predominantly as a reductase, regenerating active hormone from inactive 11-keto metabolites (Figure 1.4). These results are supported by studies in 11β-HSD1 deficient transgenic mice, which cannot convert 11-dehydrocorticosterone to corticosterone *in vivo* (Kotelevtsev *et al.* 1997).

11β-HSD1 reactivates glucocorticoids in tissues with few MR but abundant GR, suggesting that the physiological role of 11β-HSD1 is to amplify local glucocorticoid concentrations in glucocorticoid target tissues (Seckl & Walker 2001). For example, in the liver this enzyme might be important in maintaining local glucocorticoid levels even when plasma levels are low. This has been supported by several studies; 11β-HSD1 knockout mice show impaired gluconeogenic responses to fasting (Kotelevtsev *et al.* 1997) and pharmacological inhibition of 11β-HSD in humans results in enhanced insulin sensitivity (Walker *et al.* 1995a). In the lung, its other major site of expression, 11β-HSD1 plays a role in surfactant production (Hundertmark *et al.* 2002), and in the detoxification of substances such as tobacco-derived carcinogens (Maser & Oppermann 1997). Amplification of glucocorticoid action by 11β-HSD1 is also important in the brain. 11β-HSD1 is highly expressed in hippocampal neurones (Rajan *et al.* 1996) and other areas of the brain (Moisan *et al.* 1990a; Moisan *et al.* 1990b; Sakai *et al.* 1992), where it acts as a reductase to amplify glucocorticoid action (Rajan *et al.* 1996). Studies in 11β-HSD1 deficient mice have shown that this enzyme has damaging effects on cognitive

function (Yau *et al.* 2001) and may also influence negative feedback regulation of the HPA axis by glucocorticoids (Kotelevtsev *et al.* 1997; Harris *et al.* 2001).

In addition to glucocorticoid metabolism, 11β-HSD1 can catalyse the inter-conversion of the oxysterols 7-ketocholesterol and 7β-hydroxycholesterol (Hult *et al.* 2004). There is evidence that reduction of 7-ketocholesterol to 7β-hydroxycholesterol by 11β-HSD1 occurs, since *in vivo* inhibition of 11β-HSD1 in rats resulted in accumulation of 7-ketocholesterol in the liver and plasma (Schweizer *et al.* 2004). This indicates that substrate competition for enzyme activity between the oxysterols and glucocorticoids may occur *in vivo*.

Physiology of 11β-HSD2. 11β-HSD2 is a high affinity NAD-dependent dehydrogenase, which converts active glucocorticoids into inactive 11-keto metabolites (Figure 1.4). It is found primarily in mineralocorticoid target tissues, such as the kidney, sweat glands, salivary glands and colon (Stewart & Krozowski 1999). In these sites 11β-HSD2 is constitutive; its synthesis and activity are not highly regulated. The major role of 11β-HSD2 in aldosterone target tissues is to protect MR from illicit occupation by glucocorticoids. Consequently, transgenic disruption of 11β-HSD2 recapitulates the major features of SAME (Kotelevtsev *et al.* 1999).

11β-HSD2 is also expressed at sites where MR are not abundant and do not require protection from cortisol; for example, in the placenta throughout most of gestation (Stewart *et al.* 1995; Waddell *et al.* 1998). In this site, 11β-HSD2 is thought to protect the foetus from excessive exposure to maternal glucocorticoids (Brown *et al.* 1996). This is important, as excessive gestational exposure to glucocorticoids retards foetal growth and may 'programme' the development of hypertension and hyperglycemia, and thus an increased risk of diabetes and CHD, in later life (Seckl 1997). 11β-HSD2 is also found in several adult tissues (e.g. lung (Suzuki *et al.*, 1998), heart (Walker *et al.* 1991) and blood vessel wall (Walker *et al.* 1991; Christy *et al.* 2003)) that are not classic targets for mineralocorticoids. Cardiac 11β-HSD2 may prevent fibrosis resulting from the stimulation of MR by glucocorticoids (Konishi *et al.* 2003). The role of 11β-HSD2 in the blood vessel wall is discussed later (section 1.3).

1.2.3.3 Tissue-specific pre-receptor metabolism as a regulatory mechanism

Improved understanding of the roles of the 11β-HSD isozymes has emphasised the physiological importance of tissue-specific metabolism of glucocorticoids. Indeed, it is increasingly apparent that the actions of glucocorticoids are not regulated solely by systemic factors such as circulating plasma concentrations (modulated by hormone synthesis and peripheral metabolism), binding by plasma proteins (such as CBG), and receptor expression and density in target tissues. Rather than merely representing a clearance mechanism, pre-receptor metabolism by 11β-HSD types 1 and 2 in target tissues is now recognised to be an essential component in regulating tissue-specific responses to glucocorticoids (Seckl & Walker 2001). Consequently, in addition to its role in rare conditions such as SAME, tissue-specific pre-receptor metabolism of glucocorticoids is now thought to contribute to the pathogenesis of common conditions such as obesity, insulin resistance, essential hypertension and cardiovascular disease.

1.2.4 Glucocorticoids and cardiovascular disease

1.2.4.1 Increased glucocorticoids and cardiovascular risk

Excess circulating glucocorticoid levels are associated with metabolic and cardiovascular complications such as central obesity, insulin resistance, hyperglycemia, dyslipidemia and hypertension. Indeed, these conditions often limit the usefulness of glucocorticoid therapy for inflammatory or immune disease, and are the most important causes of morbidity and mortality in patients with Cushing's syndrome (Ross & Linch 1982). Activation of the HPA axis with increased cortisol secretion can be observed in non-Cushing's patients, perhaps caused by programming events during early life (Seckl 2004) or psychosocial stress (Bjorntorp 1991). This form of hypercortisolemia has also been associated with hypertension, hyperglycemia and dyslipidemia in several studies (Walker 2006). Interestingly, the cluster of metabolic abnormalities caused by elevations in glucocorticoid secretion and plasma concentration is strikingly similar to features of the metabolic syndrome (Reynolds & Walker 2003; Wake & Walker 2004). However, patients displaying the principal feature of the metabolic syndrome, obesity, have normal or low circulating glucocorticoid levels (Migeon *et al.* 1963; Ljung *et al.* 1996). This suggests that if

glucocorticoids are involved in the aetiology of the metabolic syndrome, tissue-specific alterations in metabolism and sensitivity may be the contributory factor, rather than altered circulating hormone levels.

Tissue sensitivity to cortisol can vary between individuals and between tissues, and evidence suggests that increased tissue sensitivity to glucocorticoids is associated with cardiovascular risk factors (Walker *et al.* 1996; Walker *et al.* 1998; Panarelli *et al.* 1998; Andrews *et al.* 2002). In addition to differences in GR expression, increased sensitivity may be due in part to polymorphisms in the GR gene that change receptor function (van Rossum & Lamberts 2004), or to variations in 11β-HSD activity. For example, obesity is associated with a tissue-specific dysregulation of 11β-HSD1 activity; studies in obese Zucker rats (Livingstone *et al.* 2000) and humans (Rask *et al.* 2001; Rask *et al.* 2002; Wake *et al.* 2003) have shown enzyme activity to be increased in adipose tissue but decreased in the liver. Furthermore, transgenic mice over-expressing 11β-HSD1 selectively in adipose tissue (Masuzaki *et al.* 2001) or liver (Paterson *et al.* 2004) display features of the metabolic syndrome, whilst transgenic deletion of 11β-HSD1 improves hepatic insulin sensitivity, glucose tolerance and lipid profiles (Morton *et al.* 2001). Since 11β-HSD2 deficiency causes hypertension in humans (Stewart *et al.* 1996) and mice (Kotelevtsev *et al.* 1999), milder reductions in renal enzyme activity could contribute to essential hypertension. A clinical study showed an increased half-life of $11\alpha^{3}$[H]-cortisol in a subgroup of patients with essential hypertension (Walker *et al.* 1993), and investigations into the 11β-HSD2 genotype have revealed a polymorphism associated with higher cortisol:cortisone metabolite ratios that is more common in men with blood pressure sensitive to salt loading (Lovati *et al.* 1999).

Therefore, increased glucocorticoid action in humans is associated with the cardiovascular risk factors of metabolic syndrome, such as obesity. For this reason, treatments that will reduce glucocorticoid action are being developed. Therapeutic inhibition of 11β-HSD1 would lower hepatic and adipose cortisol concentrations, enhancing insulin sensitivity, reducing gluconeogenesis and reducing adiposity (Sandeep & Walker 2001; Seckl & Walker 2001). This may be of benefit in patients with diabetes or obesity. Indeed, selective inhibitors of 11β-HSD1 have been tested in several rodent models, where they have been found to decrease obesity

(Hermanowski-Vosatka *et al.* 2005; Berthiaume *et al.* 2007), and improve hyperglycemia and insulin sensitivity (Alberts *et al.* 2002; Alberts *et al.* 2003; Hermanowski-Vosatka *et al.* 2005).

On the basis that glucocorticoids elicit adverse influences on cardiovascular risk factors, it would seem that the action of these hormones is detrimental to the development of CVD. On the other hand, the anti-inflammatory effects of these hormones would be expected to inhibit the development of atherosclerotic lesions. Hence, the net effect of glucocorticoids on CVD is not predictable.

1.2.4.2 Glucocorticoids and atherosclerosis

Perhaps unsurprisingly, given their anti-inflammatory properties, glucocorticoid treatment can decrease macrophage accumulation in animal models of atherogenesis (Asai *et al.* 1993; Tauchi *et al.* 2001). In addition, these hormones prevent incorporation of modified LDL into macrophages *in vitro* (Asai *et al.* 1993) and reduce cholesterol accumulation in the aorta of mice susceptible to atherosclerosis (Tauchi *et al.* 2001). This contrasts with the association between glucocorticoid excess and increased atherosclerosis in humans. Anti-inflammatory glucocorticoid therapy is associated with atherogenesis in patients with inflammatory disease (Maxwell *et al.* 1994; del Rincon *et al.* 2004; Davis, III *et al.* 2005); however it is difficult to attribute this effect solely to glucocorticoids given the underlying inflammatory conditions (Bernatsky *et al.* 2005; Davis, III *et al.* 2007). Pharmacoepidemiological studies have found a correlation between glucocorticoid therapy and adverse cardiovascular events, most strongly with the incidence of heart failure and myocardial infarction (Souverein *et al.* 2004; Wei *et al.* 2004). This effect was not altered by adjustment for the underlying disease, or for components of the metabolic syndrome. This suggested that glucocorticoids may not only increase cardiovascular risk through adverse changes in systemic metabolic factors, but also by actions in the blood vessel wall. Increases in endogenous glucocorticoids have also been linked to atherosclerosis; studies in Cushing's patients have found intimal thickening, increased stiffness and a higher incidence of atherosclerotic plaques in the carotid artery (Faggiano *et al.* 2003) which remain five years after successful cure of the disease (Colao *et al.* 1999). In a recent pilot clinical study, higher plasma cortisol levels in response to stress were associated with coronary artery stenosis

(Alevizaki *et al.* 2007). Coronary artery disease has also been linked to a polymorphism in GR that increases sensitivity to glucocorticoids (Lin *et al.* 2003).

Consistent with the evidence that excess glucocorticoids promote atherosclerosis in humans, selective inhibition of 11β-HSD1 in ApoE knockout mice fed a high fat diet significantly delayed the progression of atherosclerotic lesion development (Hermanowski-Vosatka *et al.* 2005). Furthermore, there was a suggestion of a direct protective effect of enzyme inhibition in the blood vessel wall, with a decrease in vascular MCP-1 expression observed (Hermanowski-Vosatka *et al.* 2005).

1.2.4.3 Systemic vs. local actions of glucocorticoids in cardiovascular disease

Whilst their anti-inflammatory actions suggest that glucocorticoids would reduce the development of vascular lesions, they can also cause adverse systemic changes in cardiovascular risk factors and may contribute to the development of atherosclerosis, as described above. What remains unclear is whether their influence on atherogenesis is due to direct interactions with the blood vessel wall, or to indirect changes in systemic factors, such as blood pressure or circulating lipid levels. It is unlikely that glucocorticoids promote atherosclerosis solely through adverse effects in the vessel wall, and components of local glucocorticoid action (e.g. on VSMCs or inflammatory cells) may prevent lesion formation. Therefore, it would be premature to assume that reducing glucocorticoid action at all sites, for example via systemic 11β-HSD1 inhibition, would be beneficial in the treatment of atherosclerosis. Since their role in the vascular response to injury remains unclear, a more detailed analysis of the local actions of glucocorticoids on the blood vessel wall is required.

1.3 Intra-vascular glucocorticoid actions and metabolism

Glucocorticoids can directly alter vascular function and structure, the inflammatory response to arterial injury, and the growth of new blood vessels (Figure 1.5). These actions may contribute to the link between glucocorticoids and cardiovascular disease (Hadoke *et al.* 2006).

1.3.1 Vascular glucocorticoid receptors and metabolism

The ability of glucocorticoids to interact with the vascular wall is indicated by the presence of both GR and MR in freshly isolated vessels

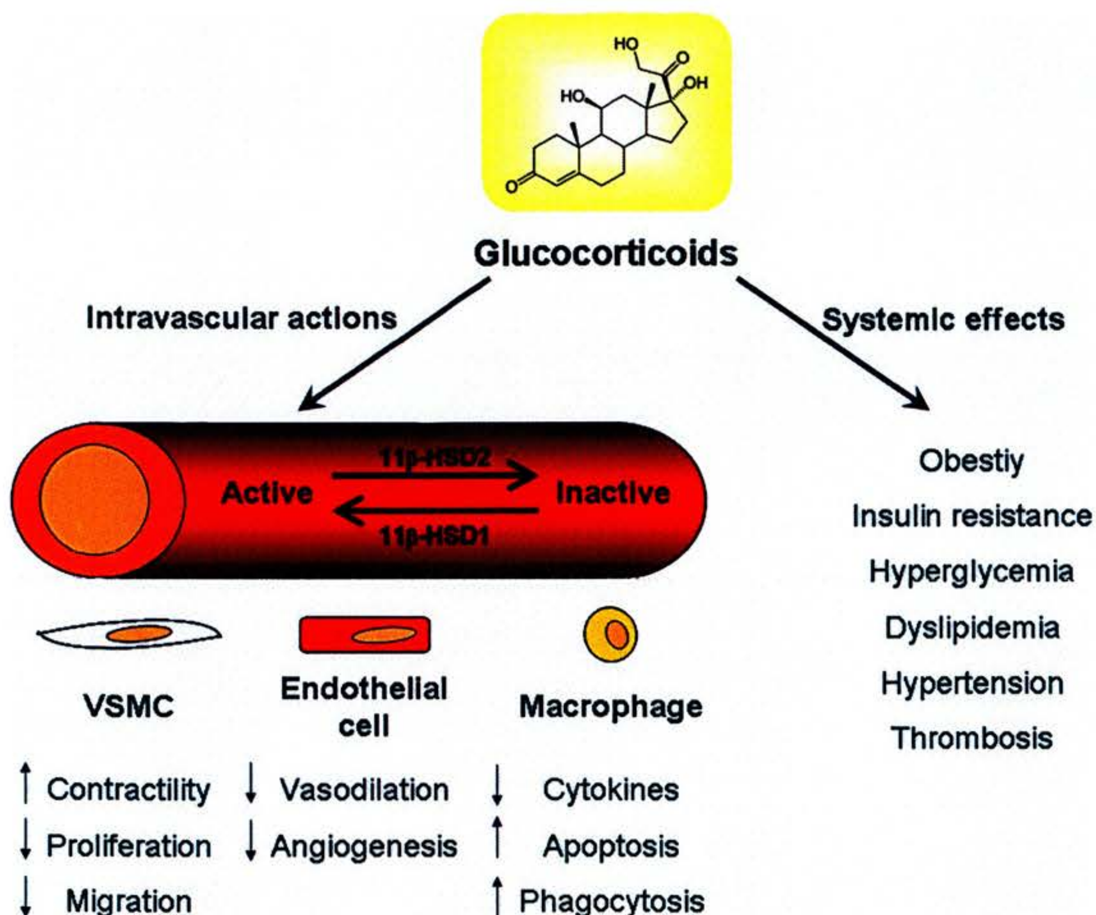


Figure 1.5: Effect of glucocorticoids on cardiovascular physiology and disease

Glucocorticoid hormones present within the vasculature have effects on vascular smooth muscle cells (VSMCs), endothelial cells and macrophages to influence artery function, structure and the response to inflammation. It is likely that these processes will be modulated by inter-conversion of active and inactive glucocorticoids in vascular tissue by the isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 2). Glucocorticoids can also have systemic actions on non-vascular organs, such as the liver, adipose tissue and kidney, to cause adverse changes in cardiovascular risk factors.

(Kornel *et al.* 1982; Christy *et al.* 2003) and in cultured VSMCs (Meyer & Nicholls 1981; Scott *et al.* 1987) and endothelial cells (Inoue *et al.* 1999; Golestaneh *et al.* 2001). Vascular GR are functional, since antagonism with RU38486 blocks induction of ACE activity in rat aortic endothelial cells (Sugiyama *et al.* 2005). Similarly, antagonism of MR inhibits angiotensin-II-induced hypertrophy of VSMCs (Hatakeyama *et al.* 1994) and aldosterone-induced swelling of endothelial cells (Oberleithner *et al.* 2003). The distribution of receptors for glucocorticoids may vary between vascular beds; for example MR has been detected in VSMCs from rabbit aorta and pulmonary artery, but not in smaller arteries (Lombes *et al.* 1992). It remains to be established whether membrane binding sites for glucocorticoids are present in the vasculature.

As in other tissues, the interaction of glucocorticoids with their receptors in the vasculature is unlikely to be regulated by circulating concentrations alone. The expression of both 11β-HSD1 and 11β-HSD2 suggests that pre-receptor metabolism of glucocorticoids may influence steroid action within the vessel wall (Figure 1.5), although there is some controversy over the cellular localisation of these enzymes and the direction of their activity (Morris *et al.* 2003). In the mouse aorta 11β-HSD1 is localised to VSMC whilst 11β-HSD2 is present only in endothelial cells (Christy *et al.* 2003), however studies in cultured human and rat cells have suggested that both isoforms exist in VSMCs (Hatakeyama *et al.* 1999; Cai *et al.* 2001) and endothelial cells (Brem *et al.* 1998). Therefore, the cellular distribution of these enzymes may vary between species, and with the anatomical location of the vessel (Hadoke *et al.* 2006). In addition, there is evidence that 11β-HSD activity varies in arteries from different vascular beds, with activity increasing as artery diameter decreases (Walker *et al.* 1991). Therefore, variations in cellular distribution and activity suggest that intra-vascular glucocorticoid metabolism is not the same in all blood vessels, and may vary between species.

1.3.2 Glucocorticoid actions in the blood vessel wall

1.3.2.1 Modulation of vascular tone

Hypertension caused by glucocorticoid excess cannot be entirely attributed to renal MR agonism, since these hormones can increase blood pressure in rats devoid of

renal mass (Langford & Snavely 1959), and normalisation of renal sodium handling with spironolactone in humans does not totally normalise blood pressure (Williamson *et al.* 1996). Furthermore, selective GR agonists, such as dexamethasone, increase blood pressure in the absence of an increase in sodium retention or plasma volume (Whitworth 1992). This suggests that increased cardiac output or an increase in peripheral vascular resistance may contribute to the development and maintenance of hypertension in response to glucocorticoids. Indeed, enhancement of vascular contractility by glucocorticoids has been implicated in the development of hypertension (Brem 2001).

Although it is well established that glucocorticoids contribute to maintenance of vascular tone *in vivo* (Figure 1.5), the mechanisms involved have yet to be confirmed. There is much evidence that glucocorticoids directly enhance vascular contraction, with several possible mechanisms suggested. Glucocorticoid-dependent potentiation of noradrenaline- and angiotensin II-mediated VSMC contraction (Pirpiris *et al.* 1992) has variously been attributed to up-regulation of receptors in vascular smooth muscle, altered intracellular signalling downstream from receptor activation and modulation of the activity and synthesis of vasoactive substances (Walker & Williams 1992; Kornel *et al.* 1993; Ullian 1999). Alternatively, enhanced contraction may be secondary to impaired endothelium-dependent relaxation. Glucocorticoids can impair endothelium-dependent vasodilation to acetylcholine (Walker *et al.* 1995b; Mangos *et al.* 2000), and reduce the release of vasodilators (e.g. prostaglandins, NO) (Rosenbaum *et al.* 1986; Wen *et al.* 2000; Whitworth *et al.* 2002), whilst increasing the release of vasoconstrictors (e.g. angiotensin II, endothelin-1) (Mendelsohn *et al.* 1982; Morin *et al.* 1998), from endothelial cells.

Whilst 11β–HSD2 deficiency in SAME results in glucocorticoid-induced renal MR activation and severe hypertension, there is also evidence to show that changes in 11β–HSD activity within the vasculature can contribute to alterations in blood pressure. In most pharmacological studies *in vitro*, inhibition of 11β–HSD dehydrogenase activity is associated with an enhanced response to vasoconstrictors, whereas inhibition of the reductase reaction produces attenuated contraction (Morris *et al.* 2003). Whilst interpretation of these results may be complicated by the fact that

some 11β–HSD inhibitors can directly influence vascular function by damaging the endothelium (Ullian *et al.* 1996), they are supported by the demonstration that inhibition of 11β-HSD1 activity with antisense oligonucleotides diminishes the ability of 11-dehydrocorticosterone to enhance phenylephrine-mediated contraction (Souness *et al.* 2002). *In vivo,* 11β–HSD activity is impaired in vessels isolated from hypertensive rats (Takeda *et al.* 1993; Takeda *et al.* 1994), whilst enhanced vascular contractility to glucocorticoids and catecholamines is observed in humans with congenital deficiency of 11β-HSD2 (Walker *et al.* 1992b). Observations in mice with transgenic deletion of 11β-HSD2 (Kotelevtsev *et al.* 1999) have further confirmed the role of glucocorticoid metabolism in the control of blood pressure and vascular function. 11β-HSD2 knockout mice are hypertensive (Kotelevtsev *et al.* 1999), and have enhanced vasoconstrictor responses to noradrenaline with impaired NO-mediated endothelium-dependent vasodilation (Hadoke *et al.* 2001). The cause for the changes in vascular reactivity and endothelial dysfunction are unknown, but are unlikely to be due to renal sodium retention and increased blood pressure since chronic renal mineralocorticoid excess does not replicate the changes in vascular function (Hadoke *et al.* 2001). Data from 11β-HSD2 knockout mice suggested that the observed changes in vascular reactivity were due to a loss in 11β-HSD2-mediated protection of GR within endothelial cells and, hence, glucocorticoid-mediated endothelial cell dysfunction (Hadoke *et al.* 2001). However, studies in isolated vessels from these mice failed to confirm this (Christy *et al.* 2003), suggesting that some other indirect mechanism causes the vascular abnormality *in vivo*. On the other hand, transgenic deletion of 11β-HSD1 has no effect on blood pressure (Kotelevtsev *et al.* 1997) or vascular contractility (Hadoke *et al.* 2001), and this enzyme does not seem to influence vascular reactivity *in vivo*. Therefore, studies in the 11β-HSD1 knockout mouse do not confirm what pharmacological *in vitro* studies had suggested.

1.3.2.2 Influence on vascular inflammation

The anti-inflammatory effects of glucocorticoids *in vivo* are mediated through interactions with both arterial cells and inflammatory cells circulating in the vascular lumen, and by promoting the resolution of inflammation (see section 1.2.1.1 and Figure 1.5). GR are present in the inflammatory cells (e.g. macrophages) that are involved in vascular lesion formation (Liu *et al.* 1999). The expression of 11β-HSD1

in VSMCs (Cai *et al.* 2001), T and B lymphocytes (Zhang *et al.* 2005), and its induction on the differentiation of monocytes into macrophages (Thieringer *et al.* 2001), suggests that regeneration of active glucocorticoids in cells of the vessel wall and lumen may contribute to the regulation of vascular inflammation during lesion formation. Indeed, pro-inflammatory cytokines have been shown to selectively up-regulate 11β-HSD1 activity in cultured VSMCs (Cai *et al.* 2001) and THP-1 cells, a human macrophage-like cell line (Thieringer *et al.* 2001), indicating that a mechanism for local feedback inhibition of inflammation may exist in the vasculature. However, studies in intact vessels *in vitro* and in mice subjected to inflammatory stimuli did not replicate this finding (Dover *et al.* 2007). This may be explained by phenotypic differences between proliferating cells in culture and quiescent cells in intact vasculature, anatomical differences, local glucocorticoid concentrations, or modulation of the inflammatory response by neighbouring tissues (Hadoke *et al.* 2006).

The role of 11β-HSD1-mediated glucocorticoid regeneration in the resolution of inflammation has been demonstrated *in vivo*, after the induction of an acute inflammatory response in mice via thioglycolate injection (Gilmour *et al.* 2006). Cells elicited into the peritoneum showed an early and dramatic up-regulation of 11β-HSD1 activity, which remained high until the resolution of inflammation (Gilmour *et al.* 2006). The demonstration that inactive 11-keto glucocorticoids can promote macrophage phagocytosis of apoptotic neutrophils to the same extent as active glucocorticoid (an effect which is dependent on 11β-HSD1), and the fact that 11β-HSD1$^{-/-}$ mice showed a delayed clearance of exogenous apoptotic neutrophils after thioglycolate injection (Gilmour *et al.* 2006), indicated a functional role for this enzyme in the early resolution of acute inflammation (Chapman *et al.* 2006). This idea is supported by a recent investigation which demonstrated that macrophages from 11β-HSD1$^{-/-}$ mice showed increased production of inflammatory cytokines after LPS stimulation *in vitro*, and that 11β-HSD1$^{-/-}$ mice showed an exaggerated response to LPS challenge *in vivo* (Zhang & Daynes 2007). Current studies, using a mouse model of arthritis, have also indicated that regeneration of active glucocorticoids by 11β-HSD1 plays a role during more chronic inflammation (Chapman & Seckl 2007).

Therefore, macrophage 11β-HSD1 activity may also be involved in regulating the inflammatory response to acute and chronic vascular injury.

1.3.2.3 Inhibition of angiogenesis

The growth of new blood vessels (angiogenesis) is essential during embryonic development. It also occurs physiologically during adult life, in the female reproductive tract and during wound healing. Excessive angiogenesis contributes to the pathology of several diseases, such as cancer, certain retinopathies and rheumatoid arthritis (Carmeliet 2003). It is a complex process, under the control of various activators and inhibitors, and modulation of angiogenesis represents a therapeutic target for the treatment of several conditions (Carmeliet 2003). Glucocorticoids inhibit angiogenesis (Folkman *et al.* 1983), but the mechanisms by which they do so remain unclear (Hadoke *et al.* 2006). A recent study has demonstrated that generation of endogenous glucocorticoids by 11β-HSD1 acts to regulate angiogenesis, and that glucocorticoid-mediated inhibition of angiogenesis is GR-dependent (Small *et al.* 2005). Therefore, in addition to well-recognised effects on artery function and inflammation, glucocorticoids can also act within the vessel wall to influence its structure, and this process is modulated by the metabolism of these hormones (Figure 1.5).

1.3.2.4 Neointimal lesion formation

Inhibition of either the inflammatory response to injury (Miller *et al.* 2001), or VSMC proliferation and migration (Dzau *et al.* 2002), inhibits vascular lesion development in a variety of models. Since glucocorticoids can inhibit both inflammation, and smooth muscle cell proliferation (Longenecker *et al.* 1982; Longenecker *et al.* 1984; Berk *et al.* 1988) and migration (Goncharova *et al.* 2003; Ribichini *et al.* 2007) (Figure 1.5), their ability to directly influence neointimal lesion formation has been examined both in animals and humans. Importantly, as for their role in atherogenesis, it is likely that the influence of glucocorticoids on neointimal formation is a balance between their beneficial actions on the vessel wall, and adverse effects on systemic cardiovascular risk factors.

Glucocorticoid administration prevents neointimal lesion formation in animal models of acute vascular injury. Glucocorticoids inhibit the response to cuff placement on

the femoral artery of the mouse (Pires *et al.* 2005), balloon injury in the carotid arteries of the rat (Villa *et al.* 1994; Guzman *et al.* 1996) and in similar models of injury in the rabbit (Van Put *et al.* 1995; Petrik *et al.* 1998; Valero *et al.* 1998; Ribichini *et al.* 2007). Similarly, dexamethasone release from coated metal stents inhibited neointimal lesion formation in the dog femoral artery (Strecker *et al.* 1998). The mechanisms underlying this inhibition of neointimal proliferation are unclear but given the central role of inflammation in the response of the vessel wall to injury (Wainwright *et al.* 2001), it is likely that intra-vascular glucocorticoid-mediated inhibition of inflammation could attenuate both the initiation and progression of neointimal proliferation. Indeed, in a model of balloon arterial injury in cholesterol-fed rabbits, dexamethasone inhibited early macrophage accumulation in the vessel wall, possibly by reducing the expression of MCP-1 (Poon *et al.* 2001). Glucocorticoid administration has also been shown to reduce cytokine release after stenting in rabbits (Ribichini *et al.* 2007). Inhibition of the inflammatory response to acute vascular injury is consistent with the ability of glucocorticoid treatment to decrease atherosclerotic lesion development in animal models via effects on inflammatory cells (Asai *et al.* 1993; Tauchi *et al.* 2001). The anti-proliferative actions of glucocorticoids, which inhibit the growth of VSMCs in culture (Longenecker *et al.* 1982; Longenecker *et al.* 1984; Berk *et al.* 1988), and their ability to inhibit VSMC migration (Goncharova *et al.* 2003; Ribichini *et al.* 2007), may also contribute to the reduction of neointimal lesion development. Furthermore, glucocorticoids can inhibit thrombin-stimulated release of mitogens (PDGF and heparin-binding epidermal growth factor (HB-EGF)) from human aortic VSMCs (Nakano *et al.* 1993). This may be highly relevant since PDGF has been implicated in both the induction of migration and the expression of inflammatory chemoattractant molecules by VSMC following vascular injury (Marmur *et al.* 1992).

It should be noted that glucocorticoids have not inhibited lesion growth in all animal models. Dexamethasone had no effect on neointimal proliferation in response to balloon injury in iliac arteries of hypercholesterolemic rabbits (Karim *et al.* 1997), or in response to stenting in porcine coronary arteries (Lincoff *et al.* 1997). These negative results were reflected in disappointing results from early clinical trails using

glucocorticoids as anti-restenotics. For example, one large randomised clinical trial found that a single dose of methylprednisolone did not inhibit restenosis after coronary angioplasty (Pepine *et al.* 1990), whereas a small study showed that local delivery of methylprednisolone acetate before stent implantation did not reduce neointimal growth in lesions with high risk of restenosis (Reimers *et al.* 1998). Furthermore, a study investigating the effect of anti-inflammatory agents on the vascular response to injury found that patients treated with glucocorticoids, in combination with colchicine, actually exhibited increased risk of coronary aneurysm after stent placement (Rab *et al.* 1991). This was attributed to altered vessel wall healing in the presence of anti-inflammatory agents. More recently, however, clinical trials using glucocorticoids to inhibit restenosis after coronary stenting have demonstrated beneficial results on luminal narrowing (Versaci *et al.* 2002; Han *et al.* 2006).

The negative results in early clinical trials could be attributed to differences between animal models and the clinical condition or, alternatively, to inadequacies in the clinical trials (e.g. small sample size, inappropriate patient selection, dose, duration of treatment, route of administration). It is also likely that the well-recognised adverse systemic effects of increased glucocorticoids on cardiovascular risk factors (section 1.2.4), may have offset the benefits of their anti-inflammatory and anti-proliferative actions in the vessel wall. This is supported by positive results from more recent trials, using stents to deliver dexamethasone locally to the vessel wall (Han *et al.* 2006). However, it should also be noted that glucocorticoids possess properties that can promote vascular cell proliferation. As described above (section 1.3.2.1) glucocorticoids can attenuate the activity of NO (Walker *et al.* 1995b; Mangos *et al.* 2000), itself a potent inhibitor of cell growth, and stimulate release of growth factors (e.g. endothelin-1) (Morin *et al.* 1998). Glucocorticoids also stimulate ACE expression in VSMCs (Fishel *et al.* 1995) and endothelial cells (Mendelsohn *et al.* 1982). Increased ACE activity leads to increased generation of angiotensin II, a stimulant of VSMC growth both *in vitro* (Berk et al. 1989) and *in vivo* (Griffin *et al.* 1991). The influence of angiotensin II generation on lesion development has been highlighted by the demonstration that ACE inhibition limits neointimal lesion formation following balloon injury of the rat carotid artery and aorta (Powell *et al.*

1989; Capron *et al.* 1991). Alternatively, changes in plasma lipids could influence the ability of glucocorticoids to interact with vascular cells. Lipoprotein (a) can down-regulate glucocorticoid receptor gene expression in human VSMCs, thus inhibiting any protective actions of glucocorticoids and, possibly, representing a novel atherogenic mechanism (Sato *et al.* 1995).

1.4 Hypothesis & aims

1.4.1 Hypothesis

Vascular lesions develop as a result of chronic, uncontrolled inflammatory and proliferative responses to injury in the vascular wall. Pharmacological administration of glucocorticoids, which have both anti-inflammatory and anti-proliferative properties, inhibits the formation of neointimal lesions in animal models. Furthermore, the potential of these hormones to treat restenosis after coronary interventions in humans is being investigated. It is not known, however, whether the availability of endogenous glucocorticoids in the vessel wall influences lesion development after vascular injury. In addition, the interaction between systemic and local actions of glucocorticoids on the vascular response to injury has not been determined in detail. Therefore it was proposed that:

(a) endogenous glucocorticoids act in the vessel wall to directly inhibit neointimal lesion formation, and

(b) this effect is modulated via the regulation of glucocorticoid availability within the vessel wall by 11β-HSD1.

1.4.2 Aims

In order to address this hypothesis, the aims of this project were:

(i) To develop *in vivo* and *in vitro* models of neointimal lesion formation following femoral artery injury in the mouse.

(ii) To determine whether the inflammatory response to injury influences glucocorticoid levels by altering 11β-HSD1 reductase activity in the vessel wall.

(iii) To determine the influence of glucocorticoid administration on the formation of neointimal lesions.

(iv) To investigate the influence of endogenous glucocorticoids and their metabolism by 11β–HSD1 on the formation of neointimal lesions using both an enzyme inhibitor and mice with selective transgenic disruption of 11β-HSD1.

Chapter 2

Materials & Methods

2.1 Materials

Surgery: All surgical instruments and equipment were obtained from Fine Science Tools, Interfocus Ltd., Haverhill, U.K., except the straight sprung angioplasty guidewire, (Cook Inc., Bloomington, U.S.A.) and Mersilk 6-0 sutures with 15 mm cutting needle (Ethicon, Livingston, U.K.). Isoflurane was from Merial Animal Health Ltd., Harlow, U.K. Vetergesic (0.3mg/ml buprenorphine) was from Alstoe Animal Health, York, U.K. Lignocaine (1% lidocaine hydrochloride) was from Hameln Pharmaceuticals Ltd., Gloucester, U.K.

Chemicals, drugs and reagents: All chemicals and drugs were from Sigma-Aldrich, U.K., except the 11β-HSD1 inhibitor compound 815 which was from Enamine, Ukraine, and IL-1β which was from R&D Systems, U.K.

All solvents (including HPLC grade) were from Fisher Scientific, U.K. All histological chemicals and solutions were from Sigma-Aldrich, U.K., except Harris' haematoxylin (Thermo Shandon, U.K.) and Weigert's iron haematoxylin A and B solutions (Bios Europe Ltd., U.K.). DPX mounting medium was from VWR International, U.K.

Monoclonal primary antibody against smooth muscle cell α-actin (clone 1A4) was from Sigma-Aldrich, U.K. Polyclonal rabbit anti-human primary antibodies against von Willebrand factor and fibrinogen were from Dako Cytomation, U.K. Monoclonal rat anti-mouse primary antibody against Mac-2 (clone M3/38) was from Cedarlane, Canada. Polyclonal rabbit anti-human primary antibody against proliferating cell nuclear antigen was from Santa Cruz Biotechnology, U.S.A.

Vectastain ABC kits (containing blocking serum, biotinylated secondary antibody and ABC reagent), avidin biotin blocking kit, levamisole solution, alkaline phosphatase substrate (VECTOR red) and diaminobenzidine substrate kits were obtained from Vector Laboratories, U.K. Proteinase K ready-to-use antigen retrieval solution and normal IgG fraction (rabbit and rat) were from Dako Cytomation, U.K.

All myography drugs were from Sigma-Aldrich, U.K. and salts from V.W.R International, U.K. All PCR reagents were from Promega, U.K., except primers, 1Kbp DNA ladder and 10x TAE buffer, which were from Invitrogen, U.K.

2.1.1 Buffers and solutions

2.1.1.1 Histology and immunohistochemistry

0.2% acetic acid: 1 ml of acetic acid was diluted in 499 ml distilled water.

Alkaline tap water: 400 ml tap water plus 2-3 drops of ammonia.

Gomori's aldehyde fuchsin: 5 g of pararosaniline base was dissolved in 500 ml of 60% ethanol. To this 1 ml of hydrochloric acid (HCl) and 2 ml of fresh (within 6 months of purchase) paraldehyde were added, and the mixture was allowed to blue at room temperature for at least two days. Before use the solution was filtered, and each new batch was tested for optimum staining time.

Gomori's trichrome: 3 g of phosphotungstic acid was dissolved in 500 ml of distilled water. To this 3 g of chromotrope 2R and 1.5 g of fast green FCF were added and mixed until dissolved, then 5 ml of acetic acid were added. The solution was filtered and tested for optimum staining time before use.

3% Hydrogen peroxide (H_2O_2): A commercial solution of 30% H_2O_2 was diluted 1:10 in phosphate buffered saline (PBS).

74 O.P.: A solution of 99.5% ethanol and 0.5% methanol.

2% oxalic acid: 10 ml of a commercial 100% solution diluted in 490 ml of distilled water.

Phosphate buffered saline (PBS): 1 tablet was dissolved per 200 ml distilled water.

5% phosphotungstic acid: 250 ml of a commercial 10% solution was diluted in 250 ml of distilled water.

0.3% potassium permanganate in 0.3% aqueous sulphuric acid: Prepared from stock solutions of 1% potassium permanganate (5 g dissolved in 500 ml distilled water) and 3% sulphuric acid (15 ml concentrated sulphuric acid made up to 500 ml with distilled water), made up to the appropriate volume with distilled water.

1x Tris buffered saline (TBS)/Tween: 61 g Tris base and 90 g NaCl were dissolved in 1 litre of distilled water and the pH adjusted to 7.6. 5 ml of Tween 20 was added to give a 10x solution of 0.5M Tris base, 9% NaCl, 0.5% Tween 20. The solution was then diluted 1:10 before use.

100mM Tris-HCl pH 8.2: 12.11 g of Tris base was dissolved in 950 ml distilled water. Concentrated HCl was used to adjust the pH to 8.2 and the volume made up to 1 litre.

Weigert's iron haematoxylin: Equal volumes of Weigert's iron haematoxylin solution A and solution B were mixed and filtered before use.

2.1.1.2 Myography

High potassium physiological salt solution (KPSS): KPSS was prepared in the same way as PSS, except with the omission of NaCl and the addition 9.22 g of KCl, giving a final concentration of 1M KCl (125mM K^+).

Physiological salt solution (PSS): 6.95 g NaCl, 0.35 g KCl, 0.54 g $CaCl_2.6H_2O$, 0.29 g $MgSO_4.7H_2O$, 2.10 g $NaHCO_3$, 0.161 g KH_2PO_4, 0.0096 g K_2EDTA and 0.991 g D-glucose were dissolved in 1 litre of distilled water and the pH adjusted to 7.4. This gave a solution with final concentrations of: 100mM NaCl, 0.005mM KCl, 0.002mM $CaCl_2.6H_2O$, 0.001mM $MgSO_4.7H_2O$, 0.02mM $NaHCO_3$, 0.001mM KH_2PO_4, 0.00003mM K_2EDTA and 0.005mM D-glucose.

2.1.1.3 Enzyme Activity Assays

C buffer: 63 g glycerol, 8.77 g NaCl, 186 mg EDTA and 3.03 g of Tris were dissolved in 500 ml distilled water and the pH adjusted to 7.7.

Homogenising buffer: 100 g of glycerol, 300 mg of Tris and 186 mg of EDTA were dissolved in 500 ml of distilled water. The pH was adjusted to 7.5 and when ready for use 7.7 mg of Dithiothreitol was added to 50 ml of buffer.

HPLC mobile phase: A mixture of filtered HPLC grade water (60%), acetyl nitrile (15%) and methanol (25%).

Krebs' buffer: Krebs' buffer with a final concentration of 118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$ and 25mM $NaHCO_3$ was prepared and the pH adjusted to 7.4.

Sucrose buffer: 42.79 g of sucrose was dissolved in 500 ml of distilled water to give a 0.25M solution. The pH was adjusted to 7.5.

2.1.1.4 Corticosterone radioimmunoassay

Borate buffer: 8.25 g of boric acid, 2.7 g of NaOH and 3.5 ml concentrated HCl were dissolved in 1 litre of distilled water and the pH adjusted to 7.4. This gave a solution with a final concentration of 130mM boric acid and 67.5mM NaOH. 5 g of bovine serum albumin (BSA) were then added, and the final solution stored at -20°C until use.

2.1.1.5 Polymerase chain reactions

1x Tris acetate EDTA (TAE) buffer: 100 ml of a 10x commercial solution was diluted in 900 ml distilled water.

2.1.2 Drugs and radiolabelled steroids

Acetylcholine: 55 mg of acetylcholine (Fwt: 181.7) were dissolved in 30 ml distilled water to give a 0.01M solution (10^{-2}M). 500 µl aliquots were stored at -20°C until use.

Corticosterone: 345 mg corticosterone (Fwt: 346.5) were dissolved in 1 ml ethanol to give a stock solution of 995µM corticosterone.

[^{3}H] Corticosterone: Commercial (GE Healthcare, U.K.) stock solutions of [^{3}H] corticosterone in ethanol with a concentration of 12.6nmol/ml were stored at -20°C until use.

Cortisol: Cortisol (Hydrocortisone, Fwt: 362.5) was incorporated into silastic pellets, as described below in section 2.4.2.2.

[^{3}H] 11-Dehydrocorticosterone: [^{3}H] 11-Dehydrocorticosterone was synthesised in-house (see section 2.10.1), re-suspended in ethanol to give concentrations of approximately 10-15nmol/ml and stored at -20°C until use.

Dexamethasone: 5 mg of dexamethasone (Fwt: 392.5) were dissolved in 2 ml of ethanol, to which 48 ml of 0.9% saline were added. This gave a solution with a final concentration of 0.1mg/ml. In a 30 g mouse, for example, the required dose of 1mg/kg would require 0.03 mg dexamethasone and therefore 0.3 ml of solution; an appropriate volume for sub-cutaneous injection.

11β-HSD1 inhibitor: 216 mg of compound 815 (Fwt: 271.4) were dissolved in 72 ml of 5% cyclodextrin (3.6 g in 72 ml distilled water). This gave a solution with a

final concentration of 3mg/ml. In a 30 g mouse, for example, the required dose of 30mg/kg would require 0.9 mg of drug and therefore 0.3 ml of solution, an appropriate volume for oral gavage.

IL-1β: 5 μg of IL-1β were dissolved in 5 ml PBS containing 0.1% BSA, to give a final concentration of 1μg/ml. 200 μl aliquots were stored at -20°C.

Noradrenaline: 96 mg of noradrenaline (Fwt: 319.3) were dissolved in 30 ml distilled water to give a 0.01M solution (10^{-2}M). 500 μl aliquots were stored at -20°C until use.

Phenylephrine: 61 mg of phenylephrine (Fwt: 203.7) were dissolved in 30 ml distilled water to give a 0.01M solution (10^{-2}M). 500 μl aliquots were stored at -20°C until use.

RU38486: RU38486 (Mifepristone, Fwt: 429.6) was incorporated into silastic pellets, as described below in section 2.4.2.3.

Sodium nitroprusside: 79 mg of sodium nitroprusside (Fwt: 261.9) were dissolved in 30 ml distilled water to give a 0.01M solution (10^{-2}M). 500 μl aliquots were stored at -20°C until use.

2.2 Animals

Male C57B16 (Harlan Olac, U.K.) and 11β-HSD1$^{-/-}$ (bred in-house at the Biomedical Research Facility, Little France, Edinburgh, U.K.) mice weighing 25-30 grams were used in all experiments. Animals were maintained under controlled conditions of light (lights on 0800h-2000h) and temperature (21-22°C), and allowed free access to standard chow (Special Diet Services, U.K.) and drinking water. Animal experiments were carried out under Home Office license. Mice were killed by either Schedule 1 or decapitation, according to Home Office guidelines.

2.3 Models of neointimal proliferation

2.3.1 Intra-luminal wire injury of the femoral artery *in vivo*

A model of vascular injury and neointimal proliferation was developed in the mouse femoral artery (chapter 3). The final, refined surgical procedure was carried out as follows (see also Figures 2.1a and b).

Mice were weighed and then anaesthetised by isoflurane inhalation with suitable analgesic cover (0.05mg/kg buprenorphine given as 0.03mg/ml Vetergesic by sub-cutaneous injection). Femoral artery isolation was performed under a surgical microscope using aseptic technique: all surgical instruments were sterilized by autoclaving prior to experiments being carried out, and a bead sterilizer was used between animals.

Femoral artery injury was carried out by adaptation of the method of Sata *et al* (2000). After shaving the inner surface of the hind limb and making a small skin incision at the top of the leg, the rectus femoris and vastus medialis muscles were separated along the ligament by blunt dissection to expose the femoral bundle. The femoral artery and vein were isolated from the nerve both proximally and distally to the branch point of the popliteal artery (which is located between the rectus femoris and vastus medialis muscles). Blood flow in the femoral artery and vein was interrupted with temporary ligatures (6-0 silk suture) proximal and distal to this branch point, and the popliteal artery was isolated and ligated distally with 6-0 suture. After removing connective tissue from the arteries around the branch point the popliteal artery was dilated with lignocaine (1% lidocaine hydrochloride) and a flexible angioplasty guidewire (straight sprung 0.014" diameter) was introduced via an arteriotomy. After loosening the proximal temporary ligature, the guidewire was advanced 5-10 mm into the common femoral artery towards the iliac artery, left in place for 1 minute and then removed. The popliteal artery was then ligated with 6-0 suture proximally to the arteriotomy site, and blood flow restored in the common femoral artery by removing the two temporary ligatures. This induced endothelial denudation and stretching of the artery wall, followed by reperfusion of blood over the injured area. The wound was closed using 6-0 sutures and the animal was allowed to recover.

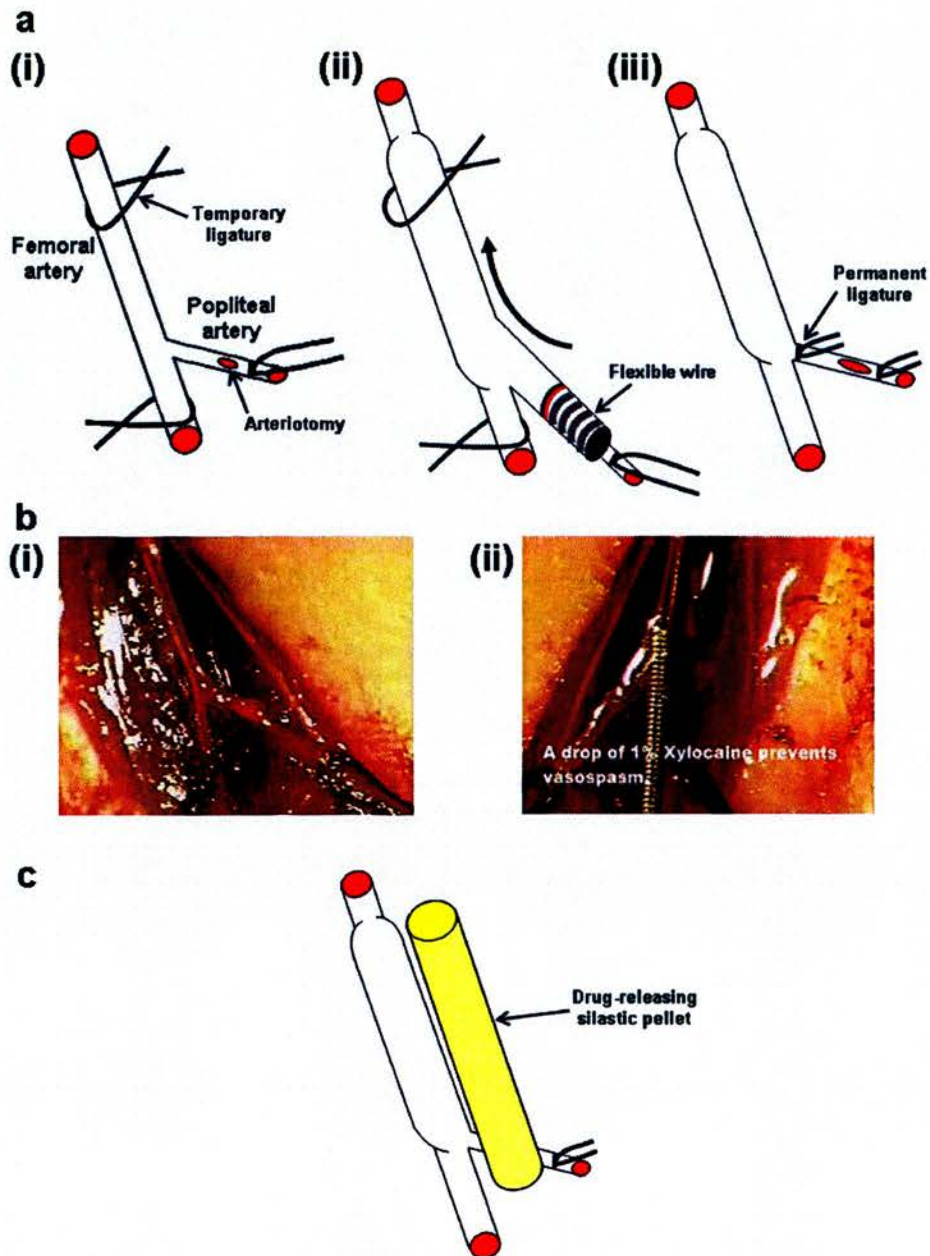


Figure 2.1: Surgical model of wire-induced injury to the mouse femoral artery

Temporary ligatures were placed on the isolated femoral artery for vascular control during the procedure, and the popliteal branch was ligated distally: a (i) and b (i). A flexible wire was inserted into the popliteal branch via an arteriotomy, and advanced into the femoral artery towards the iliac artery, causing stretching of the vessel wall: a (ii) and b (ii). After removal of the wire, the popliteal branch was ligated proximally to the arteriotomy site: a (iii). In some experiments, drug-releasing silastic pellets were then placed next to the injured artery: c. Photographs reproduced with kind permission from Dr. M. Sata.

2.3.2 Ligation-induced injury of the femoral artery *in vivo*

To induce neointimal proliferation in the femoral artery by side-branch ligation, mice were anaesthetised and prepared for surgery as described above (section 2.3.1). After exposing the popliteal artery by separating the rectus femoris and vastus medialis muscles through blunt dissection, and separating the femoral artery and vein from the nerve, the popliteal branch was then ligated with 6-0 silk suture immediately adjacent to the branch point with the main femoral artery (see Figure 1a(iii) for site of permanent ligature). The skin incision was closed with 6-0 silk sutures, and the mice allowed to recover. Ligation-induced injury was used in some experiments as an alternative model of neointimal proliferation, in the contra-lateral leg of mice undergoing intra-luminal wire injury.

2.3.3 *In vitro* model of neointimal proliferation

An attempt was made to set up a sterile tissue culture model of neointimal proliferation, in order to allow the influence of local glucocorticoid and 11β-HSD activity on lesion development to be investigated specifically within the vessel wall. An adaptation of the method of Guerin *et al* (2004) was used.

Mice were subjected to intra-luminal wire injury of the femoral artery *in vivo*, as described above (section 2.3.1). Immediately following injury, mice were killed by cervical dislocation and the left (injured) and right (control) femoral arteries were carefully excised, and placed in sterile tubes containing tissue culture medium (1:1 mixture of medium for smooth muscle cells and endothelial cells; Promocell, U.K.) at room temperature. Vessels were then transferred to 6 well plates and cultured in the same medium under sterile conditions at 37°C in a 5% CO_2 atmosphere for 21 days. Tissue culture medium was changed every 2 days.

2.4 Administration of drugs

2.4.1 Systemic glucocorticoid administration

To assess the effect of an increase in systemic glucocorticoid on neointimal proliferation, mice received 1mg/kg/day dexamethasone or vehicle alone (4% ethanol/ 0.9% saline) by sub-cutaneous injection. Dosing was started at least two hours before wire injury and continued daily. Injections were given in the morning

for 21 days. The dexamethasone and vehicle solutions were coded, so that drug administration was carried out under blinded conditions.

2.4.2 Local administration of drugs

To investigate the influence of locally-released compounds on the response to injury, silastic pellets (Silastic medical grade elastomer base and curing agent; Dow Corning, U.S.A.) were implanted proximally to the operation site after wire-induced injury (Figure 2.1c). Such implants release their impregnated contents at a constant rate *in vivo* for at least three weeks (Soro *et al.* 1997) and have been used to demonstrate the local inhibitory effect of glucocorticoids on angiogenesis *in vivo*, in a sub-cutaneous polyurethane sponge implantation model (Small *et al.* 2005).

2.4.2.1 Vehicle

1 g of silastic elastomer was mixed with 100 mg of curing agent, and centrifuged at 13,000 rpm for 1 minute. This mixture was then taken up into a 1 ml syringe and injected into fine bore polythene tubing (internal diameter 0.86 mm, outer diameter 1.27 mm; SIMS Portex Ltd., U.K.) using a 19 gauge needle. The elastomer was allowed to cure for at least 24 hours at 25°C, and was then cut into pellets weighing approximately 10 mg before being removed from the tubing.

2.4.2.2 Cortisol

275 mg of cortisol were mixed with 1 g of silastic elastomer and centrifuged at 13,000 rpm for 1 minute. 100 mg of curing agent were added, mixed and centrifuged again at 13,000 rpm for 1 minute. The rest of the protocol was then followed as for vehicle pellets. A 10 mg pellet contained approximately 2 mg cortisol.

2.4.2.3 RU38486

166 mg of the GR antagonist RU38486 were mixed with 300 mg of silastic elastomer and centrifuged at 13,000 rpm for 1 minute. 33 mg of curing agent were added, mixed and centrifuged again at 13,000 rpm for 1 minute. The rest of the protocol was then followed as for vehicle pellets. A 10 mg pellet contained approximately 3.3 mg RU38486.

2.4.3 11β-HSD1 inhibitor

To assess the effect of systemic inhibition of 11β-HSD1 on neointimal proliferation, mice were dosed with 30mg/kg of a specific inhibitor (compound 815

(Hermanowski-Vosatka *et al.* 2005)) or vehicle alone (5% cyclodextrin) twice daily by oral gavage. Loading doses were given one day before surgery, and dosing was then continued in the morning and afternoon for 14 days. The 11β-HSD1 inhibitor and vehicle solutions were coded, so that drug administration was carried out under blinded conditions.

2.5 Preparation of vessels for histological analysis

At the end of experiments in which vessel structure was to be examined, left and right femoral arteries were carefully cleaned of surrounding tissue and excised, from the bifurcation of the iliac artery to the branch point of the popliteal artery. Arteries were fixed in 10% neutral buffered formalin for up to 24 hours, and then stored in 70% ethanol. Arteries were dehydrated through a graded alcohol series and embedded in paraffin using an automatic tissue processing machine (Shandon, U.K.) and embedding station (Shandon, U.K.). Transverse sections (4 μm) were cut on a microtome, and a set of eight serial sections were mounted from a water bath onto separate slides (Superfrost plus; VWR International, U.K.) every 100 μm along the entire length of the vessel.

2.6 Histology

2.6.1 Haematoxylin and eosin

Sections were dewaxed in xylene and rehydrated through absolute ethanol and two changes of 74 O.P. to water. Sections were placed in filtered Harris' haematoxylin for 5 minutes, or until desired staining was achieved, and then blued in running tap water for 1 minute. After washing in alkaline tap water for 1 minute, staining of nuclei was assessed microscopically. Sections were then rinsed in 70% ethanol before being placed in alcoholic eosin Y for 30 seconds. After washing in tap water, sections were dehydrated through two changes of 74 O.P. and absolute ethanol, cleared in xylene and mounted with DPX.

2.6.2 United States trichrome stain

Staining was carried out either manually or using an automated staining system (Varistain Gemini; Thermo Shandon, U.K.). Sections were dewaxed in xylene and rehydrated through absolute and 95% ethanol to water. Freshly prepared 0.3% potassium permanganate in 0.3% aqueous sulphuric acid was then applied and, after rinsing in water, sections were decolourised in 2% aqueous oxalic acid. Sections were washed in tap water, rinsed in 70% alcohol and then immersed in filtered Gomori aldehyde fuchsin, which stains elastic fibres deep purple. After differentiating in 70% alcohol and washing well in water, the staining of elastin was checked microscopically (if slides were being stained by hand), before filtered Weigert's iron haematoxylin was applied for 5 minutes to stain cell nuclei. The slides were then left to blue in running tap water, before 5% aqueous phosphotungstic acid was applied, which prepares the tissue for the trichrome stain. After rinsing in water, sections were immersed in filtered Gomori trichrome stain for 20 minutes (which stains platelets light purple, collagen fibres green and muscle red). Finally, the slides were rinsed in 0.2% aqueous acetic acid and then dehydrated and cleared in xylene, before coverslipping with DPX mountant.

2.7 Morphometric analysis

In order to measure the extent of neointimal proliferation in injured arteries, images of stained sections were digitised using a Photometrics CoolSnap camera (Tucson, U.S.A.) coupled to a light microscope (Zeiss Axioskop) via a microcolour liquid crystal turnable RGB filter (Cambridge Research and Instrumentation Inc., Woburn, U.S.A.). Microcomputer Imaging Device software (MCID; Imaging Research Inc., St. Catharines, Canada) was used to integrate the microscope and camera, and to carry out image analysis of the digitised images. Analysis was performed on sections every 100 μm along each artery, and the parameters measured were mean area (μm^2) inside the external elastic lamina (EEL), internal elastic lamina (IEL) and lumen. These measurements could then be used to calculate the area of the media (area inside EEL – area inside IEL) and the area of any neointima present (area inside IEL – area inside lumen). The section with the largest area of neointima was chosen to

represent each artery when calculating the mean neointimal area in a group. Additional measurements included perimeter (μm) and average diameter (μm) of the EEL, IEL and lumen. All analysis was carried out under blinded conditions; each artery sample was coded to conceal the treatment that it had been subjected to.

2.8 Immunohistochemistry

Representative sections were stained immunohistochemically to allow the identification of the following cell types or proteins.

2.8.1 Smooth muscle cells

Slides were deparaffinised and rehydrated, then mounted in Sequenza cassettes (Thermo Shandon, U.K.) using 1x TBS/Tween. A 1:200 dilution of alkaline phosphatase-conjugated monoclonal primary antibody against smooth muscle cell α-actin was made up in 1x TBS/Tween, and 200 μL was applied to sections which were then incubated overnight at 4°C. Sections of mouse aorta incubated with or without primary antibody served as positive or negative controls, respectively (Figure 2.2). To visualise sites of antibody binding, an alkaline phosphatase substrate kit with a red chromagen (VECTOR red) was diluted in 100mM Tris-HCl pH 8.2; levamisole was also added to block endogenous alkaline phosphatase activity. Sections were washed thoroughly in 1x TBS/Tween and then tap water. After counterstaining in haematoxylin and blueing in tap water, sections were then dehydrated, cleared and mounted in DPX.

2.8.2 Endothelial cells

Sections were deparaffinised and rehydrated, then mounted in Sequenza cassettes using distilled water. Epitope retrieval was carried out by incubating slides with 4-5 drops of ready-to-use proteinase K solution for 15 minutes at room temperature. After washing in PBS, sections were blocked with diluted goat serum for 30 minutes at room temperature and avidin biotin blocking was then carried out using a kit. Sections were incubated for 1 hour at room temperature with rabbit anti-human primary antibody against von Willebrand factor, diluted to 1:500 in goat serum.

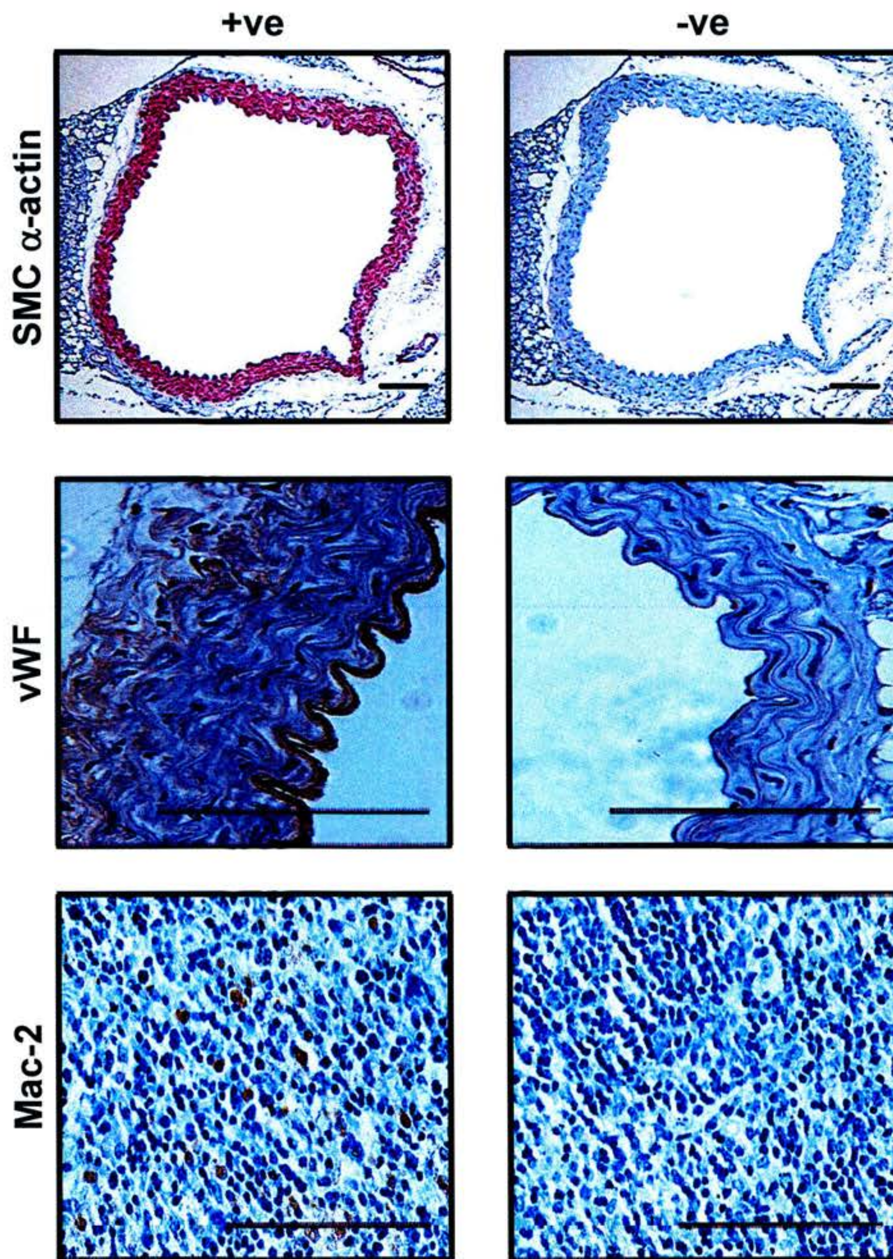


Figure 2.2: Immunohistochemistry controls

Representative positive (+ve) and negative (-ve) controls, carried out in parallel with immunohistochemistry experiments. Sections of mouse aorta (smooth muscle cell α-actin, von Willebrand factor (vWF)) or spleen (Mac-2) incubated with primary antibody acted as positive controls. Aorta with no primary antibody (smooth muscle cell α-actin) or normal rabbit IgG (vWF), and spleen with normal rat IgG (Mac-2) served as negative controls. Scale bar = 100 μm.

Sections of mouse aorta incubated with primary antibody served as a positive control, while sections either without primary antibody or incubated with normal rabbit IgG fraction (diluted to the same concentration as primary antibody) acted as negative controls (Figure 2.2).

After rinsing with PBS, endogenous peroxidise activity was inhibited by adding 3% H_2O_2 to the slides for 10 minutes. Sections were rinsed in PBS and then a goat anti-rabbit biotinylated secondary antibody was applied for 30 minutes. After washing and incubating with ABC solution for a further 30 minutes, sites of immunoreactivity were detected using the horseradish peroxidase substrate diaminobenzidine (DAB). Sections were washed in tap water, counterstained and blued, and then dehydrated, cleared and mounted in DPX.

2.8.3 Macrophages

Sections were deparaffinised and rehydrated, incubated with 3% H_2O_2 for 10 minutes and then washed in running tap water for 10 minutes. Slides were mounted in Sequenza cassettes using PBS and then incubated with diluted rabbit serum for 30 minutes. After washing with PBS, sections were incubated overnight at 4°C with rat anti-mouse primary antibody against Mac-2, diluted to 1:6000 in rabbit serum. Sections of mouse spleen incubated with primary antibody served as a positive control, while sections either without primary antibody or incubated with normal rat IgG fraction (diluted to the same concentration as primary antibody) acted as negative controls (Figure 2.2). Slides were then washed with PBS and incubated for 1 hour with rabbit anti-rat biotinylated secondary antibody. After washing and incubating with ABC solution for a further 30 minutes, sites of immunoreactivity were detected using DAB. Sections were washed in tap water, counterstained and blued, and then dehydrated, cleared and mounted in DPX.

2.8.4 Proliferating cells

Sections were deparaffinised and rehydrated, incubated with 3% H_2O_2 for 15 minutes and then washed in PBS for 5 minutes. Slides were mounted in Sequenza cassettes using PBS and then incubated with diluted goat serum containing avidin for 30 minutes. After washing with PBS, sections were incubated overnight at 4°C with rabbit anti-human primary antibody against proliferating cell nuclear antigen, diluted

to 1:50 in PBS containing biotin. Sections of mouse testis incubated with primary antibody served as a positive control, while sections either without primary antibody or incubated with normal rabbit IgG fraction (diluted to the same concentration as primary antibody) acted as negative controls. Slides were then washed with PBS and incubated for 1 hour with goat anti-rabbit biotinylated secondary antibody. After washing and incubating with ABC solution for a further 30 minutes, sites of antibody binding were detected using DAB. Sections were washed in tap water, counterstained and blued, and then dehydrated, cleared and mounted in DPX.

2.8.5 Fibrinogen

Sections were deparaffinised and rehydrated, then mounted in Sequenza cassettes using distilled water. Epitope retrieval was carried out by incubating slides with 4-5 drops of ready-to-use proteinase K solution for 5 minutes at $37^{o}C$. After cooling to room temperature and washing in PBS, sections were blocked with diluted goat serum for 30 minutes at room temperature. Sections were incubated overnight at $4^{o}C$ with rabbit anti-human primary antibody against fibrinogen, diluted to 1:200 in goat serum. Sections of femoral artery containing thrombus incubated with primary antibody served as a positive control, while sections either without primary antibody or incubated with normal rabbit IgG fraction (diluted to the same concentration as primary antibody) acted as negative controls.

After rinsing with PBS, endogenous peroxidise activity was inhibited by adding 3% H_2O_2 to the slides for 10 minutes. Sections were rinsed in PBS and then a goat anti-rabbit biotinylated secondary antibody was applied for 30 minutes. After washing and incubating with ABC solution for a further 30 minutes, sites of immunoreactivity were detected using DAB. Sections were washed in tap water, counterstained and blued, and then dehydrated, cleared and mounted in DPX.

2.9 Small vessel wire myography

To determine the functional characteristics of the mouse femoral artery, arterial rings were mounted on 40 μm, parallel, intra-luminal wires in a small vessel wire myograph for measurement of isometric force. Myograph chambers contained PSS at 37°C, perfused with 95% O_2/ 5% CO_2. In some vessels the endothelium was removed by rubbing the luminal surface with a human hair.

After the length of each arterial ring was measured, normalization was carried out by stepwise incremental stretches of the vessel and application of the LaPlace relation ($P = T/r$, where P is the trans-luminal pressure, T is the wall tension and r is the internal radius of the vessel) to determine the internal diameter under an intra-luminal pressure of 13.3kPa (L100). Arteries were then stretched to their optimum resting level (0.9L100) (Falloon *et al.* 1995). Before each set of experiments, arteries were contracted twice with NA-K (a mixture of 10^{-5}M noradrenaline in KPSS), once with 10^{-5}M noradrenaline alone, once with KPSS alone and finally once again with NA-K. Cumulative concentration-response curves were then obtained for phenylephrine ($1x10^{-9}$M to $3x10^{-4}$M) and KPSS (10mM K^+ to 125mM K^+). Vasodilator concentration-response curves were obtained for acetylcholine ($1x10^{-9}$M to $3x10^{-4}$M) and the NO donor sodium nitroprusside ($1x10^{-9}$M to $3x10^{-5}$M) in vessels pre-contracted with sufficient phenylephrine to achieve around 80% of the maximum contraction.

Contractions to phenylephrine are expressed as a percentage of the maximum response to KPSS in that vessel (to control for vessel size), and contractions to KPSS are expressed as force per unit length of vessel (mN/mm). Relaxation is expressed as a percentage of the pre-contraction obtained with phenylephrine in that vessel. To assess sensitivity to agonists, the negative logarithm of the concentration required to produce 50% of maximum responses was calculated (pD_2 for contraction, -log IC_{50} for relaxation). The maximum response (E_{max}) and pD_2/ -log EC_{50} for each agonist was compared between groups.

2.10 Measurement of 11β-HSD1 activity

In vivo 11β-HSD1 acts predominantly as a reductase, catalysing the conversion of inactive 11-dehydrocorticosterone to active corticosterone (Jamieson *et al.* 2000). However, in homogenised cell preparations it is capable of bi-directional activity, and the dehydrogenase direction predominates (Lakshmi & Monder, 1988). Traditionally, *in vitro* activity assays have measured 11β-HSD1 activity in the dehydrogenase direction using homogenised tissue preparations, as described for liver (section 2.10.4). Using this technique it is possible to determine between the activities of the two isozymes of 11β-HSD, by exploiting their cofactor preference for either NADP or NAD. This has been achieved in the rat (Walker *et al.* 1992a), but the mouse 11β-HSD isozymes do not show such selective cofactor specificity (Hadoke *et al.* 2001). Therefore, in the intact vessel assay described (section 2.10.2), activity was measured in the reductase direction.

2.10.1 Synthesis of [^{3}H] 11-dehydrocorticosterone

Two rat placentas were homogenised in 1 ml homogenising buffer, and stored at -80°C until use. 120 μl of [^{3}H] corticosterone were dried under nitrogen at 60°C and reconstituted in 50 μl of ethanol. A reaction mixture containing this 50 μl of [^{3}H] corticosterone, 200 μl of 25mM NAD cofactor, 300 μl placental homogenate and 4.45 ml of C buffer was prepared in a glass vial, vortexed, and incubated at 37°C for 4 hours. Steroids were extracted by three serial additions of 4 ml ethyl acetate to the reaction mixture, split into eight volumes. After each addition the sample was vortexed and centrifuged at 1700 rpm for 10 minutes at 4°C. The upper organic layer was transferred to a fresh glass tube; all eight volumes were dried nitrogen into one sample, reconstituted in 100 μl of ethanol and then stored at -20°C until use. 1 μl aliquots of [^{3}H] corticosterone and the [^{3}H] 11-dehydrocorticosterone product were analysed by high performance liquid chromatography (HPLC) to assess purity and concentration of the [^{3}H] 11-dehydrocorticosterone. Concentration was calculated by using the following equation, where [^{3}H] 11-dehydrocorticosterone is A and [^{3}H] corticosterone is B:

Concentration of [^{3}H] A = (Area of [^{3}H] A peak / Area of [^{3}H] B peak) x concentration of [^{3}H] B nmol/ml.

2.10.2 Intact vessels *in vitro*

11β-reductase activity was measured in control and injured femoral arteries, and aortic rings, using an adaptation of the method of Souness *et al* (2002). Vessels were incubated at 37°C in 1 ml culture medium (DMEM + 1% antibiotic/ antimycotic mix; Invitrogen, U.K.) containing 10pmol [^{3}H] 11-dehydrocorticosterone. Sections of mouse liver served as a positive control. As a negative control, [^{3}H] 11-dehydrocorticosterone was incubated in a well without tissue, and medium alone in a well was included in the experiment as a blank. The entire length of each femoral artery was incubated in an individual well, while aortic ring incubations were performed in duplicate for each animal.

Following incubation, the culture medium was removed at several time-points and stored at -20°C before extraction of steroids (section 2.10.3) and analysis by HPLC (section 2.10.5). It has been shown previously that aortic rings only retain 2-3% of the added radioactivity in these experiments (Souness *et al.* 2002), so only the supernatant, and not the vessels themselves, was included in the extraction.

At the end of the experiment the tissue samples were blotted on tissue paper to remove excess medium, and then weighed. Enzyme activity was calculated as the amount of product ([^{3}H] corticosterone) formed in pmol per mg of tissue over time, after subtraction of conversion in negative control samples.

2.10.3 Extraction of steroids from culture medium

To extract a purified preparation of steroids from the culture medium collected during intact vessel activity assays, samples were run through C_{18} Sep Pak columns (Waters Millipore, U.K.) under gravity (Small *et al.* 2005). Firstly the columns were prepared by using 5 ml of HPLC grade methanol to separate the C_{18} chains, and 5 ml HPLC grade water as a wash. The samples were then run onto the column and washed on with 5 ml HPLC grade water. Samples were eluted from the columns and collected into glass tubes with 2 ml HPLC grade methanol. This sample was then dried under nitrogen at 60°C and dissolved in 800 μl HPLC mobile phase.

2.10.4 Liver homogenates

Liver microsomes were prepared by homogenising approximately 300 mg liver in 1 ml sucrose buffer. To remove membrane debris the homogenate was centrifuged at

1000 rpm for 10 minutes at 4°C. The supernatant was removed into a fresh tube and centrifuged at 30,000 rpm for 30 minutes at 4°C to remove nuclear debris. This supernatant was removed into a fresh tube and centrifuged at 55,000 rpm for 60 minutes at 4°C. The resultant microsomal pellet was resuspended in 400 μl of sucrose buffer. The protein concentration of each sample was determined via the Bradford method, using a colorimetric protein assay kit (Bio-Rad, U.K.) and a standard curve constructed from dilutions of BSA (Bio-Rad, U.K.).

11β-Dehydrogenase activity was measured in liver microsomes using an adaptation of the method of Livingstone *et al* (Livingstone *et al.* 2000). Reactions were prepared in duplicate with a final volume of 250 μl. This contained 180 μl of liver microsomes (dissolved in Krebs' buffer to a final protein concentration of 20μg/ml), 50 μl of cofactor mix (dissolved in Krebs' buffer to give a final concentration of 2mM NADP and 0.2% glucose) and 20 μl of substrate mix (dissolved in 25% ethanol: 75% Krebs' buffer, to give a final concentration of 2μM corticosterone [10nM [^{3}H] corticosterone and 1.99μM corticosterone]). Reactions containing no cofactor served as negative controls, and tissue blanks were prepared containing only Krebs' buffer, substrate and cofactor. Reactions were incubated for 2 hours at 37°C, and the reaction was then stopped by the addition of 10 volumes of ethyl acetate. In this way, measurements of enzyme activity were carried out in the linear range of product formation with time (approximately 30% conversion of substrate to product). The tubes were vortexed and the upper organic phase containing steroids was removed to a fresh tube and dried down under nitrogen at 60°C. Steroids were then dissolved in mobile phase and analysed by HPLC as described (section 2.10.5).

Enzyme activity was expressed as reaction velocity: amount of product ([^{3}H] 11-dehydrocorticosterone) formed in pmol per μg protein per minute.

2.10.5 High performance liquid chromatography

The HPLC system consisted of an auto-sampler and mobile phase pump (Waters, U.K.), a symmetry C_8 column (length 15 cm, internal diameter 4.6 mm, pore size 5 μm; Waters, U.K.) and a radioactivity monitor linked to a scintillation fluid pump (Berthold, U.K.). The system was controlled by the computer programme Chromeloen v6.7 (Dionex, U.K.). Steroid samples extracted from experiments using

liver homogenates and intact vessels were re-suspended in 800 μl mobile phase and 50-200 μl of each sample were injected into the HPLC system to give optimal peak shape and height (Figure 2.3, 2.4). The flow rate of the mobile phase was 1.5ml/min and the flow rate of the scintillant (Gold Flow, Meridian, U.K.) was 3ml/min to achieve optimal mixing in the column and counting efficiency. The column temperature was 40°C to improve peak shape and maintain stability of retention times. Radioactive standards for corticosterone and 11-dehydrocorticosterone were injected at the start of each run to confirm peak identity. The approximate retention time for [^{3}H] 11-dehydrocorticosterone was 10-12 minutes and for [^{3}H] corticosterone was 15-18 minutes. Peaks were approximately 2 min 30 seconds wide and peak height was at least 50-fold higher than background. Representative chromatograms obtained from a typical 11β-reductase assay (Figure 2.3), and a typical 11β-dehydrogenase assay (Figure 2.4), are shown. Following chromatography, the area under each peak was integrated using the Chromeleon software and used to quantify the percentage conversion of [^{3}H] 11-dehydrocorticosterone to [^{3}H] corticosterone (11β-reductase activity) or [^{3}H] corticosterone to [^{3}H] 11-dehydrocorticosterone (11β-dehydrogenase activity). Since the starting concentration of substrate for each reaction was known, the total amount of product formed in each sample could then be calculated.

2.11 Analysis of plasma steroid levels

2.11.1 Cortisol

Plasma samples from mice implanted with a unilateral cortisol-releasing silastic pellet and contra-lateral vehicle control were analysed for cortisol using a radioimmunoassay kit (ICN Biomedicals Inc, U.S.A.). Cortisol standards of known concentration (0, 1, 3, 10, 30 and 100μg/dl) and plasma samples were added in duplicate to tubes coated with a rabbit anti-cortisol antibody. Following this, [^{125}I] cortisol was added to each tube and the mixture was then vortexed and incubated at 37°C for 45 minutes.

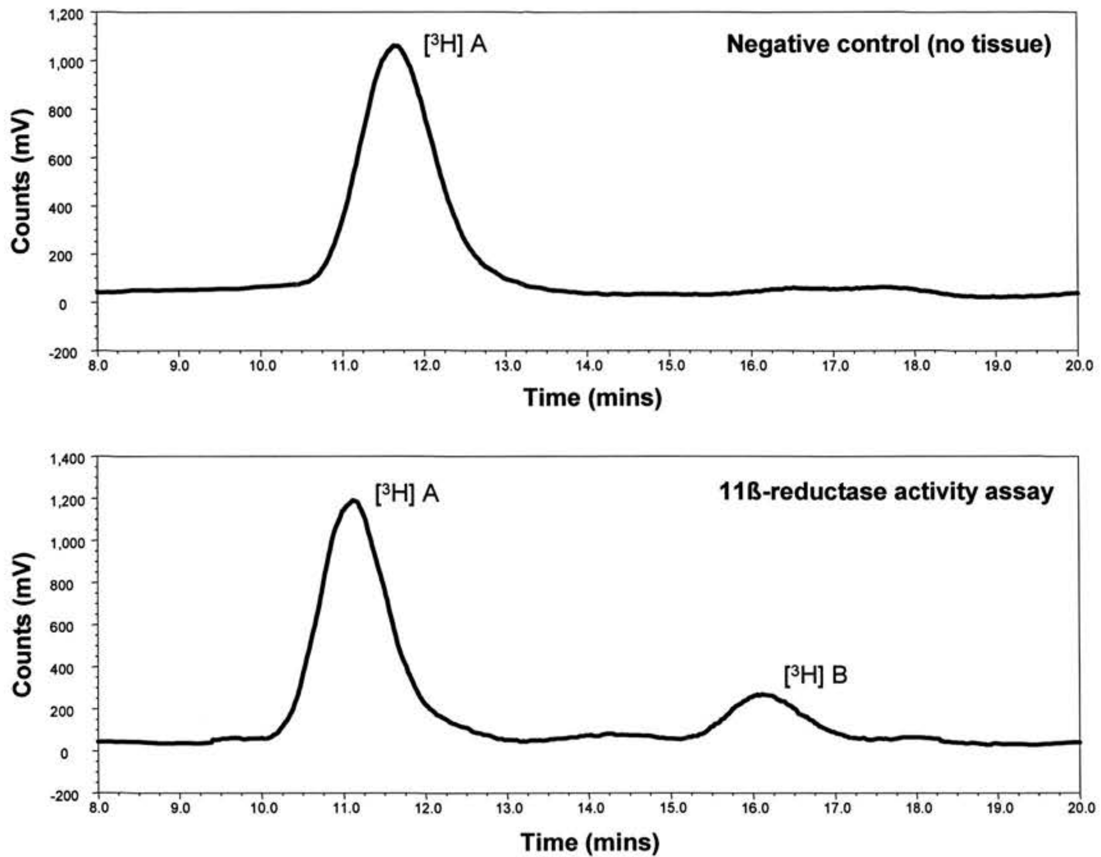


Figure 2.3: Chromatograms from 11β-reductase activity assay in intact vessels

Two representative high performance liquid chromatography (HPLC) chromatograms, showing peaks for [^{3}H] 11-dehydrocorticosterone ([^{3}H] A; retention time 10-12 minutes) and [^{3}H] corticosterone ([^{3}H] B; retention time 15-18 minutes). The chromatograms show a negative control experiment (top), where 10pmol [^{3}H] A was incubated for 24 hours with culture medium alone, and an 11β-reductase activity assay (bottom), where [^{3}H] A was incubated under the same conditions with an intact femoral artery. In the negative control there is no conversion of [^{3}H] A to its 11β-reduced metabolite [^{3}H] B. However when vascular tissue is present metabolism to [^{3}H] B occurs, indicating the presence of 11β-reductase activity.

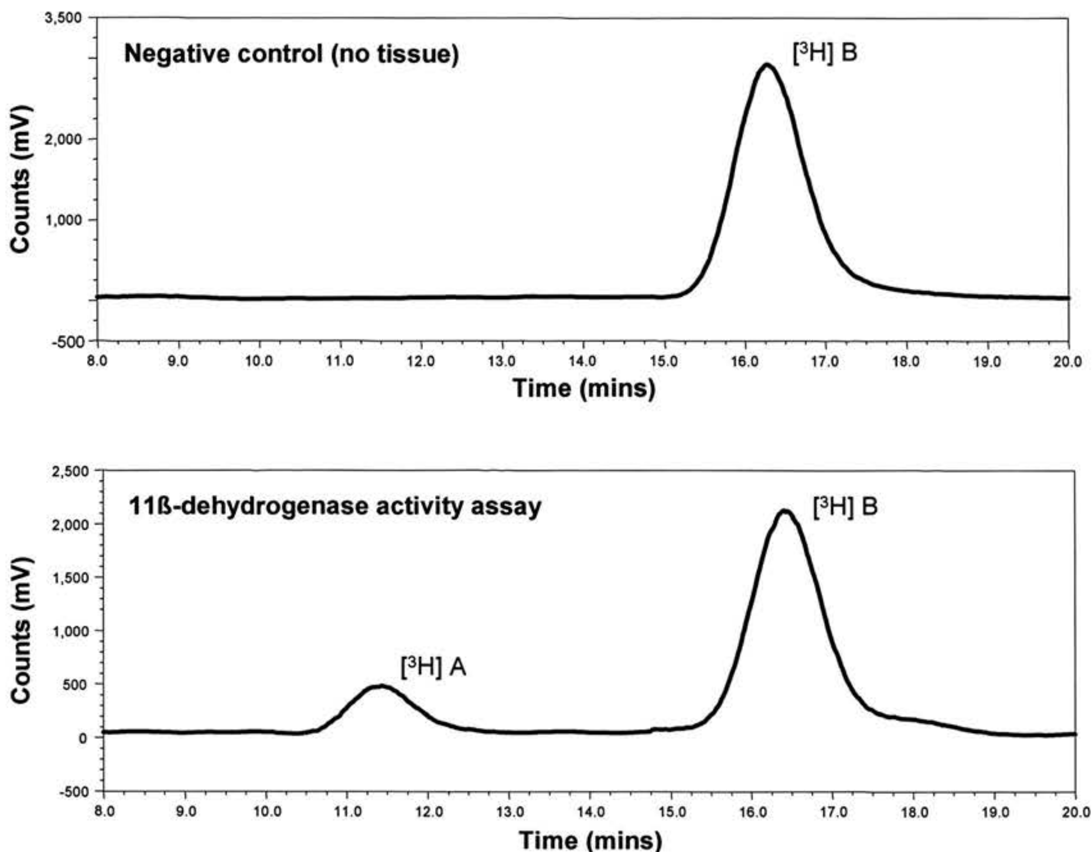


Figure 2.4: Chromatograms from 11β-dehydrogenase activity assay in liver microsomes

Two representative high performance liquid chromatography (HPLC) chromatograms, showing peaks for [^{3}H] 11-dehydrocorticosterone ([^{3}H] A; retention time 10-12 minutes) and [^{3}H] corticosterone ([^{3}H] B; retention time 15-18 minutes). The chromatograms show a negative control experiment (top), where 10nM [^{3}H] B was incubated for 2 hours with buffer and cofactor only, and an 11β-dehydrogenase activity assay (bottom), where [^{3}H] B was incubated with buffer, cofactor and liver microsomes. In the negative control there is no conversion of [^{3}H] B to its metabolite [^{3}H] A. However when liver microsomes are present metabolism to [^{3}H] A occurs, indicating the presence of 11β-dehydrogenase activity.

The contents of each tube were discarded and the radioactivity remaining was counted in a gamma counter, to determine the amount of antibody-bound [^{125}I] cortisol. Since the radioactive and non-radioactive forms of antigen compete for antibody binding sites, the more non-radioactive cortisol in the sample, the less radioactive cortisol will remain bound. To calculate the percentage bound [^{125}I] cortisol in each standard and sample, the average counts per minute (cpm) was divided by the average cpm for the zero standard and multiplied by 100. A standard curve of percentage bound [^{125}I] cortisol against cortisol concentration was constructed with results obtained from the standards, and the levels of cortisol in each sample could then be determined from this. At 50% displacement on the cortisol standard curve, cross reactivity for corticosterone was 3% and cross reactivity for cortisone was 1.4%.

2.11.2 Corticosterone

Plasma samples from mice implanted with a unilateral RU38486-releasing silastic pellet and contra-lateral vehicle control, and mice implanted with bilateral vehicle pellets, were analysed for corticosterone by radioimmunoassay. Plasma samples were diluted 1:10 in borate buffer, and denatured at 80°C for 30 minutes to release corticosterone from CBG. In this way, the assay measured total plasma corticosterone. 20 μl of diluted and denatured sample were then added in duplicate to a 96 well plate. Corticosterone standards of known concentration (0, 0.3, 0.6, 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320nM) were also added in duplicate to the plate. A rabbit anti-corticosterone primary antibody (diluted 1:100 in borate buffer; kindly provided by C. Kenyon), [^{3}H] corticosterone (3 μl in 6 ml borate buffer and adjusted to 10,000 cpm) and an anti-rabbit secondary antibody linked to scintillation proximity assay (SPA) beads (diluted according to manufacturers instructions; GE Healthcare, U.K.) were then added to each well. The plate was sealed and inverted to mix the contents of each reaction, then incubated at room temperature for 16 hours in the dark. Since the SPA beads ensured that only [^{3}H] corticosterone bound to primary antibody would be detected, there was no need to separate bound from unbound antigen. The radioactivity in each well was counted in a beta counter and, to calculate the percentage bound [^{3}H] corticosterone in each standard and sample, the average cpm was divided by the average cpm for the zero standard and multiplied by

100. A standard curve of percentage bound [^{3}H] corticosterone against corticosterone concentration was constructed with results obtained from the standards, and the levels of corticosterone in each sample could then be determined from this. Finally, the result was multiplied by 10 to account for the initial dilution of each plasma sample.

2.12 Genotyping by polymerase chain reactions

To confirm the genotype of 11β-HSD1$^{-/-}$ mice bred to use in these studies, polymerase chain reactions (PCR) were carried out. Using primers and the enzyme *Taq* polymerase, specific sequences in the genomic DNA from transgenic and C57Bl6 mice were amplified in a number of cycles, during which the DNA template is repeatedly denatured, annealed with the sequence-specific primers and replicated by *Taq* polymerase. Primers Ex1-3F1 and Ex1-3R1 allowed detection of the wild type 11β-HSD1 allele, while Ex1-3F1 and Yuri2oligo4 allowed detection of the disrupted transgenic 11β-HSD1 allele with integrated targeting vector (Table 2.1; (Kotelevtsev *et al.* 1997)). Following 36 cycles the amplified DNA was then separated by size using gel electrophoresis; the wild type 11β-HSD1 allele product was approximately 1.7Kbp and the targeted 11β-HSD1 allele product was approximately 1.4Kbp. This difference in size between transgenic and C57Bl6 mice allowed identification of 11β-HSD1$^{-/-}$ mice.

Total DNA was extracted from tail tips removed from 11β-HSD1$^{-/-}$ and C57Bl6 animals using a DNeasy Tissue kit (Qiagen, U.K.). PCR reactions containing 1μl undiluted DNA, 1x Green Go*Taq* reaction buffer, 6mM $MgCl_2$, 10mM each of dATP, dCTP, dGTP and dTTP, 1pmol/μl each of Ex1f, Ex1r and Yuri2oligo4 primers and 0.1U/μl Go*Taq* DNA polymerase were made up to a total volume of 20 μl with nuclease-free water.

Reactions were carried out on an Eppendorf Mastercycler Gradient (Eppendorf, Germany) with a heated lid. Samples were heated to 95°C for 5 minutes for initial denaturation, and then underwent 36 cycles of PCR amplification (denaturation at 95°C for 1 minute, primer annealing at 61°C for 1 minute, and elongation by *Taq* at

Primer	Sequence
Ex1-3F1	TTC TTC GTG TGT CCT ACA GG
Ex1-3R1	CCC GCC TTG ACA ATA AAT TG
Yuri2oligo4	CAC TGC ATT CTA GTT GTG GTT TGT CC

Table 2.1: Primer sequences for 11β-HSD1 genotyping by polymerase chain reaction

Using the primers above and the enzyme *Taq* polymerase, specific sequences in the genomic DNA from 11β-HSD1$^{-/-}$ and C57Bl6 mice were amplified and then separated by size using gel electrophoresis. Primers Ex1-3F1 and Ex1-3R1 allowed amplification of the wild type 11β-HSD1 allele, while Ex1-3F1 and Yuri2oligo4 allowed amplification of the targeted 11β-HSD1 allele in transgenic mice. This allowed identification of 11β-HSD1$^{-/-}$ or wild type genotype in the mice used in these studies.

$72^{o}C$ for 2 minutes 10 seconds). Reactions were then maintained at $72^{o}C$ for a further 10 minutes to ensure elongation of products to full length and cooled to $4^{o}C$ before gel electrophoresis.

To prepare a 1% gel, 2 g of agarose were melted in 200 ml 1x TAE buffer. After the addition of 2 μl of ethidium bromide, the gel was allowed to set in a mould at room temperature. The gel was then placed in a tank containing 1x TAE, and 10 μl of 1Kbp DNA ladder was loaded into the first well to allow determination of product size. The total volume of each PCR reaction was loaded into individual wells and the gel electrophoresed at 140 volts for approximately 1 hour, or until the loading buffer band was ¾ of the way down the gel. The gel was photographed under UV light at λ260nm to allow visualization of PCR product bands.

2.13 Statistics

All data are expressed as mean ± standard error of the mean (S.E.M). Values quoted for n refer to the number of different mice used in each experiment. Data were analysed by unpaired t test or Analysis of Variance (ANOVA) followed by post hoc tests, as appropriate. $p<0.05$ was considered significant.

Chapter 3

Femoral artery injury as a model to study neointimal proliferation in the mouse

3.1 Introduction

The major aim of the work described in this thesis was to assess the impact of both exogenous and endogenous glucocorticoids on neointimal lesion development after vascular injury. In order to allow use of the murine model of genetic 11β-HSD1 inactivation, it was necessary to introduce and clearly describe an appropriate model of neointimal lesion formation in the mouse.

Several well-described models of neointimal proliferation have been developed in the mouse (see chapter 1.1.4.3), including: wire-induced intra-luminal injury (Lindner *et al.* 1993), carotid artery ligation (Kumar & Lindner 1997), electrical injury (Carmeliet *et al.* 1997c) and perivascular cuff placement (Moroi *et al.* 1998). A model of intra-luminal injury was considered most appropriate for investigating the influence of exogenous and endogenous glucocorticoids on neointimal lesion formation. The model of neointimal proliferation developed was adapted from the method published by Sata *et al* (2000). This involves inserting a flexible angioplasty guidewire into the femoral artery via a small muscular side-branch. The wire stretches and denudes the artery and the injury caused results in rapid medial cell apoptosis, followed by the growth of a neointimal lesion composed of smooth muscle cells. It is not known how this vascular injury and subsequent neointimal proliferation affects contractile and relaxant function of the artery. Furthermore, since inflammation is critical to neointimal lesion formation (Wainwright *et al.* 2001), and 11β-HSD1 activity can be up-regulated by inflammatory stimuli in VSMCs (Cai *et al.* 2001), it is possible that intra-vascular injury may influence 11β-HSD1 activity in the vessel wall.

It is also possible to study the growth of neointimal lesions in isolated vessels *in vitro* (Pederson & Bowyer 1985; Soyombo *et al.* 1990; Voisard *et al.* 1999; Guerin *et al.* 2004). This allows investigation of the role that cells within the vascular wall, such as the endothelium and VSMCs, play in the development of vascular lesions. The introduction of such a model would allow the impact of glucocorticoids, and their generation by 11β-HSD1, on the vascular response to injury to be assessed in isolation from systemic effects.

Therefore, the specific aims of this chapter were:

(i) To introduce and develop a model of wire injury to the femoral artery based on the technique of Sata *et al* (2000).

(ii) To determine the time-dependent changes in arterial structure, function and 11β-HSD1 activity following intra-luminal injury.

(iii) To generate a model of neointimal proliferation *in vitro*.

3.2 Method development

3.2.1 Wire-induced injury of the mouse femoral artery

3.2.1.1 Animals

Male C57B16 mice (Harlan Olac, U.K.) were used in all studies in this chapter. Initially, animals were grouped by age and used between 10 and 16 weeks. However, it was found that some mice in this age-range were too small and insertion of the wire into the femoral artery was very difficult. Subsequently mice weighing 25-35 grams were used.

3.2.1.2 Surgical procedure

To induce acute intra-luminal vascular injury and produce neointimal proliferation in the mouse femoral artery, a surgical procedure was set up according to the method of Sata *et al* (2000), and then refined (for final method see chapter 2.3). The process was aided by a video of the operation provided by this group. For all studies in this chapter unilateral wire injury was carried out in the left femoral artery, with the right femoral artery acting as an uninjured contra-lateral control. Sham operation was performed in a group of mice (n= 3), by carrying out every part of the injury procedure except insertion of the flexible guidewire.

Methodological modifications. Initially, general anaesthesia was induced by inhalation of either halothane or isoflurane. Isoflurane was chosen for the refined method, as it induced a more rapid and stable anaesthesia, and reduced mortality during the operation. Surgery was performed on cork matting to reduce heat loss, and mice were allowed to recover in a heated chamber.

When placing the proximal temporary ligature used for vascular control, it was found that the most effective position was above the superficial caudal epigastric artery, which descends from the common femoral artery proximally to the popliteal artery. This not only halted blood flow in the main femoral artery but also helped to retract a large fat pad near the inguinal ligament, improving access to the femoral artery. In contrast to the method of Sata *et al* (2000), it was found that separation of the femoral artery from the vein was not necessary and this was discontinued in later studies to reduce damage to the vessel wall (particularly the adventitia).

3.2.1.3 Effect of vascular injury surgery on mice

Surgery was generally well tolerated, although mice occasionally died under anaesthesia (5/ 67 during model development). This problem was virtually abolished by switching from halothane to isoflurane anaesthesia. Mice quickly regained consciousness (recovery time $\leq$ 20 min) with few side-effects (some limping was evident for a few days after surgery). Only two out of 67 mice died spontaneously after recovery, with no obvious cause of death. A tendency to chew sutures did not prevent the skin from healing well in most animals.

3.2.1.4 Collection of arteries for histological analysis

To assess the time-course of neointimal proliferation and the composition of neointimal lesions after wire injury of the femoral artery, mice were killed by cervical dislocation either immediately or 2, 7, 14, 21 or 28 days after surgery (n= 5-6 per time-point). Sham operated animals were killed after 28 days (n= 3). Left and right femoral arteries were carefully excised and fixed in 10% neutral buffered formalin for up to 24 hours. After this time arteries were stored in 70% ethanol. The arteries were then dehydrated in a graded alcohol series, embedded in paraffin and cut into transverse sections: sets of eight 4 μm serial sections were taken at 100 μm intervals along the entire arterial segment.

3.2.1.5 Histological staining

Histological staining (haematoxylin and eosin or United States trichrome) confirmed the development of neointimal lesions following wire injury of the femoral artery (Figure 3.1). Initially, serial cross sections were stained with haematoxylin and eosin using an established protocol (chapter 2.6.1). In order to improve distinction of the IEL and EEL for quantitative analysis of neointimal lesion size, a trichrome staining method was then introduced. The United States trichrome stain (chapter 2.6.2) differentiates elastin, collagen and muscle in tissue samples (Hadoke *et al.* 1995). Identification and quantification of the neointima, media and adventitia was more straightforward in sections stained with the United States trichrome stain than in

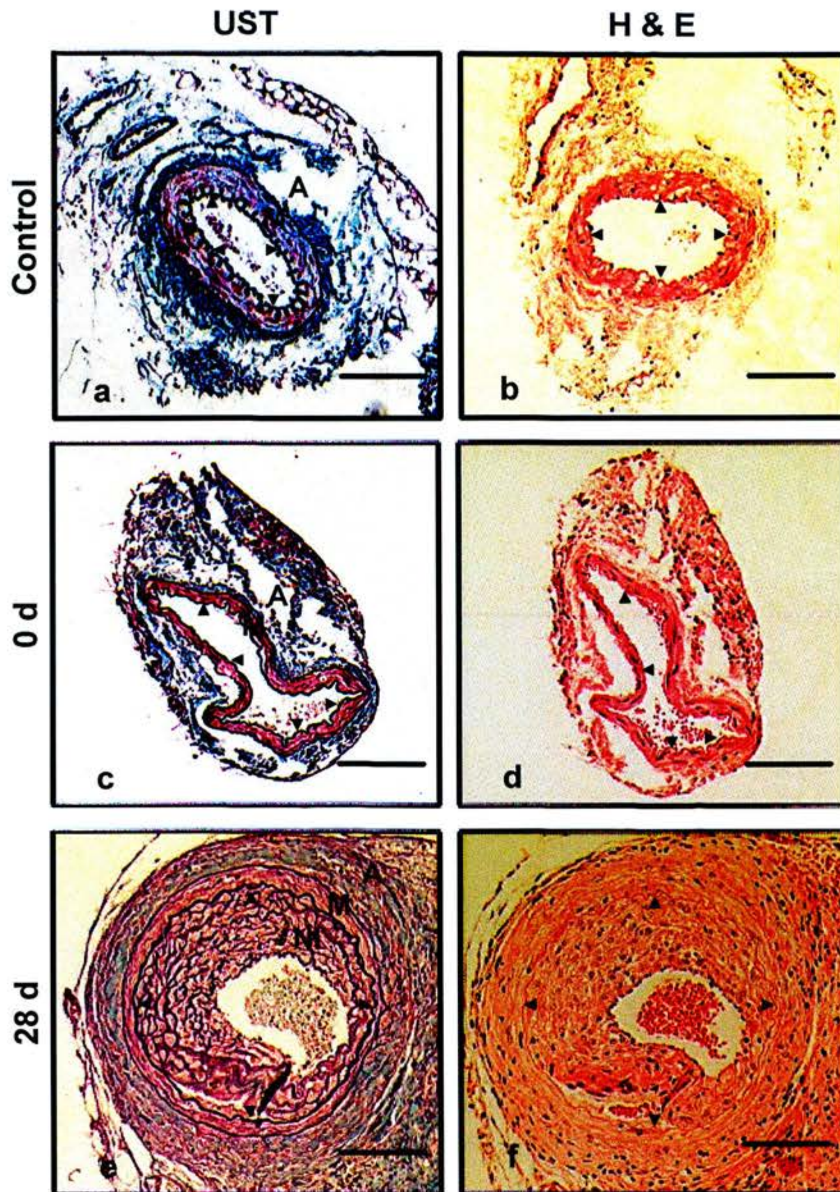


Figure 3.1: Comparison of histological staining methods

Representative serial sections of control (uninjured) femoral artery, and arteries immediately (0d) and 28 days after injury, stained with either United States trichrome (UST; a,c,e) or haematoxylin and eosin (H & E; b,d,f). Distinction of the internal elastic lamina (IEL) and external elastic lamina (EEL), and so identification of the layers of the artery wall, is easier in sections stained with UST than in those stained with H & E. Immediately after injury (c,d) the artery wall shows signs of stretch- the IEL is less convoluted and the medial layer is thinner. 28 days after injury (e,f) neointimal proliferation has occurred and a large neointimal lesion occludes the lumen. Red blood cells can also be seen in the lumen of the vessels. A: adventitia, M: media, NI: neointima. Arrow heads indicate IEL. Scale bar = 100 μm.

those stained with haematoxylin and eosin, as it provided clear staining of the IEL and EEL (Figure 3.1). Once this method was established it was used to analyse serial sections in all samples, either manually or using an automated staining system (Varistain Gemini Thermo Shandon, U.K.).

3.2.1.6 Effects of wire injury on morphology of the femoral artery wall

To measure changes in artery morphology after injury, images of sections stained with the United States trichrome stain were digitised using a light microscope coupled to a colour camera (chapter 2.7). To quantify neointimal lesion size, image analysis software was used to make measurements of mean area (μm^2) inside the EEL, IEL and lumen, in sections every 100 µm along each artery. Due to variation in the distance of wire advancement into the femoral artery during surgery (5-10 mm), the length of neointimal lesions induced by injury was variable between animals (data not shown). Therefore, the total lesion area was not quantified; instead the section with the largest area of neointima was chosen to represent each arterial sample. Additional measurements made included perimeter (µm) and average diameter (µm) of the EEL, IEL and lumen.

Femoral arteries isolated immediately after injury (0d) showed evidence of severe stretch, with the perimeters of the EEL and IEL increased compared with uninjured controls (Table 3.1). The IEL was no longer as convoluted as in an uninjured artery and the lumen was larger, although this did not become significant until 7 days after injury (Figures 3.2, 3.3 and Table 3.1). While the EEL of the vessel wall remained intact after injury, some damage to the IEL was observed. Small breakages were seen in all arteries at 0d. Furthermore, removal of the IEL was seen in 3/ 5 arteries at 0d, always in areas near to the site of wire insertion (Figure 3.4).

Two days after injury, the artery wall remained stretched (Table 3.1). There was evidence of damage to the IEL in 3/ 5 arteries at this time-point. Insertion of the wire also caused medial thinning, which was evident 2d and 7d after injury (Figures 3.2, 3.3) but this did not reach significance (Table 3.1).

Neointimal lesions formed as a result of injury in a time-dependent manner, first becoming evident at 7d, and continuing to increase in size until 21d, when growth

	EEL circumference (µm)	IEL circumference (µm)	Luminal area (µm²)	Neointimal area (µm²)	Medial area (µm²)
Control	952 ± 61	866 ± 71	26176 ± 4855	0 ± 0	34123 ± 2254
0d	1461 ± 101*	1405 ± 92 *	66611 ± 26620	0 ± 0	35262 ± 2854
2d	1488 ± 195 *	1410 ± 198 *	49635 ± 19753	0 ± 0	21384 ± 3106
7d	1649 ± 50 **	1583 ± 39 ***	83243 ± 11314*	45901± 11621	28488 ± 4138
14d	1792 ± 69 ***	1663 ± 75 ***	50348 ± 10733	111605 ± 19733**	54032 ± 7693
21d	1854 ± 175 ***	1762 ± 141 ***	26475 ± 6747 +	167654 ± 40486***	59226 ± 783 *
28d	1679 ± 56 ***	1612 ± 31 ***	16641 ± 5482 +	143203 ± 10332***	42548 ± 2136

Table 3.1: Morphometric analysis of wire-injured femoral arteries

Femoral arteries, collected at various time-points after injury, were analysed using digitised cross sections and image analysis software. Injured arteries were compared with uninjured contra-lateral controls at 0d. The measurements made indicated that wire injury caused an increase in external elastic lamina (EEL) and internal elastic lamina (IEL) circumference which was maintained up to 28d, and that luminal area became larger at early time-points. Data are mean ± S.E.M, n= 6 for control, 7, 14, 21 and 28 days, n= 5 for 0 and 2 days. Each parameter analysed by one way ANOVA with Tukey's post hoc test: * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$ vs. control, $^{+}p<0.05$ vs. 7d.

N.B. Luminal, neointimal and medial area at each time-point are also shown in Figure 3.3.

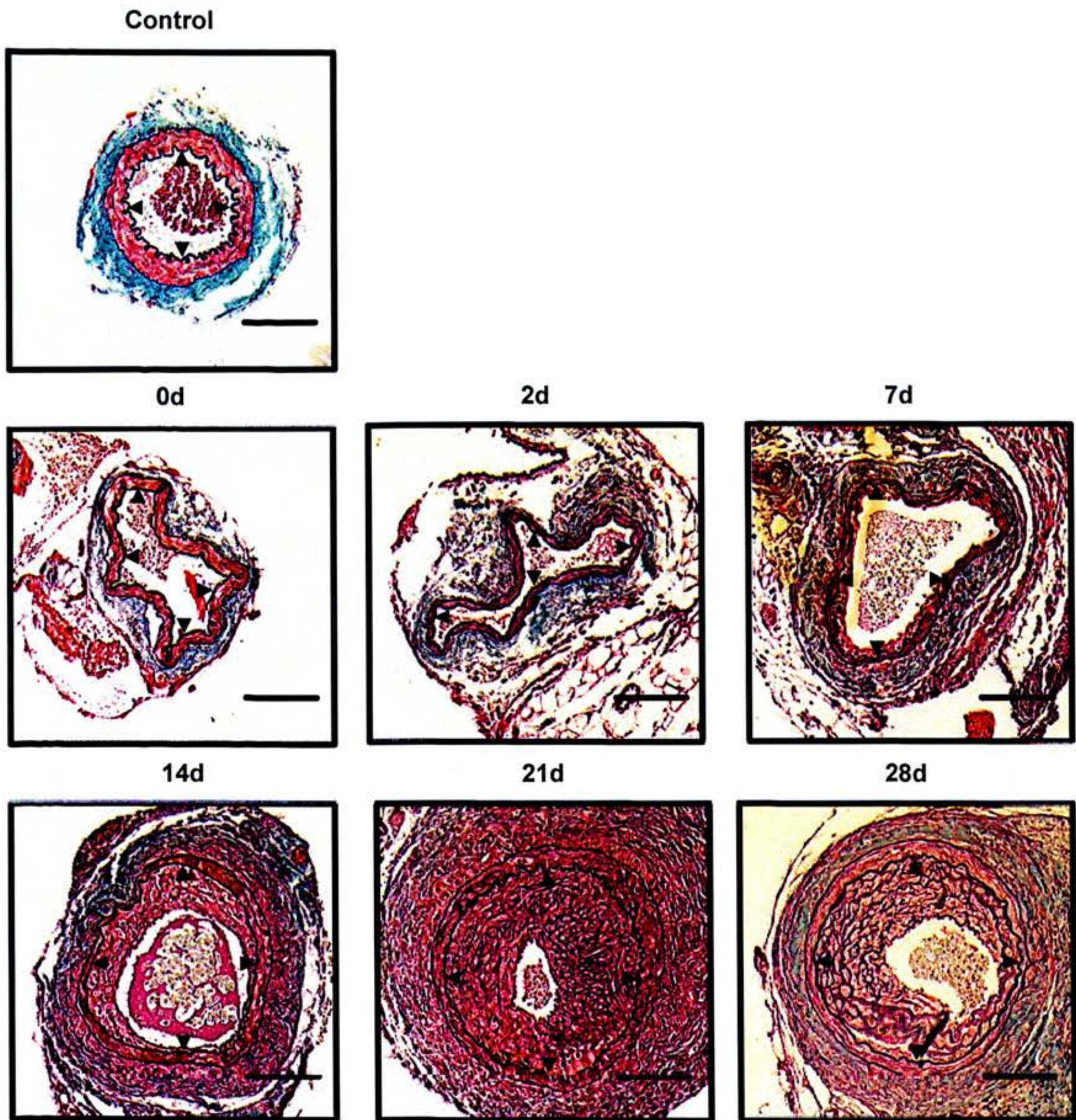


Figure 3.2: Neointimal proliferation after wire injury of the mouse femoral artery

After injury, arteries were excised at the time-points indicated and stained with the United States trichrome stain. A control uninjured vessel is also shown for comparison. Immediately (0d) and 2d after injury the internal elastic lamina is stretched and the medial layer is thinner than in the control. An absence of nuclei suggests that the endothelium has been denuded. Neointimal proliferation is first observed at 7d and continues until lesions peak in size at 21d. Neointimal lesions stain strongly for elastic fibres. Remodelling of the adventitia can also be observed. Arrow heads indicate internal elastic lamina. Scale bar = 100 µm.

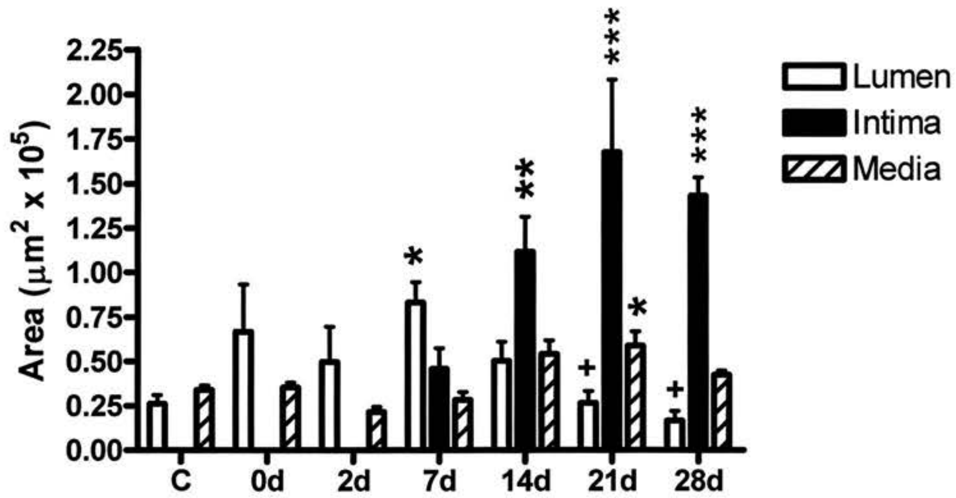


Figure 3.3: Quantification of luminal, neointimal and medial size over time

After wire injury of the femoral artery, morphometric analysis was carried out on digitised cross sections at the time-points shown. The graph shows that neointimal proliferation begins 7d after injury, becoming significant from 14d onwards. Luminal area is larger than uninjured controls at early time-points, and then decreases in parallel with lesion growth. Medial area shows an initial trend towards a decrease which is not significant, and then becomes larger than controls at 21d. Data are mean ± S.E.M, n= 6 for control, 7, 14, 21 and 28 days, n= 5 for 0 and 2 days. Analysed by one way ANOVA with Tukey's post hoc test: * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ vs. control, $^{+}p< 0.05$ vs. 7d.

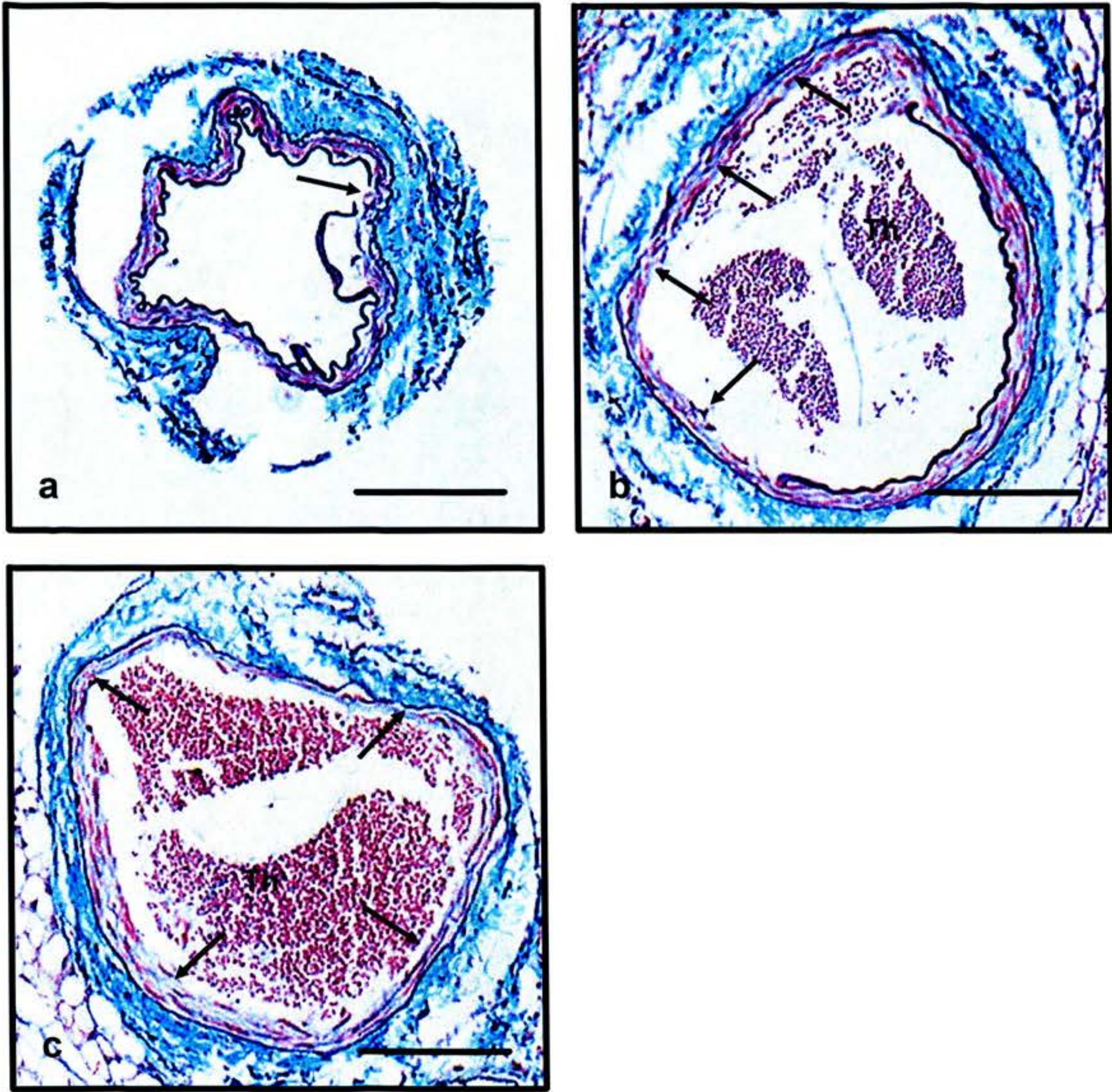


Figure 3.4: Damage to internal elastic lamina caused by wire insertion

Representative sections of arteries immediately after injury, showing types of potential damage caused to the internal elastic lamina (IEL) by wire insertion. Indicated by arrows, IEL breaks (a), partial stripping (b) and complete removal (c) were all observed, close to the site of wire insertion. Thrombosis (Th) in response to IEL removal can be seen in (b) and (c). Scale bar = 100 µm.

peaked (Figures 3.2, 3.3). In parallel to neointimal proliferation, medial size gradually increased and was significantly larger than control after 21 days (Figure 3.3 and Table 3.1). Luminal size decreased as neointimal lesion size increased (Figure 3.3 and Table 3.1). Expansion of the femoral artery, as assessed by an increase in the perimeter of the IEL and EEL, persisted over the time-course of these experiments (Table 3.1).

3.2.1.7 Thrombosis after wire injury of the femoral artery

Occlusive thrombus, which completely blocked the lumen of the vessel, was seen in 18% of the arteries studied from 0 to 28 days during the model development (6/ 34).

Since there was minimal reperfusion in the arteries collected immediately after injury, only a very slight thrombotic response was seen. Thrombosis was usually associated with areas of IEL breakage or removal (Figure 3.4). Vessels 2d after injury had been reperfused, and in two cases this resulted in the formation of an occlusive thrombus over the area of IEL damage (Figure 3.5). In both arteries this was seen in areas near to the site of wire insertion and branch ligation, leaving traces of moderate thrombus in proximal sections before disappearing further up the vessel. In the third artery with IEL damage, the IEL had been removed in areas near to the site of wire insertion but this resulted in mural thrombus rather than occlusion (Figure 3.5). In areas of the vessels without IEL damage, evidence of platelet adhesion to the stretched artery wall could be seen (Figure 3.5).

At later time-points some evidence of thrombosis could still be seen. As at earlier time-points, extensive damage and IEL removal were sometimes observed where the wire was inserted into the artery. This could be associated with occlusive thrombus (one at 7d, one at 14d and two at 21d) or more often with mural thrombus, which became organised into fibro-proliferative neointimal lesions. However neointimal lesions which were not of thrombotic origin were seen in all arteries, extending proximally along the vessel, away from the site of wire insertion.

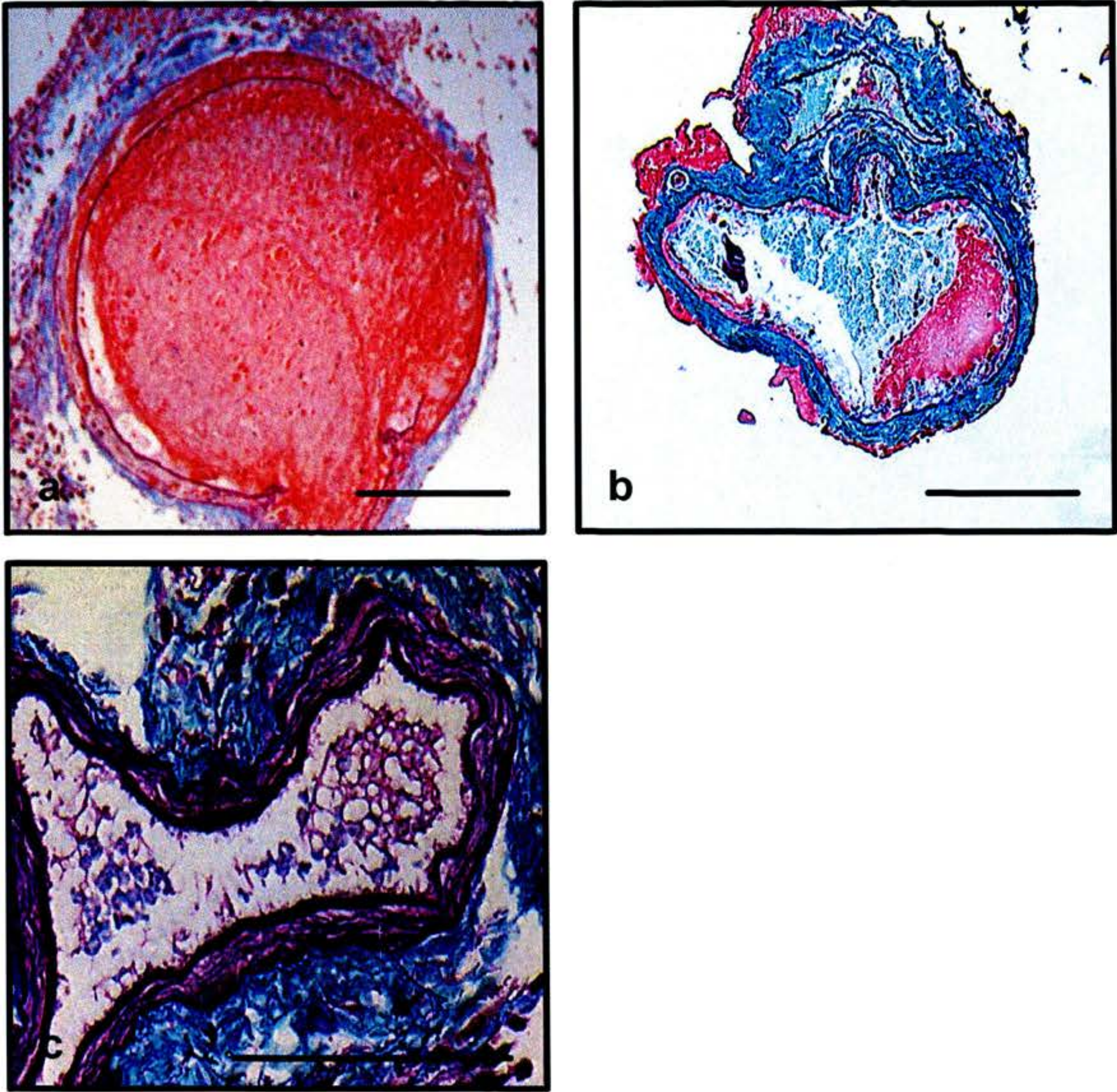


Figure 3.5: Types of thrombosis caused by wire insertion

Representative sections of arteries 2 days after injury, showing types of thrombus formed in response to wire insertion. Occlusive (a) and mural (b) thrombi were observed over areas of IEL damage, whereas evidence of platelet adhesion (c) was seen in the intact stretched artery wall. Scale bar = 100 µm.

3.2.1.8 Cellular components of neointimal formation after wire injury

The composition of neointimal lesions in serial sections of injured arteries was assessed using antibodies against smooth muscle cell α-actin (alkaline-phosphatase conjugated), von Willebrand factor (vWF), Mac-2 and proliferating cell nuclear antigen (PCNA). The protocol for each antibody is described in chapter 2.8. An attempt was also made to identify endothelial cells using an antibody against CD31 (BD Pharmingen, U.K.) but staining was unsuccessful. It is likely that this was due to antigen masking in the formalin-fixed tissue sections used.

The area occupied by immunoreactivity for smooth muscle cell α-actin and Mac-2 was quantified in the neointima and media of injured arteries by measurement on digitised cross sections at each time-point, and expressed as a percentage of the total area of the respective vessel layer. The number of nuclei staining for PCNA in the neointima and media at each time-point was counted under a light microscope, and is expressed as a percentage of the total number of nuclei in the respective vessel layer.

Neointimal lesions stained positively for elastic fibres (Figure 3.2), whilst staining for smooth muscle cell α-actin confirmed that VSMCs were a major cellular component of advanced neointimal lesions (Figure 3.6). Smooth muscle cells were first evident in lesions at 14d and peaked in area at 21d (Figures 3.6, 3.7). Injury also caused a dramatic reduction in medial smooth muscle cells, which was first apparent at 2d and persisted across the time-course of neointimal development (Figures 3.6, 3.7).

vWF is a glycoprotein present in blood plasma and produced by the endothelium, megakaryocytes (in the α-granules of platelets) and subendothelial connective tissue. An absence of cellular nuclei and a lack of strong staining for vWF indicated that the endothelial layer had been denuded in arteries at 0d and 2d after injury (Figures 3.2, 3.8). Granular staining was observed on the luminal surface of arteries at 2d, suggesting the adherence of platelets after injury (Figure 3.8). Expression of vWF over the luminal surface of neointimal lesions was first observed at 7d, when coverage was patchy and limited to individual cells. Lesions from 14d to 28d showed

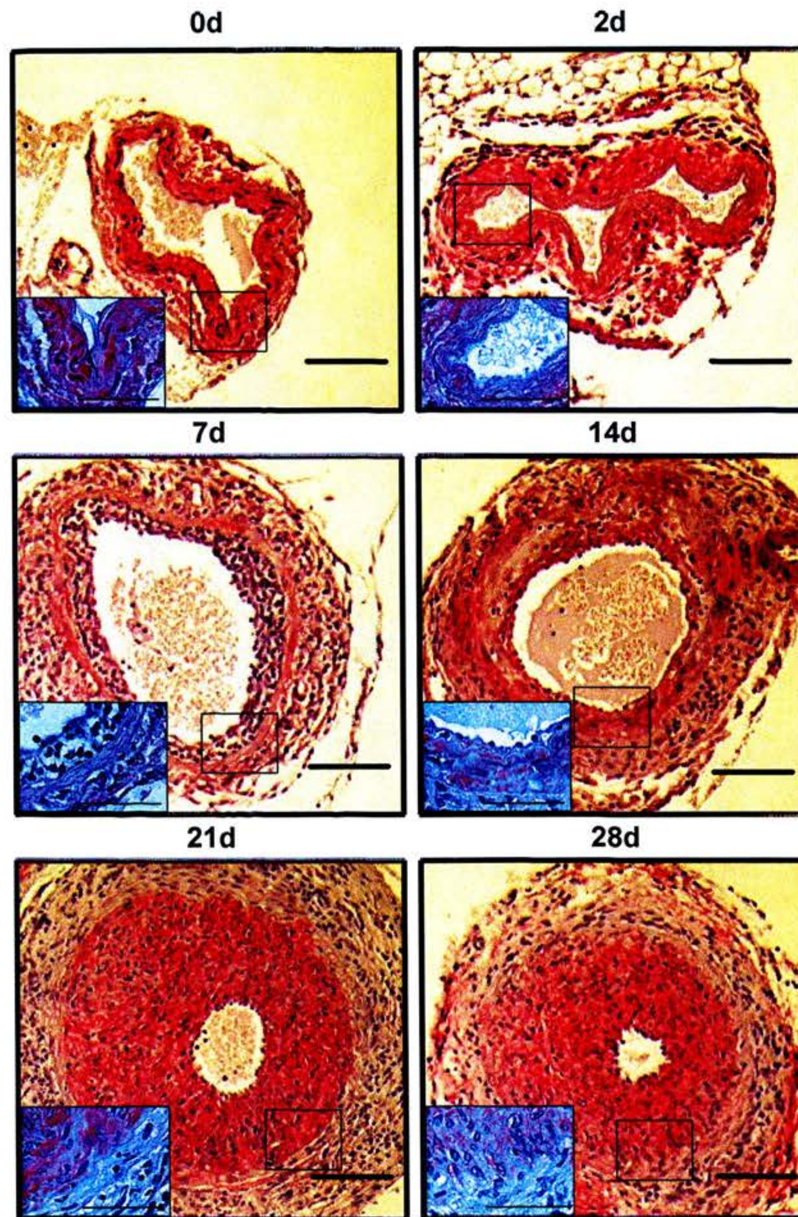


Figure 3.6: Expression of smooth muscle cell α-actin after vascular injury

Representative sections of wire-injured femoral arteries at the time-points shown stained with primary antibody against smooth muscle cell α-actin, and detected with VECTOR red. Immunoreactivity in neointimal lesions was first detected at 14d, and indicated that smooth muscle cells are a major component of advanced lesions at 21d and 28d. Staining also showed that there is a dramatic loss of smooth muscle from the media of the artery wall 2d after injury which persisted over the course of neointimal proliferation. Inserts at higher magnification confirm an absence of cellular nuclei in the media at 2d and 7d, and re-appearance of medial nuclei from 14d onwards. Box indicates area shown in insert at high magnification. Scale bar = 100 μm at low magnification and 50 μm at high magnification.

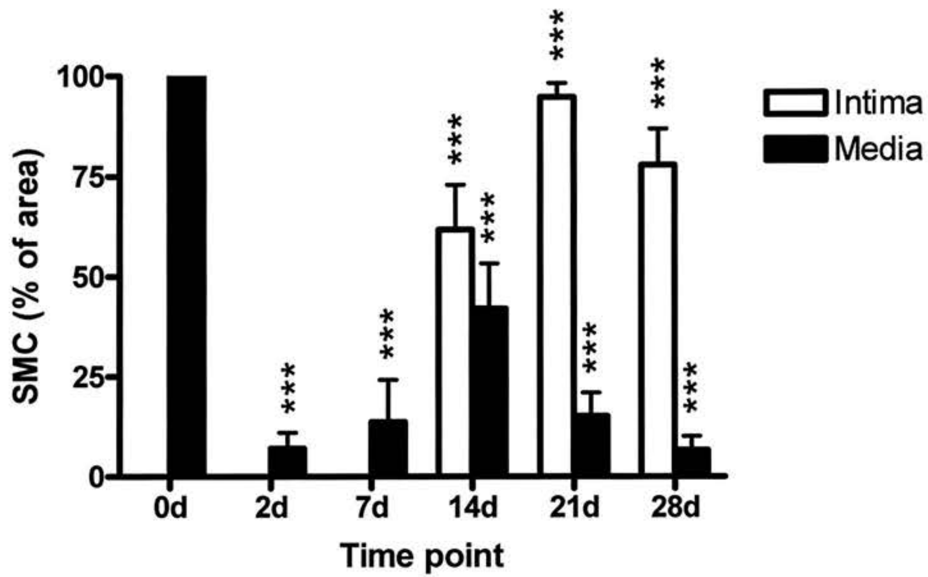


Figure 3.7: Quantification of neointimal and medial smooth muscle cell α-actin

The area stained for smooth muscle cell α-actin in the neointima and media of injured arteries was measured on digitised cross sections at each time-point, and expressed as a percentage of the total area of the respective vessel layer. Measurements confirmed that smooth muscle cell α-actin was first expressed in neointimal lesions at 14d and peaked at 21d. Immunoreactivity in the media showed a dramatic reduction at 2d, a partial recovery at 14d and a further reduction at 28d. Data are mean ± S.E.M, n= 6 for 7, 14, 21 and 28 days, n= 5 for 0 and 2 days. Analysed by one way ANOVA with Tukey's post hoc test: *** p<0.001 vs. 0d.

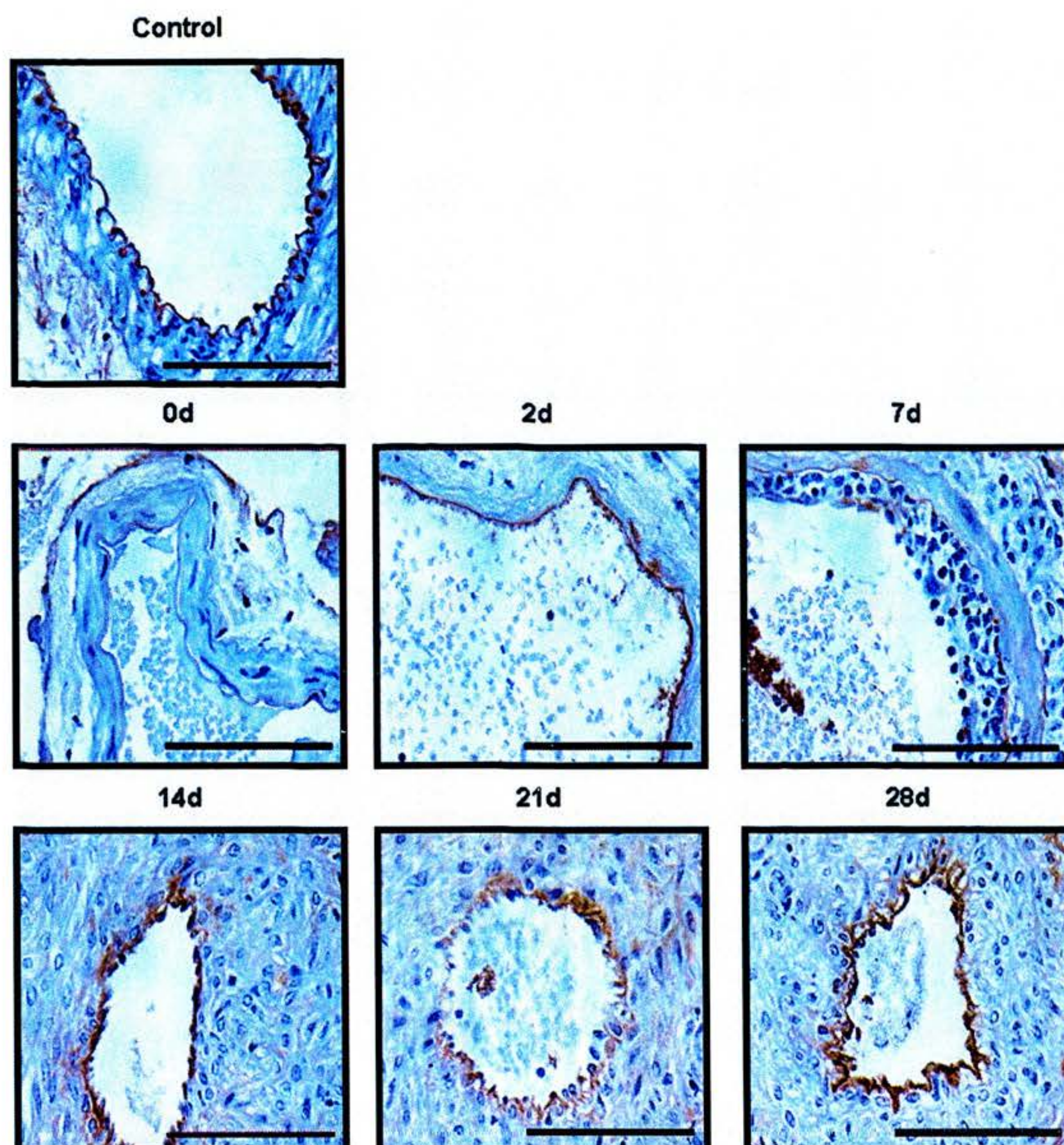


Figure 3.8: Expression of von Willebrand factor after wire-induced injury of the femoral artery

Wire-injured femoral arteries were collected at the time-points shown and representative sections (n= 3 per time-point) were stained with primary antibody against von Willebrand factor (vWF), detected with DAB. A control uninjured vessel is also shown for comparison. No cellular staining for vWF was observed at 0d, indicating the endothelium had been removed by wire injury. Immunoreactivity was detected in a granular pattern at 2d, indicating the adherence of platelets to the vessel wall after injury. Cells expressing vWF were first detected on the luminal surface of neointimal lesions at 7d, and from 14d onwards strong expression was observed in a thickened layer on the luminal surface of advanced lesions. Scale bar = 100 μm.

strong expression of vWF in a thickened layer of cells over the luminal surface (Figure 3.8).

Immediately after injury, no staining for macrophages was seen in the vessel wall or lumen; however, by 2d sites of Mac-2 expression (a macrophage cell surface antigen) could be seen exclusively in the cells of the adventitia (Figure 3.9). At 7d a large proportion of cells within the neointima stained positively for Mac-2, and occasionally cells on the luminal surface of lesions showed positive staining (Figures 3.9, 3.10). Macrophages could also be seen in the media at this time-point, and were still observed in the adventitia. At 14d smaller sites of Mac-2 expression were observed in the adventitia, media and neointima than at 7d (Figures 3.9, 3.10). Advanced neointimal lesions at 21d and 28d showed small and discrete areas of Mac-2 expression, with staining also seen in certain areas of the media immediately under the IEL, and in some cells in the adventitia (Figures 3.9, 3.10).

PCNA, a marker of cellular proliferation, is expressed in the G_1 and S phases of the cell cycle. No PCNA expression was seen in the vessel wall immediately after injury, and by 2 days expression was detected in the cells of the adventitia only (Figure 3.11). PCNA was first seen in the neointima and media 7 days after injury, and reached maximal levels in the neointima at 14 days (Figures 3.11, 3.12). PCNA was also expressed in the cells of the adventitia at these time-points. After this time, PCNA expression decreased in all layers of the vessel wall (Figures 3.11, 3.12).

Evidence of adventitial remodelling was seen after wire-induced injury, with a large number of cellular nuclei, smooth muscle cells, macrophages, proliferating cells and elastic fibres observed outside of the EEL (Figures 3.2, 3.6, 3.9, 3.11).

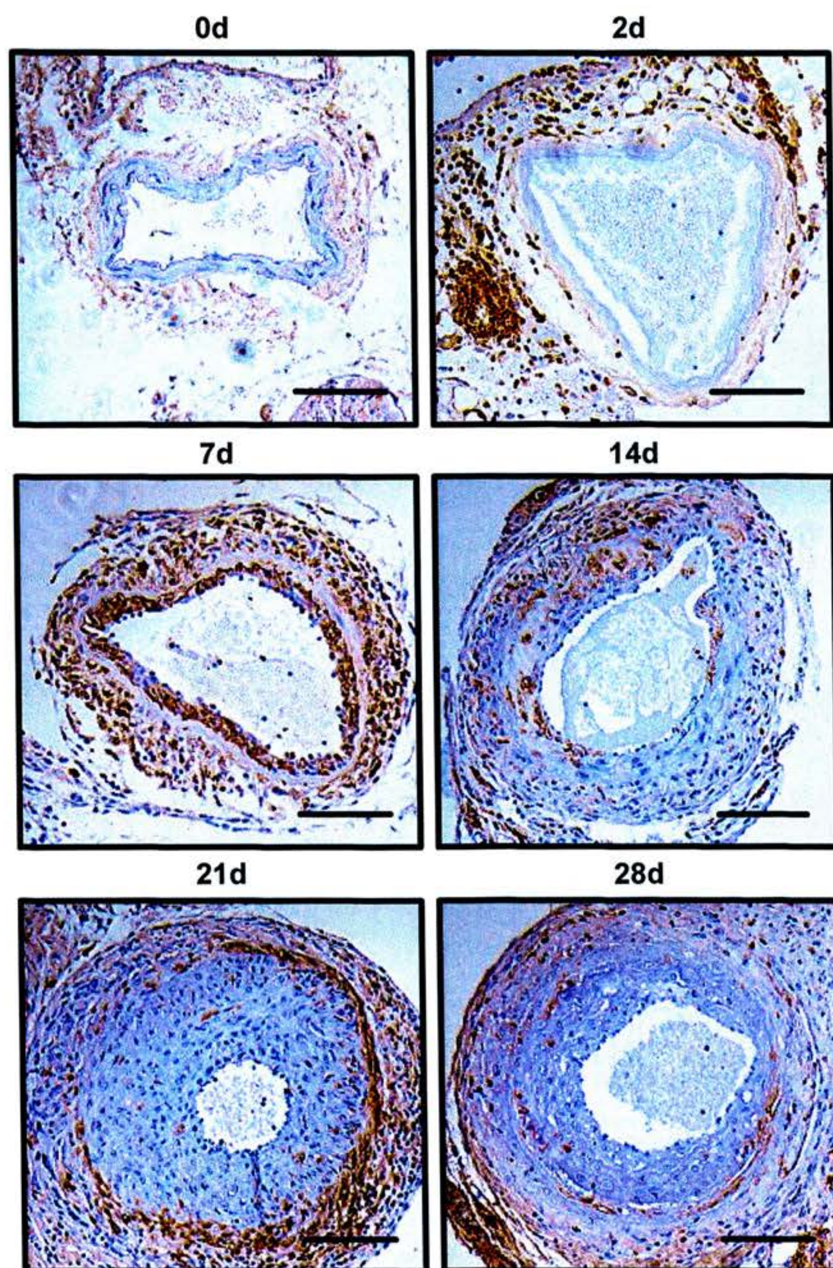


Figure 3.9: Expression of Mac-2 in the vessel wall after wire-induced injury

Wire-injured femoral arteries were collected at the time-points shown and representative sections stained with primary antibody against Mac-2, detected with DAB. No staining for Mac-2 was observed at 0d, and staining was detected exclusively in the cells of the adventitia at 2d after injury. Immunoreactivity was detected in a large proportion of cells in the neointima at 7d, after which time expression was limited to small discrete sites in neointimal lesions. Mac-2 was also detected in the media from 7d onwards, with high levels of expression seen under the internal elastic lamina at 21 days. Scale bar = 100 µm.

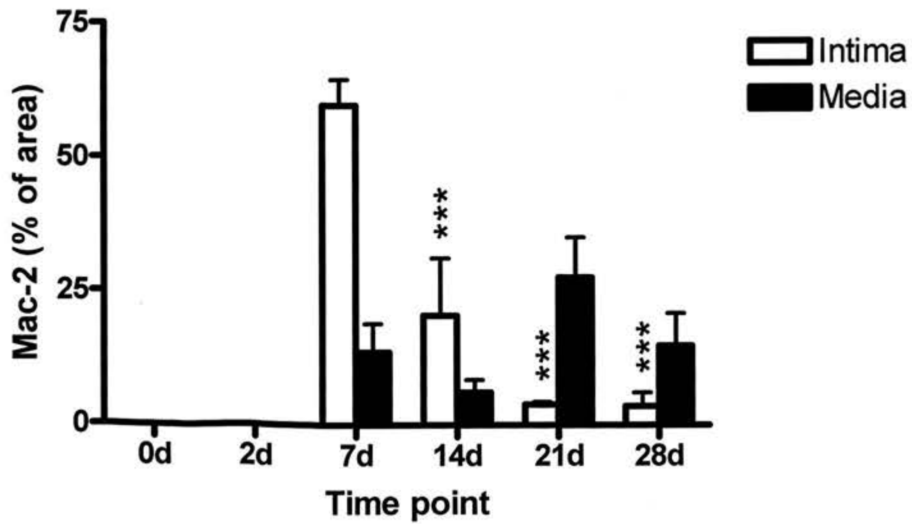


Figure 3.10: Quantification of neointimal and medial Mac-2 content over time

The area stained for Mac-2 in the neointima and media of wire-injured femoral arteries was measured on digitised cross sections at each time-point, and is expressed as a percentage of the total area of the respective vessel layer. Measurements confirmed that Mac-2 was highly expressed in neointimal lesions at 7d, after which time expression decreased, and occurred in small areas only in advanced lesions. Immunoreactivity was also detected in the media from 7d onwards. Data are mean ± S.E.M, n= 4. Analysed by one way ANOVA with Tukey's post hoc test: *** $p<0.001$ vs. 7d.

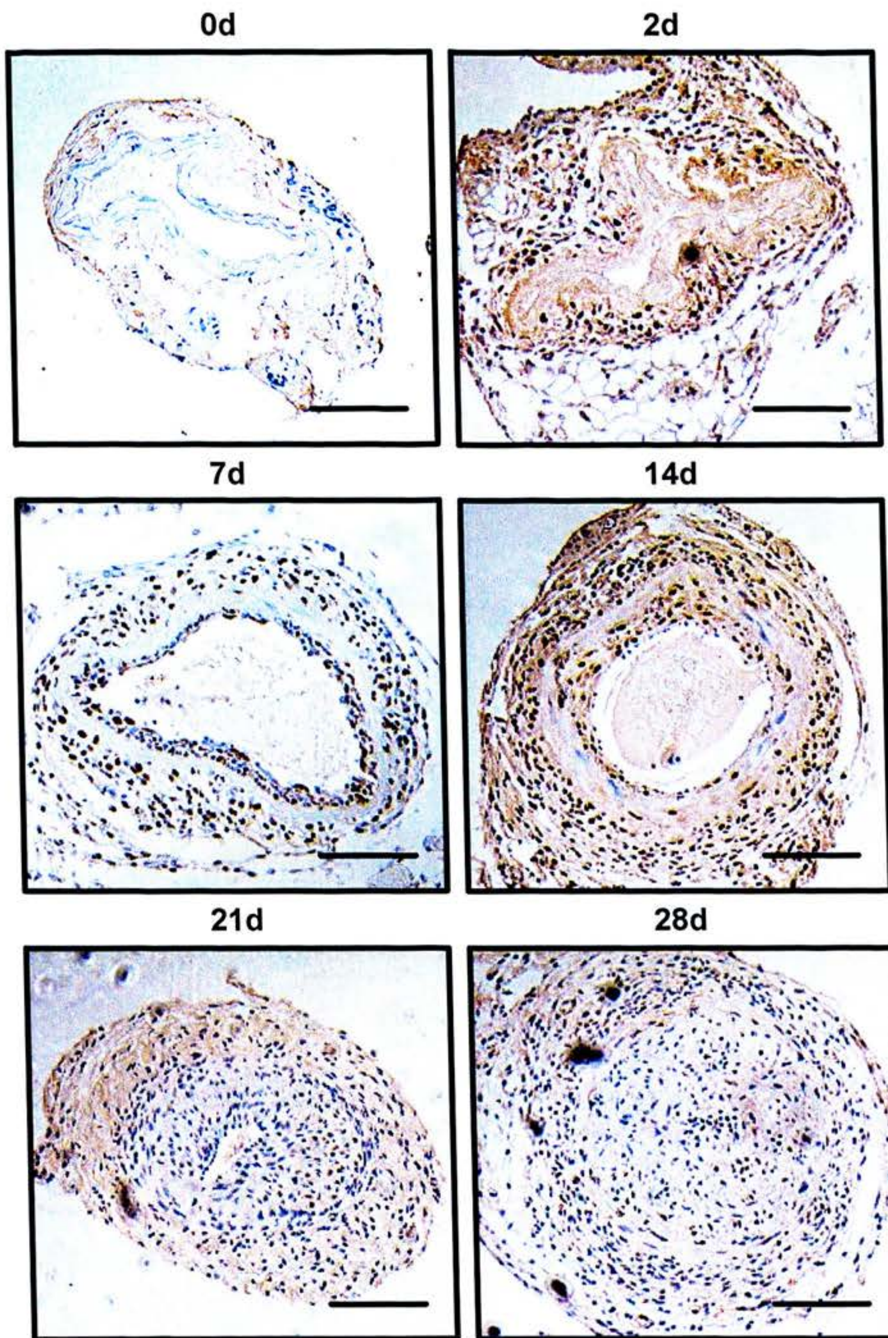


Figure 3.11: Detection of PCNA in the vessel wall after wire-induced injury

Representative sections of wire-injured femoral arteries collected at the time-points shown were stained with primary antibody against proliferating cell nuclear antigen (PCNA), and detected with DAB. No staining for PCNA was observed at 0d, and staining was detected in nuclei in the adventitia at 2d after injury. PCNA was first seen in the neointima and media 7 days after injury, and was more abundant at 14 days. After this time, PCNA expression decreased in all layers of the vessel wall. Scale bar = 100 µm.

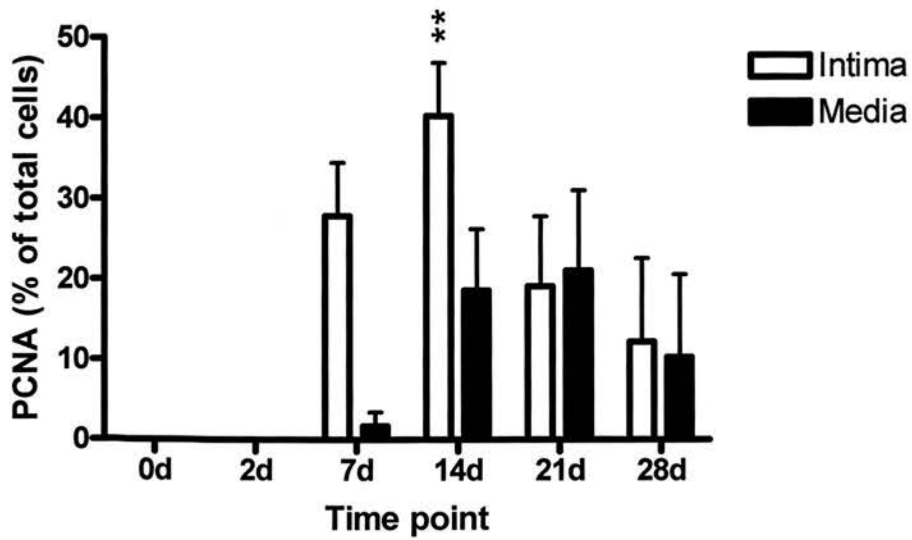


Figure 3.12: Quantification of neointimal and medial PCNA expression over time

Nuclei stained for proliferating cell nuclear antigen (PCNA) in the neointima and media of wire-injured femoral arteries were counted under a light microscope at each time-point, and expressed as a percentage of the total number of nuclei in the respective vessel layer. Quantification confirmed that PCNA was first detected in neointimal lesions at 7d, peaked at 14d and then decreased. Immunoreactivity was also detected in the media from 7d onwards. Data are mean ± S.E.M, n= 4. Analysed by one way ANOVA with Tukey's post hoc test: ** $p<0.01$ vs. 0d.

3.2.1.9 Effect of sham procedure on femoral artery structure

Animals that underwent sham surgery also developed large neointimal lesions in the common femoral artery after 28 days (Figure 3.13a). These lesions were restricted to sites immediately proximal to the branch of the popliteal artery. There was no evidence of IEL damage or thrombosis in these arteries and the endothelium appeared to be intact. Although the lesions formed in sham operated arteries were sizeable, they were significantly smaller than those which formed 28 days after wire-induced injury (Figure 3.13b).

3.2.2 Neointimal proliferation induced by sham operation

3.2.2.1 Surgical protocol

In order to determine which aspect of the sham procedure was causing neointimal lesion development, groups of mice (n= 3 per group; one treatment in each leg of six mice) were subjected to different components of the operation, as follows:

(a) isolation of femoral bundle from surrounding tissue,

(b) as (a), then isolation of main femoral artery and vein from the nerve and placement of temporary ligatures for 1 minute,

(c) as (b), then popliteal artery isolated and ligated distally,

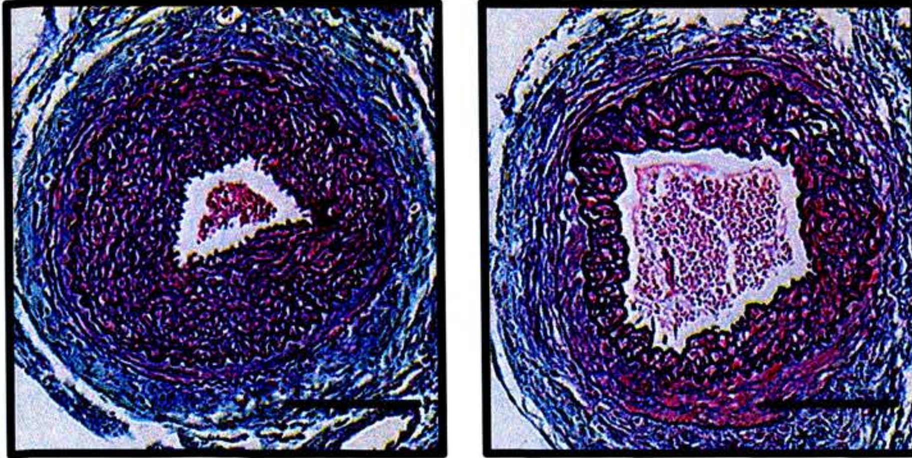
(d) as (c), then popliteal artery ligated proximally (see also Figure 3.14).

All arteries were collected after 28 days and fixed in 10% neutral buffered formalin for 24 hours. A series of transverse paraffin sections (4 μm) from each artery were stained with the United States trichrome stain and neointimal area measured on digitised cross sections.

3.2.2.2 Permanent ligation causes sham-induced neointimal proliferation

Isolation of the femoral bundle (Group a) did not cause neointimal lesion development (Figure 3.15). Isolation of the femoral artery from the nerve and placement of temporary ligatures to interrupt blood flow for 1 minute (Group b) resulted in development of a very small, discrete neointimal growth in 1/ 3 arteries.

a



b

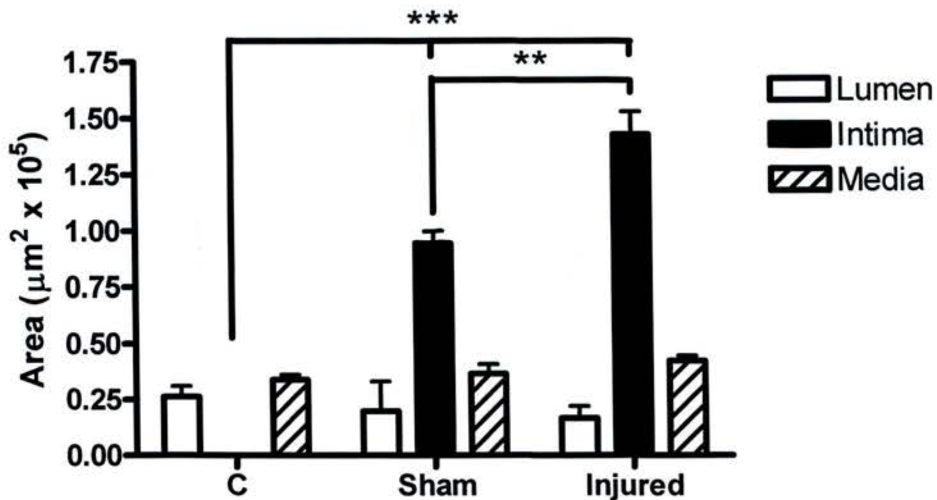


Figure 3.13: Neointimal lesion development in sham operated arteries

(a) Two representative sections showing extensive neointimal lesions formed in sham injured arteries 28 days after operation. Scale bar = 100 μm.

(b) Quantification of vessel layer area in digitised cross sections, showing the growth of neointimal lesions in both sham and injured arteries at 28d. Lesions after wire injury are significantly larger than those in sham operated arteries. Data are mean ± S.E.M, n= 6 for control and 28 days, n= 3 for sham. Analysed by one way ANOVA with Tukey's post hoc test: ** $p < 0.01$, *** $p < 0.001$.

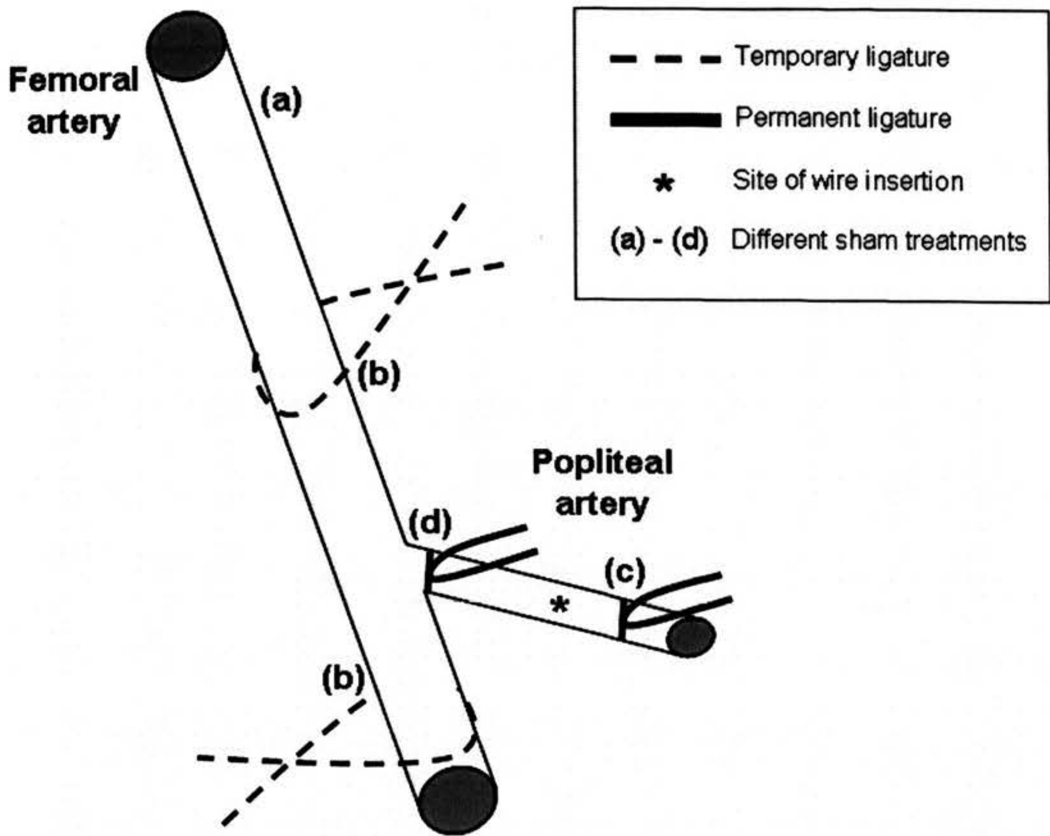


Figure 3.14: Assessment of components of the sham operation on neointimal proliferation

A schematic representation of the procedures carried out to determine which stage of the sham wire-induced injury operation induced neointimal proliferation in the femoral artery. Exposure of the femoral artery (a), placement of temporary ligatures for 1 minute (b), distal ligation of the popliteal artery (c) or proximal ligation of the popliteal artery (d) were carried out in four different groups. The site where the flexible wire would normally be inserted during the injury operation is also marked.

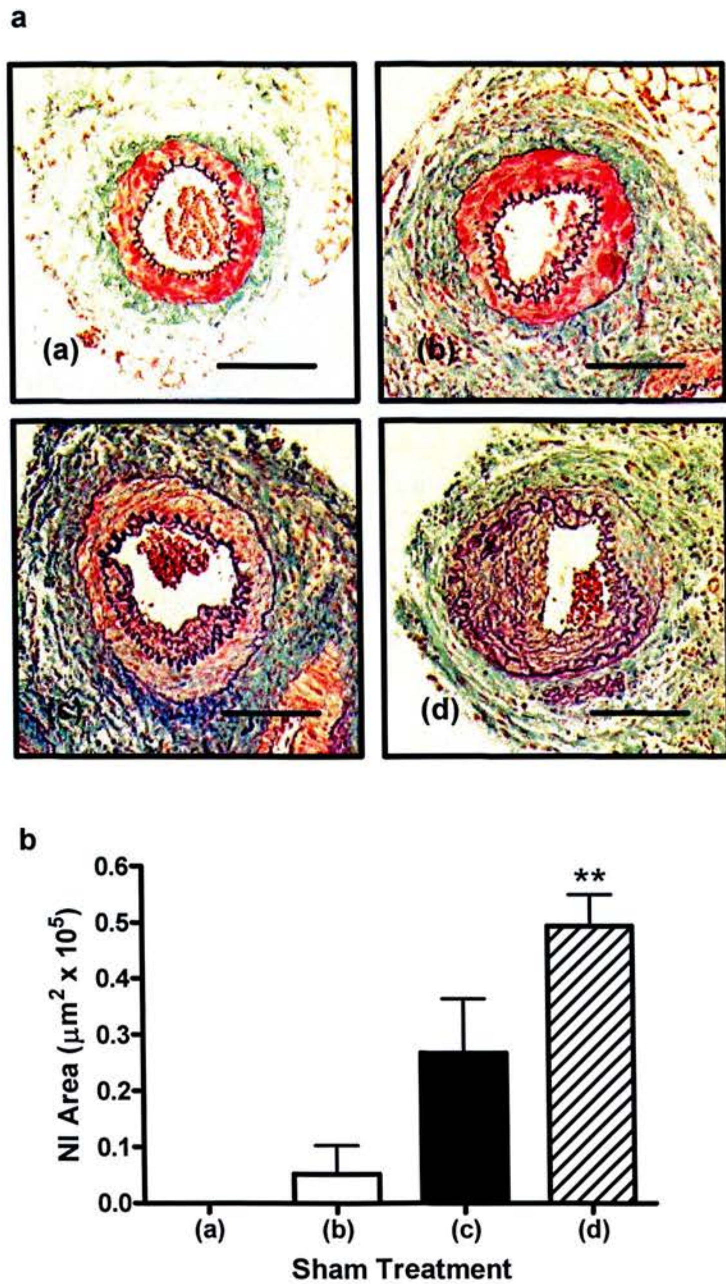


Figure 3.15: Sham-induced neointimal proliferation is caused by permanent ligation of the popliteal artery

(a) Representative sections from each sham treatment group: (a) isolation of femoral bundle, (b) placement of temporary ligatures for 1 minute, (c) distal and (d) proximal ligation of the popliteal artery. Neointimal proliferation is most extensive in (d). Scale bar = 100 µm.

(b) Quantification of neointimal area in each group, which increases from (a) through to (d). Data are mean ± S.E.M, n= 3. Analysed by one way ANOVA with Tukey's post hoc test: ** $p < 0.01$ vs. a.

In contrast, permanent ligation of the distal popliteal artery (Group c) produced intermediate-sized neointimal lesions in 3/ 3 femoral arteries. The addition of a permanent proximal ligature on the popliteal artery (Group d) caused the development of large neointimal lesions in 3/ 3 femoral arteries (Figure 3.15).

The results of this experiment prompted the inclusion of a ligation-induced neointimal proliferation model in subsequent investigations (chapter 2.3.2), where tying off the popliteal artery at its branch can be used to induce neointimal lesion development in the femoral artery. These studies also indicated that neointimal lesion growth after wire-induced injury is a complex process that involves responses not only to the wire, but also to the temporary and permanent ligatures used during the operation.

3.2.3 *In vitro* model of neointimal proliferation

An attempt was made to induce the growth of neointimal lesions in tissue culture, to allow the influence of local glucocorticoid and 11β-HSD activity on lesion development to be investigated specifically within the vessel wall (chapter 2.3.3). At the end of the culture period arteries were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Transverse serial sections were cut at 4 μm, every 100 μm along the entire length of the arterial segment.

Left femoral arteries cultured under sterile conditions for 21 days following intra-luminal wire injury showed no evidence of neointimal lesion development (Figure 3.16). Indeed, the histological appearance of these arteries was similar to those assessed immediately after injury (0d; Figure 3.2). The arterial wall appeared stretched, the artery had collapsed and there appeared to be few endothelial cells lining the luminal surface.

It is possible that the extent of injury caused to the vessel wall by wire injury *in vivo* was too severe to allow neointimal proliferation to occur *in vitro*. Also, since the method which this experiment was based on used human mammary arteries (Guerin *et al.* 2004), species differences may account for the fact that it was not successful. Further factors, such as the type of culture medium used, may have also contributed. It is clear that more research and methodological development would be needed to allow this model to be used in the future.

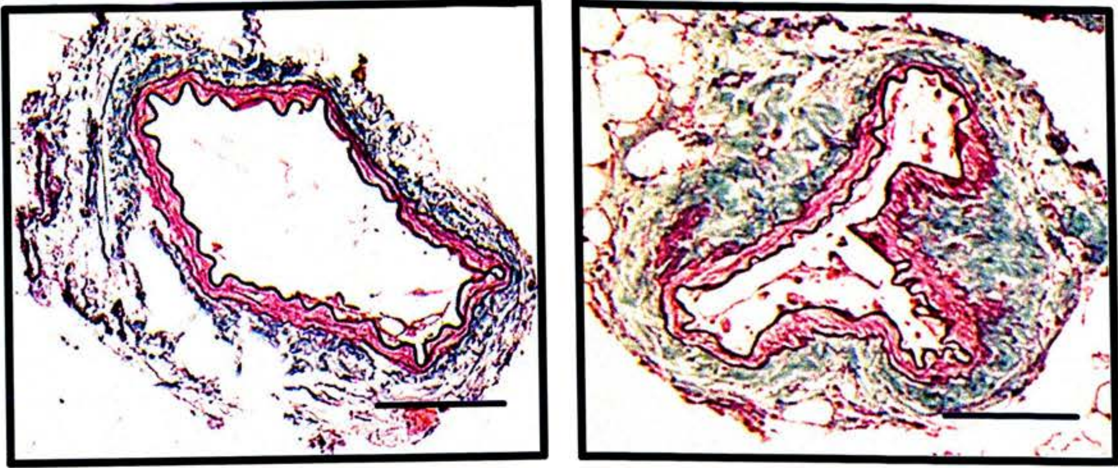


Figure 3.16: *In Vitro* model of neointimal proliferation

Representative sections of two different arteries cultured for 21 days *in vitro* after wire induced intra-luminal injury of the femoral artery. The walls of the vessels are stretched and appear to be denuded of endothelial cells. No neointimal proliferation was seen. Scale bar = 100 µm.

3.2.4 Vascular function studies

These investigations were designed to assess the impact of vascular injury and neointimal proliferation on arterial contraction and relaxation. Since there was little information in the literature at the time, an attempt was made to study the effect of intra-luminal injury on endothelial function in the mouse femoral artery. The aim was to study vascular function both immediately after injury, when it was hypothesised that endothelium-dependent responses would be abolished, and also in arteries at later time-points, in order to determine if a functional endothelium is regenerated over advanced lesions. In order to do this, functional responses were determined by mounting arterial rings (~2 mm in length) in small vessel wire myographs for measurement of isometric force (chapter 2.9). Firstly, the functional characteristics of the normal mouse femoral artery were established. Experiments were designed (n= 3-7 per group) to determine:

(a) whether functional responses were similar in left and right femoral arteries, to allow further study with no need to discriminate between the two sides,

(b) the effect of endothelial cell removal on function,

(c) the effect of storing arteries overnight on function, to assess if it is necessary to use only freshly dissected vessels in functional studies.

To denude arterial rings, the luminal surface was rubbed with a human hair after mounting in the myograph. For storage, arteries were placed in PSS at 4°C overnight. The effect of vascular injury on arterial function was assessed using arteries isolated immediately, 2 and 14 days after wire-induced injury of the femoral artery (n= 2 for each time-point).

3.2.4.1 Functional characterisation of the normal mouse femoral artery

Contraction to the α_1-adrenoceptor agonist phenylephrine in the mouse femoral artery began at a concentration of approximately $1x10^{-7}M$, showed a clear concentration-response curve and reached a maximum at around $1x10^{-4}M$ (Figure 3.17a).

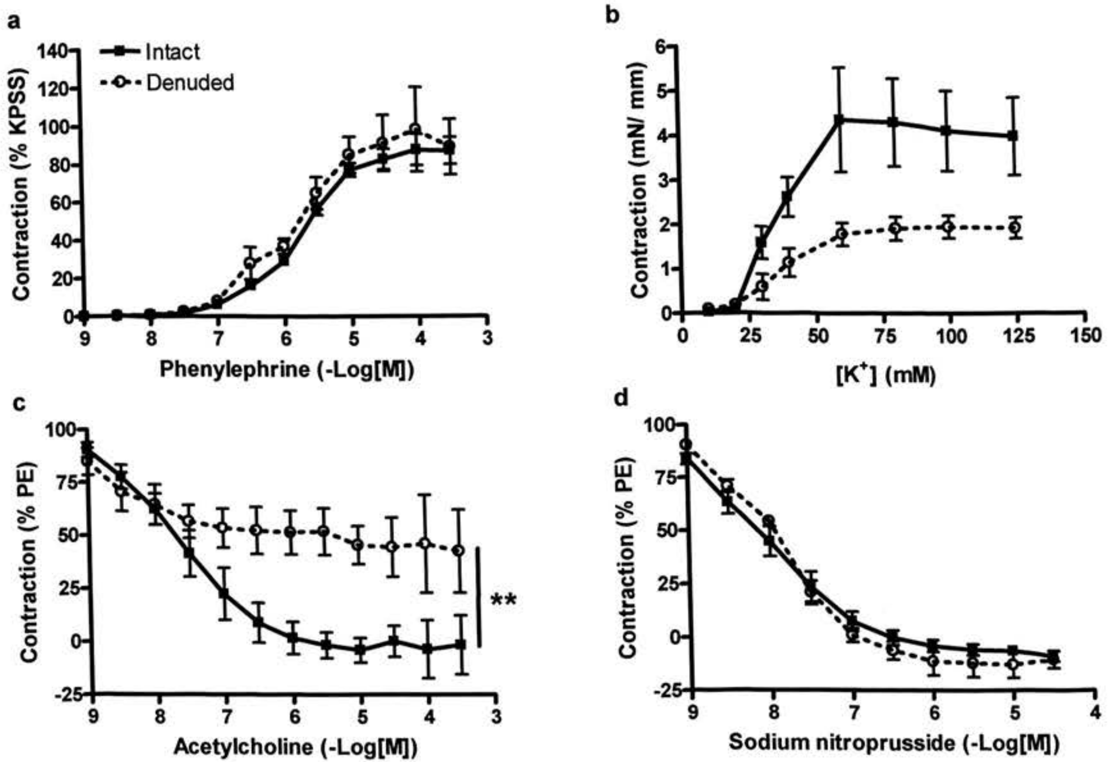


Figure 3.17: The impact of endothelial cell removal on functional responses of mouse femoral artery

Contractile responses to phenylephrine (a) and high potassium physiological salt solution (b), and relaxation to acetylcholine (c) and sodium nitroprusside (d) were measured using a small vessel myograph. Concentration-response curves to phenylephrine and sodium nitroprusside were unaffected by endothelial denudation. In contrast, the E_{max} to acetylcholine was significantly decreased in denuded arteries, and there was a trend towards a decrease in E_{max} to high potassium ($p = 0.068$). Data are mean ± S.E.M, n= 6 for intact, n= 3 for denuded. Analysed by unpaired t test: ** $p < 0.01$.

Contraction to phenylephrine was identical in the left and right femoral arteries (Table 3.2) and in intact and denuded arteries (Figure 3.17a and Table 3.2). Although the E_{max} for phenylephrine in fresh and stored arteries was not significantly different, the pD_2 in stored arteries was decreased when compared to fresh arteries (Table 3.2). The maximum contraction in response to high potassium appeared diminished in denuded arteries compared with intact arteries, although this did not quite reach significance (Figure 3.17b and Table 3.2, $p= 0.068$). The E_{max} in response to high potassium also appeared decreased in stored arteries compared with fresh arteries, although again this did not reach significance (Table 3.2, $p= 0.073$).

After pre-contraction with $1x10^{-5}$M phenylephrine (not significantly different between groups, data not shown), relaxation in response to acetylcholine in the femoral artery began at concentrations as low as $1x10^{-9}$M (Figure 3.17c), and then showed concentration-dependent increases in magnitude, until the relaxant response reached an E_{max}. Similarly to acetylcholine, relaxation in response to the endothelium-independent vasorelaxant drug sodium nitroprusside began at $1x10^{-9}$M and then showed a concentration-response relationship (Figure 3.17d). There was no significant difference in relaxation to either acetylcholine or sodium nitroprusside between the left and right femoral arteries (Table 3.2). Acetylcholine-mediated relaxation was abolished by removal of the endothelium (Figure 3.17c and Table 3.2) whereas responses to sodium nitroprusside were unaffected (Figure 3.17d and Table 3.2). Relaxation in response to acetylcholine in stored arteries was severely blunted, and the maximum relaxation reached was significantly less in stored arteries than in fresh samples (Table 3.2). In contrast, the response to sodium nitroprusside in stored arteries was not significantly affected (Table 3.2).

These experiments provided the basis for a protocol to assess contraction and endothelium-dependent and –independent relaxations in wire-injured femoral arteries. They also confirmed that there are no functional differences between the left and right mouse femoral artery, and indicated that overnight storage can have detrimental effects on endothelial function, and so should be avoided in further experiments.

	PE pD2 (-Log[M])	PE Emax (% KPSS)	KPSS pD2 (mM)	KPSS Emax (mN/mm)	ACh -logIC50 (-Log[M])	ACh Emax (% PE)	SNP -logIC50 (-Log[M])	SNP Emax (% PE)
LFA	5.72 ± 0.12	91.33 ± 6.06	33.50 ± 1.37	4.12 ± 1.01	7.32 ± 0.18	96.77 ± 7.79	7.85 ± 0.19	108.29 ± 2.09
RFA	5.64 ± 0.09	79.30 ± 9.75	34.38 ± 2	3.15 ± 0.52	7.51 ± 0.29	108.19 ± 5.59	7.81 ± 0.24	106.50 ± 3.69
Intact	5.78 ± 0.05	84.20 ± 5.87	34.57 ± 1.63	4.48 ± 1.14	7.38 ± 0.22	105.5 ± 5.56	7.99 ± 0.16	108.91 ± 2.5
Denuded	5.92 ± 0.07	91.21 ± 14.71	37.9 ± 3.84	1.96 ± 0.26	8.21 ± 0.35	59.81 ± 19.85**	7.85 ± 0.06	112.94 ± 6.07
Fresh	5.78 ± 0.05	84.2 ± 5.87	34.57 ± 1.63	4.48 ± 1.14	7.38 ± 0.22	105.5 ± 5.56	7.99 ± 0.16	108.91 ± 2.5
Stored	5.37 ± 0.09**	80.86 ± 13.35	35.04 ± 1.92	2.18 ± 0.45	7.09 ± 0.13	70.21 ± 8.27**	7.66 ± 0.24	104.2 ± 1.82

Table 3.2: Functional properties of agonists in the mouse femoral artery

Small vessel wire myography was used to determine the pD_2 (contractile agonists), $-logIC_{50}$ (relaxant agonists) and maximal response (E_{max}) to phenylephrine (PE), high potassium physiological salt solution (KPSS), acetylcholine (ACh) and sodium nitroprusside (SNP). These parameters were compared between left (LFA) and right (RFA) femoral arteries, between intact and denuded arteries and between fresh and stored arteries. Significant differences were found in the E_{max} to ACh between intact and denuded arteries and between fresh and stored arteries. There was also a significant difference in the pD_2 of PE between fresh and stored arteries. Data are mean ± S.E.M, n= 3- 7. Analysed by unpaired t test: ** $p < 0.01$.

3.2.4.2 Vascular function after intra-luminal wire injury

In a preliminary attempt to study vascular function immediately (0d), 2 and 14 days after injury, arteries failed to respond to any contractile agonists at all, and did not even contract during the standard start protocol in response to a mixture of noradrenaline and high potassium salt solution (data not shown). The process of mounting the injured arteries on wires in the myograph was technically difficult, as the artery walls were stretched and did not maintain their shape after injury.

These results indicate that the wire has damaged the vessel wall so that it can no longer contract in response to any stimulus, and that artery function has not recovered by 14 days after injury. It is likely that this is due to the loss of medial smooth muscle cells documented after wire-induced injury.

3.2.5 Effect of vascular injury and cytokine stimulation on 11β-HSD1 activity

It has been suggested that inflammatory stimuli may alter vascular 11β–HSD activity in an isozyme selective manner, thus up-regulating the generation of active glucocorticoids and conferring a mechanism for local feedback inhibition of inflammation (Cai *et al.* 2001). However, studies in normal intact vessels have failed to confirm this finding (Dover *et al.* 2007). Since local inflammation is a central component of neointimal proliferation (Miller *et al.* 2001), vascular injury may alter 11β-HSD1 activity in the vessel wall. Furthermore, the change in VSMC phenotype after injury, from quiescent to proliferating, may influence the sensitivity of 11β-HSD1 activity to up-regulation by cytokines. These hypotheses were addressed using injured and control femoral arteries collected 7 days following surgery. This time-point was chosen as immunohistochemistry demonstrated that cellular proliferation is occurring in the neointima; this has also been reported in previous studies (Sata *et al.* 2000; Reis *et al.* 2000). Aortic segments were also collected for use as additional controls.

11β-HSD1 activity was assessed in all arteries following incubation with IL-1β (10ng/mL) or vehicle (0.1% BSA in PBS) for 16 hours (n= 6). 11β-reductase activity was measured by an adaptation of the method of Souness *et al* (2002) (chapter 2.10.2). Samples of medium were collected 0, 8, 24, 32 and 48 hours after addition of

[^{3}H] 11-dehydrocorticosterone, to allow the time-course of [^{3}H] corticosterone generation to be studied (n= 6 for 24 hours, n= 3 for all other time-points). The section of femoral artery used in these experiments extended from the popliteal artery, where the wire is inserted, up to the bifurcation of the iliac artery and therefore contained the entire length of any lesion present.

Initial experiments (n= 3) measured the time-course of 11β-HSD1 activity in aorta, injured and uninjured femoral arteries. Conversion of [^{3}H] 11-dehydrocorticosterone to [^{3}H] corticosterone progressed linearly over 48 hours in the aorta (Figure 3.18a) and femoral arteries (Figures 3.18b and c). These experiments confirmed that performing enzyme activity assays after 24 hours of substrate incubation in further studies would give a measurement within the linear range of product formation with time, and not after activity had reached a plateau.

After 24 hours of [^{3}H] 11-dehydrocorticosterone incubation, 11β-reductase activity was not changed in wire-injured femoral arteries when compared with uninjured controls (Figure 3.19, p=0.33). Therefore, the hypothesis that vascular injury would act to up-regulate 11β-HSD1 activity in the vessel wall was not confirmed. To assess whether the sensitivity of vascular 11β-HSD1 activity to up-regulation by cytokines is altered under conditions of VSMC proliferation, enzyme activity was measured in injured arteries incubated with IL-1β. Exposure to IL-1β did not change 11β-HSD1 activity in injured arteries (p= 0.20) or uninjured arteries (p= 0.43) compared with respective vehicle-treated controls (Figure 3.19). However, IL-1β significantly down-regulated 11β-reductase activity in aortic segments taken from the same animals (Figure 3.19, p=0.03).

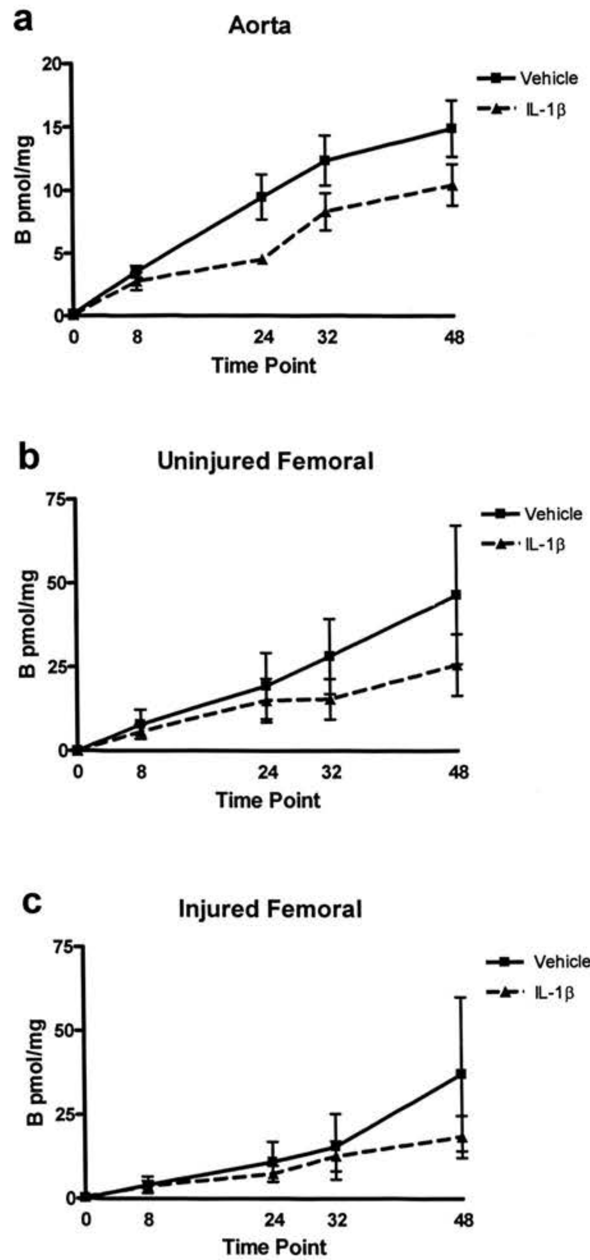


Figure 3.18: Time-course of 11β-HSD1 activity in isolated vessels

Segments of (a) aorta, and (b) uninjured or (c) injured femoral arteries were incubated for 16 hours with 10ng/ml interleukin-1β or vehicle, and then with [^{3}H] 11-dehydrocorticosterone. 11β-reductase activity over time is expressed as the amount of [^{3}H] corticosterone formed per mg of tissue during each time interval. Generation of [^{3}H] corticosterone progressed linearly with time over 48 hours in aorta and femoral arteries. Cytokine incubation caused a trend towards a decrease in 11β-reductase activity that did not reach significance, as analysed by unpaired t test. Results are mean ± S.E.M, n= 3 per group.

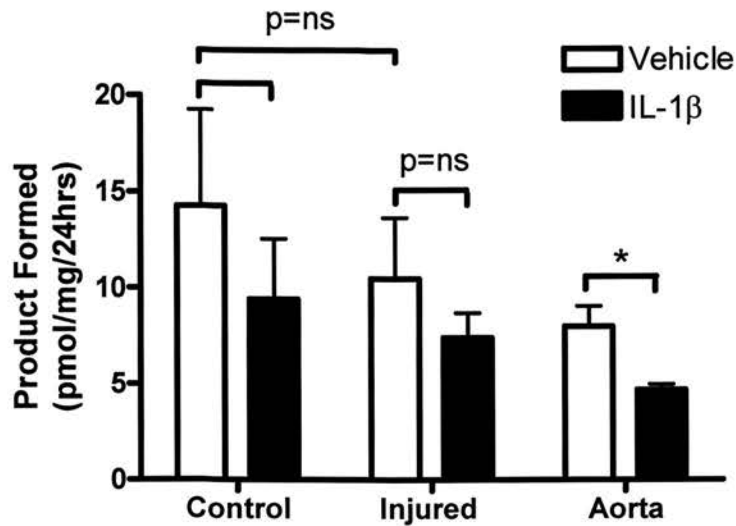


Figure 3.19 Effect of injury and inflammation on vascular 11β-HSD1 activity

Control (uninjured) and injured femoral arteries, and aortic segments, were incubated for 16 hours with 10ng/ml interleukin-1β or vehicle, and then for a further 24 hours with [^{3}H] 11-dehydrocorticosterone. 11β-Reductase activity is expressed as the amount of [^{3}H] corticosterone formed per mg of tissue over 24 hours. Wire injury of the femoral artery did not cause any change in 11β-reductase activity. Cytokine incubation did not significantly alter 11β-reductase activity in control and injured femoral arteries; however it induced a decrease in activity in aortic segments. Results are mean ± S.E.M, n= 6 per group. Differences between groups measured by unpaired t test, ns: non significant, * $p<0.05$.

3.3 Discussion

This chapter describes the introduction and development of a model of wire-induced, intra-luminal vascular injury in the mouse femoral artery (Sata *et al.* 2000). This technique induces stretching and denudation of the artery wall, followed by the time-dependent formation of neointimal lesions. Neointimal lesion formation is associated with early macrophage infiltration and cellular proliferation. Advanced lesions are elastin and smooth muscle cell-rich, and show evidence of a re-endothelialised luminal surface. Wire-induced injury causes loss of medial smooth muscle cells and abolishes vascular contractile function, but has no effect on 11β-HSD1 activity. These studies also demonstrated that permanent ligation at the branch of the popliteal artery induces neointimal proliferation in the femoral artery.

Choosing a model of vascular injury in the mouse

Although experiments using transgenic mice represent an effective way to clarify the mechanisms of neointimal lesion formation during post-angioplasty restenosis, currently there are no balloons available which can be inserted into small muscular mouse arteries (Sata *et al.* 2000). The main advantage of a wire-induced intra-luminal model of vascular injury over perivascular approaches available in the mouse (Carmeliet *et al.* 1997c; Kumar & Lindner 1997; Moroi *et al.* 1998) is that the wire stretches the vessel wall and removes the endothelium; two key mechanisms of the vascular injury induced during balloon angioplasty and stenting. Therefore, it is more relevant to use this model in the study of pathways and factors that may contribute to the pathogenesis of neointimal lesion formation.

The first model of wire injury in the mouse was developed by Lindner *et al* in 1993. In this method a wire is inserted via the external carotid artery into the common carotid artery to denude the endothelium. Smooth muscle cells are first seen in the intima 8 days after injury, and by two weeks neointimal lesions contain a similar number of smooth muscle cells as the media. There are two main advantages of wire injury in the femoral artery over the carotid. The first is that neointimal lesions produced in the carotid artery are not very extensive, and usually only grow to two or three cell layers in thickness (Lindner *et al.* 1993; Kumar & Lindner 1997). In contrast, those seen after four weeks in wire-injured femoral arteries are much larger, with intima/ media ratios comparable to those seen in larger animal models (Sata *et*

al. 2000; Roque *et al.* 2000). The second is a technical consideration: since the surgery required is relatively challenging with a high chance of vascular complications, there is more possibility of accidental death by stroke when using the carotid arteries. The femoral artery also offers the advantage of bilateral injury, and the time-course of neointimal proliferation is similar to that found in the rabbit (Doornekamp *et al.* 1996), pig (Schwartz *et al.* 1990) and baboon (Geary *et al.* 1994).

Shortly before the method of Sata *et al* (2000) was published, a similar model was reported by another group (Roque *et al.* 2000). This method involves inserting a slightly smaller wire into the femoral artery to cause endothelial denudation, rapid neutrophil accumulation and neointimal proliferation. However, this model is somewhat different from that of Sata *et al* (2000) since the wire is inserted through an arteriotomy in the main femoral artery, distal to the epigastric branch. After wire insertion and removal the main femoral artery must be ligated proximally to the arteriotomy site; therefore, blood flow to the injured artery is only partially restored. This raises the possibility that the observed neointimal proliferation may be a result of restriction of blood flow, as seen in the carotid artery ligation model (Kumar & Lindner 1997), and not of the denuding injury caused by the wire. In contrast, by inserting the wire through a small side-branch which can be tied off, blood flow to the femoral artery is completely restored using the model of Sata *et al* (2000).

The experimental model of wire- induced vascular injury

This surgical model can be carried out routinely after practice, is generally well tolerated by the animals and induces reproducible neointimal proliferation consistent with published reports. The persistence of femoral artery expansion observed over the duration of the current experiments has been reported previously by Sata *et al* (2000). The time-course of neointimal proliferation also followed a similar pattern to that seen in published models (Sata *et al.* 2000; Roque *et al.* 2000). Although neointimal lesion formation was not assessed beyond 28 days in the current investigations, previous studies have shown that lesion growth does not continue after this time in mice (Sata *et al.* 2000; Zou *et al.* 2007).

The observation that advanced neointimal lesions contained predominantly smooth muscle cells, and the indication that endothelial cell regeneration occurred over their luminal surface, is consistent with descriptions of lesion composition made by other groups (Sata *et al.* 2000; Roque *et al.* 2000). The detection of macrophages in the adventitia of injured vessels, and in small discrete areas of advanced neointimal lesions, has also been described previously (Sata *et al.* 2000). The time-course of cellular proliferation, as determined by PCNA staining, followed a similar pattern to prior studies (Reis *et al.* 2000). The adherence of platelets at early time-points to sections of the artery wall where the IEL was un-damaged has been observed by another group using a similar model (Roque *et al.* 2000). The early medial thinning and dramatic loss of VSMCs from the media of the vessel wall seen in the current studies were consistent with observations made by other groups (Sata *et al.* 2000; Reis *et al.* 2000), who have described a rapid medial cell apoptosis caused by vascular injury that occurs independently of the Fas death signalling pathway (Sata *et al.* 2001).

The observation that neointimal lesions 7d after injury contained macrophages and proliferating nuclei, with no expression of smooth muscle cell α-actin, suggests that early lesion formation occurs via the migration and proliferation of circulating cells (rather than resident VSMCs) in the arterial wall. In contrast, lesions at 14d contain smooth muscle cell α-actin positive cells in addition to macrophages and proliferating nuclei. This implies that cells derived from the circulation are undergoing proliferation and differentiation into VSMCs. Rapid medial atrophy observed after injury indicates that, for neointimal VSMCs to have originated in the arterial wall, they must derive from the adventitia or from uninjured areas of the vessel. The latter seems unlikely, however, given the extent of injury along the arterial wall. The suggestion that neointimal cells originate from the circulation (possibly from vascular progenitor cells) is consistent with the previous observation that a significant number of neointimal cells are derived from the bone marrow after wire-induced injury (Tanaka *et al.* 2003). However, it is contrary to the classically-described pathogenesis of neointimal lesion formation (Lee *et al.* 1993), whereby activation of medial VSMCs results in their migration to, and proliferation in, the intima. This mechanism of neointimal lesion formation may help to explain

why wire-injured arteries do not contract at 14d after injury, despite the presence of a smooth muscle α-actin-rich lesion; these cells are likely to be of a synthetic, rather than contractile, phenotype and may also not be ideally oriented for normal contraction of the artery (Kockx *et al.* 1993).

One limitation of the intra-luminal model of vascular injury described in this chapter is the damage induced to the IEL of the artery wall, which usually occurred in areas close to the site of wire insertion. It is likely that this was caused when difficulties were encountered introducing the wire into the popliteal artery, and more force was necessary to advance the wire along the femoral artery. The damage, which took the form of breaks in the IEL, partial stripping of the IEL away from the underlying media, or even complete removal of the IEL, left the medial layer of the vessel wall exposed. Exposure of this thrombogenic surface stimulated the formation of several types of thrombi. The formation of occlusive thrombus in 18% of all vessels studied during the model development was a higher frequency than that reported by Sata *et al* (2000) (about 6%), but falls within the range reported by Roque *et al* (2000) (5-21% depending on time-point). Non-occlusive solid thrombus on top of the exposed media and a fibrin-like mesh containing cells were also observed in vessels with IEL damage; these types of thrombi are not described in previous reports. Thrombus which formed as a result of IEL damage at the site of wire insertion often extended along the artery into areas where the wall remained intact. In addition, solid and mesh-like thrombi were sometimes seen to be undergoing infiltration by cells and gradually organising into more fibrous neointimal lesions. Therefore, in arteries where extensive IEL damage and thrombosis had occurred classification and measurement of neointimal lesions could be complicated, especially at earlier time-points (e.g. 7 and 14d) when large advanced neointimal lesions had yet to form.

Additional model of ligation-induced neointimal proliferation

The observation that large neointimal lesions formed in sham-operated arteries around the branch point of the popliteal artery was unexpected. This prompted contact with the two groups who had previously published wire-induced injury models in the mouse femoral artery. The group who developed this model (Sata *et al.* 2000) revealed that they had seen a similar neointimal response around the branch point in sham-operated mice but had chosen not to report it. The group who use a

similar model (Roque *et al.* 2000) reported that they do not use the area of femoral artery around the site of wire insertion for histology and start sectioning more proximally; therefore, they would not observe any response that may occur in sham-operated mice. By setting up a series of shams to assess the effect of each stage of the operation on neointimal proliferation, it was found that the permanent ligatures used during the procedure, in particular the tie placed proximally on the popliteal artery, induce the formation of neointimal lesions in the femoral artery.

These observations have proved very useful, as they have allowed the introduction of an alternative model of neointimal proliferation. By simply tying off the popliteal artery, neointimal lesions can be induced in the main femoral artery, presumably via a similar mechanism to the model of carotid artery ligation developed by Kumar & Lindner (Kumar & Lindner 1997). Previous studies have shown that, unlike wire-induced injury, few bone marrow-derived cells are involved in neointimal formation after ligation injury (Tanaka *et al.* 2003). In order to determine whether early lesions induced in the current model of femoral artery ligation are composed of circulating cells, arteries would have to be studied at earlier time-points after injury. This model can be used in the contra-lateral leg of mice undergoing wire-induced injury, to provide another way to measure the effect of any interventions being studied.

Conclusions

These studies describe the introduction of a model of vascular injury in the mouse, which produces structural changes in the artery wall that replicate the method on which it is based (Sata *et al.* 2000). The reproducible growth of neointimal lesions induced by this model will allow the effects of exogenous and endogenous glucocorticoids on neointimal proliferation to be investigated. The detailed analysis of the time-course and cellular composition of neointimal lesion formation performed will allow future experiments to be properly designed and interpreted.

Chapter 4

Effects of exogenous glucocorticoids on the vascular response to injury in the mouse

4.1 Introduction

The influence of glucocorticoids on the vascular response to injury *in vivo* is hard to predict, due to their adverse effects on systemic cardiovascular risk factors, and their beneficial actions locally on the vessel wall. Glucocorticoids can exert profound anti-inflammatory effects and directly inhibit smooth muscle cell proliferation (see chapter 1). This gives them the potential to inhibit neointimal proliferation of smooth muscle cells, which is stimulated primarily by the inflammatory response to vascular injury. Indeed, administration of these hormones inhibits the response to vascular injury in a range of different animal models. Glucocorticoids reduced early inflammation in a rabbit model of balloon injury (Poon *et al.* 2001), and decreased neointimal proliferation in the rat (Villa *et al.* 1994; Guzman *et al.* 1996; Nagasaki *et al.* 2004) and the rabbit (Van Put *et al.* 1995; Petrik *et al.* 1998; Valero *et al.* 1998). In a rabbit model of established atherosclerosis, oral prednisone after stenting reduced neointimal formation (Ribichini *et al.* 2007), and dexamethasone release from coated metal stents inhibited neointimal formation in the dog femoral artery (Strecker *et al.* 1998). In the mouse, one study has shown that dexamethasone decreased neointimal proliferation caused by cuff placement around the femoral artery (Pires *et al.* 2005). In addition, dexamethasone treatment can inhibit vein graft thickening in hypercholesterolemic ApoE3Leiden transgenic mice (Schepers *et al.* 2006). However, the effect of glucocorticoids on the arterial response to injury has not been studied in detail in the mouse.

Human clinical trials investigating the action of systemic glucocorticoid administration have been performed after balloon angioplasty (Stone *et al.* 1989; Pepine *et al.* 1990) and stent implantation (Rab *et al.* 1991; Lee *et al.* 1999; Versaci *et al.* 2002). The majority of these studies have yielded disappointing results, showing no beneficial effect of systemic pulse pre-treatment (Pepine *et al.* 1990; Lee *et al.* 1999), or short-term systemic administration of glucocorticoids (Stone *et al.* 1989) on restenosis rate. Indeed, one study indicated that treatment with glucocorticoids after stenting had a detrimental effect on vessel wall healing, leading to an increased incidence of coronary aneurysms (Rab *et al.* 1991). In contrast, a more recent trial reported that systemic administration of prednisone for 45 days caused a significant decrease in clinical events and restenosis rate after coronary

artery stent implantation (Versaci *et al.* 2002). This indicated that a more prolonged duration of glucocorticoid administration may be beneficial.

Local drug delivery using drug-eluting stents provides the opportunity to administer therapeutic agents over a prolonged period, while limiting potential systemic side effects. This is important due to the well-recognised, unfavourable effects of long term glucocorticoid therapy such as hypertension, impaired glucose tolerance, immunosuppression and impaired healing after myocardial infarction. A role for local administration is supported by the demonstration that application of glucocorticoids at the vessel wall inhibits neointimal proliferation in the rat (Villa *et al.* 1994; Guzman *et al.* 1996) and the mouse (Pires *et al.* 2005). Release of methylprednisolone from stents reduced neointimal formation and macrophage infiltration in a porcine model of coronary stenting (Wang *et al.* 2005). Pre-clinical studies using dexamethasone-eluting stents have shown conflicting results, with inhibition of neointimal formation seen in the dog (Strecker *et al.* 1998) but no reduction seen in the pig (Lincoff *et al.* 1997).

The outcomes of human clinical trials using dexamethasone-coated stents have also been contradictory. An early feasibility report, the Study of Antirestenosis With the BiodivYsio Dexamethasone-Eluting Stent (STRIDE) trial, indicated that dexamethasone release from coated stents may have an inhibitory effect on neointimal formation (Liu *et al.* 2003). However, in a separate pilot study, stents loaded with a high dose of dexamethasone did not significantly reduce neointimal proliferation (Hoffmann *et al.* 2004) when compared with previous results reported for bare metal stents (Kastrati *et al.* 2001). Importantly, when the effect of high-dose dexamethasone-eluting stents was evaluated in a randomised and properly controlled study, an improvement both in clinical outcomes and in restenosis rate was observed (Han *et al.* 2006). Most recently, dexamethasone-eluting stents have been shown to reduce neointimal proliferation when compared to bare metal stents, in patients with acute coronary syndrome (Konig *et al.* 2007). Dexamethasone-coated stents have also been shown to attenuate increases in adhesion molecules (Patti *et al.* 2005a) and CRP (Patti *et al.* 2005b) after stenting.

Therefore, although glucocorticoids have effects on the cells of the vascular wall that should inhibit neointimal proliferation, to date the results of studies assessing their impact on this process have been inconsistent. It is likely that species differences, as well as the protocol of steroid delivery, have contributed to the variation seen. In addition, the type of vascular injury induced has differed between studies, with models of intra-luminal (Petrik *et al.* 1998; Nagasaki *et al.* 2004; Ribichini *et al.* 2007) and perivascular injury (Van Put *et al.* 1995; Pires *et al.* 2005) used interchangeably in animal studies. However, it is also very important to consider the balance between systemic and local actions of glucocorticoids when interpreting the influence of these hormones on the vascular response to injury.

We hypothesised that glucocorticoid administration would inhibit neointimal proliferation in the mouse model of intra-luminal femoral artery injury, and that local application would avoid the adverse effects associated with systemic steroid treatment. With this in mind, the specific aims of this chapter were:

(i) To determine whether systemic glucocorticoid administration inhibits neointimal lesion formation in the mouse model of intra-luminal vascular injury.

(ii) To assess whether neointimal proliferation can be inhibited without confounding systemic effects on lesion development, by using local glucocorticoid administration.

4.2 Methods

4.2.1 Animals

Male C57B16 mice (Harlan Olac, U.K.) weighing 25-35 grams were used in all studies. To allow collection of trunk blood, animals were killed by decapitation, according to Home Office guidelines.

4.2.2 Wire-induced injury of the femoral artery

Intra-luminal vascular injury was induced in the femoral artery of mice using a wire to induce stretching and denudation of the artery wall (see chapter 2.3.1), followed by the development of neointimal lesions (see chapter 3). Wire-induced vascular injury was carried out both in the left and in the right femoral arteries of all animals.

4.2.3 Administration of drugs

4.2.3.1 Systemically-administered glucocorticoid

To increase systemic glucocorticoids and investigate the effect on neointimal proliferation, mice (n= 8 per group) received sub-cutaneous injections of 1mg/kg/day dexamethasone or vehicle (4% ethanol/ 0.9% saline; chapter 2.4.1). Dosing was started at least two hours before bilateral wire injury and continued daily (in the morning) until the end of the experiment. Body weight was recorded each day.

4.2.3.2 Locally-released glucocorticoid

To assess the action of locally-released glucocorticoid on neointimal proliferation, silastic pellets (chapter 2.4.2) were placed next to the femoral artery immediately following wire-induced injury. A pocket was created at the operation site; pellets were inserted into this pocket and kept in place when the skin incision was closed with sutures at the end of the operation. During bilateral, wire-induced injury a pellet containing cortisol was implanted in one leg, and a contra-lateral vehicle pellet (silastic elastomer only) was implanted in the other leg (n= 9). Cortisol or vehicle pellets were implanted randomly in either the right or the left leg. Each 10 mg pellet contained 2 mg cortisol (20% w/w). In an additional control group, animals underwent bilateral femoral artery injury, and vehicle pellets were placed in both legs (n= 8). To assess whether pellet implantation alone would cause neointimal proliferation, bilateral pellet implantation without any manipulation of the femoral artery was carried out in a third group of mice (n= 3).

4.2.4 Tissue collection

21 days following surgery all mice were weighed for a final time and then trunk blood was collected into heparinised tubes on wet ice. Blood samples were centrifuged at 3000 rpm for 5 minutes, and the plasma (supernatant) was removed into fresh eppendorf tubes. Organs (adrenals, thymus, spleen, kidneys, heart and liver) were removed, cleaned of connective tissue and weighed. Plasma and organs were then snap frozen on dry ice and stored at -80°C. Due to loss of samples during a freezer failure, the number of adrenal and plasma samples in the group of animals treated with a unilateral cortisol pellet was reduced from n= 9 to n= 6.

Left and right femoral arteries were cleaned of connective tissue and veins, and excised from the bifurcation of the iliac artery to the branch with the popliteal artery. Femoral arteries were immediately placed in 10% neutral buffered formalin, fixed for up to 24 hours and then stored in 70% ethanol if necessary. The arteries were dehydrated through graded alcohols, embedded in paraffin and cut into transverse sections: sets of eight 4 μm serial sections were taken at 100 μm intervals along the entire arterial segment.

4.2.5 Measurement of neointimal proliferation

To assess the extent of neointimal proliferation in these experiments, transverse sections every 100 μm along injured arteries were stained with the United States trichrome histological stain (chapter 2.6.2). The area (μm^2) of any neointima in each section was measured using a light microscope coupled to a colour camera and image analysis system (chapter 2.7). The extent of luminal narrowing was also calculated for each section (% luminal narrowing = neointimal area/ area inside IEL x 100). The section with the largest area of neointima (and therefore maximal luminal narrowing) was chosen to represent each arterial sample. All analysis was performed with the investigator blinded to which group the sample belonged to. Analysis of slides from the systemic dexamethasone experiment was performed by two independent investigators.

4.2.6 Assessing neointimal lesion composition

In arteries from animals treated with systemic dexamethasone, the United States trichrome stain suggested that neointimal lesions had a different structure than those

from vehicle-treated controls. Therefore, the cellular composition of neointimal lesions in these two groups was investigated further. Smooth muscle cell content was assessed using an alkaline-phosphatase conjugated monoclonal primary antibody against smooth muscle cell α-actin (chapter 2.8.1). A polyclonal rabbit anti-human primary antibody was used to determine the fibrinogen content of lesions (chapter 2.8.5).

4.2.7 Measurement of plasma cortisol levels

Cortisol levels in plasma samples from mice implanted with a unilateral cortisol-releasing pellet and contra-lateral vehicle control were analysed using a radioimmunoassay kit (n= 6 due to loss of 3 samples; chapter 2.11.1).

4.3 Results

4.3.1 Effect of systemic glucocorticoids on animals

Daily sub-cutaneous injections of 1mg/kg dexamethasone had obvious effects on mice; at the end of the study the group treated with glucocorticoid appeared considerably smaller and thinner than the group treated with vehicle. This observation was confirmed when body weights were compared (Figure 4.1). As expected, mice treated with vehicle showed an early weight loss after surgery, then gained weight over the 21 days of the study. In contrast, animals treated with dexamethasone failed to gain weight during the experiment and weighed significantly less than the vehicle group from 15 days onwards.

In the group treated with systemic dexamethasone, incisions made on the surface of the leg during surgery had not healed fully after 21 days. The skin around the operation site was scarred and, in some cases, the wound was not completely closed. In contrast, skin had healed fully in animals treated with vehicle. Fur re-growth was also impeded in the dexamethasone treatment group.

Upon dissection, it was apparent that certain organs which are known to change in size in response to glucocorticoids (adrenal glands, thymus and spleen) were much smaller in mice administered systemic glucocorticoid. This was verified when organ weights were compared between dexamethasone and vehicle treated groups (Figure 4.2). The adrenal glands, thymus and spleen all weighed significantly less in animals treated with systemic glucocorticoid, whereas there was no difference in weight of the heart, kidneys or liver (data not shown).

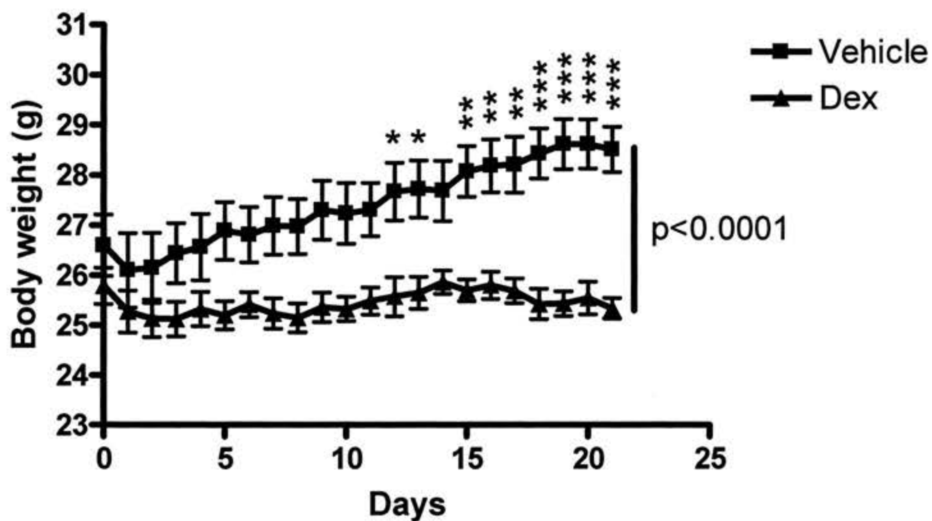


Figure 4.1: Weight change induced by systemic glucocorticoid administration

Mice were randomised to receive sub-cutaneous injections of 1mg/kg/day dexamethasone (Dex) or vehicle, for 21 days after bilateral wire-induced femoral artery injury. Vehicle-treated controls showed early weight loss after surgery, and then gained weight over the course of the experiment. In contrast, mice treated with dexamethasone failed to gain weight. Data are mean ± S.E.M, n= 8 per group. Analysed by two way ANOVA with Bonferroni post hoc test: * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$ vs. dexamethasone.

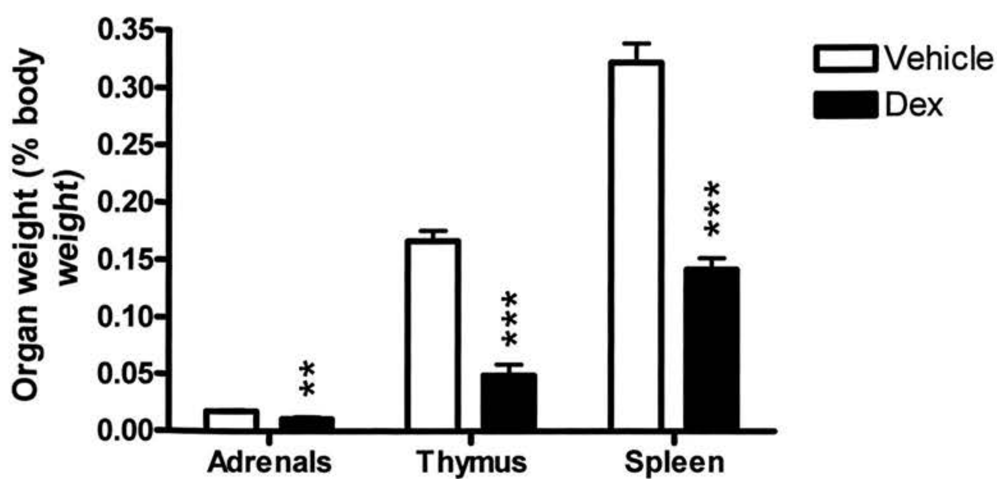


Figure 4.2: Effect of systemic dexamethasone administration on organ weights

The weight of adrenal glands, thymus and spleen were expressed as a percentage of total body weight, in animals treated with 1mg/kg/day dexamethasone (Dex) or vehicle for 21 days after bilateral wire-induced femoral artery injury. There was a significant reduction in the weights of all three organs in animals treated with systemic dexamethasone. Data are mean ± S.E.M, n= 8 per group. Organ weights compared between groups by unpaired t test: ** $p<0.01$, *** $p<0.001$ vs. vehicle.

4.3.2 Influence of systemic dexamethasone on the vascular response to injury

United States trichrome staining indicated that fibrous neointimal lesions developed after 21 days in all animals from the vehicle-treated control group (Figure 4.3a); in some arteries lesions developed over areas where the IEL had been removed during wire insertion. In the dexamethasone-treated group areas of IEL removal were also seen. In one animal a small, cellular neointima formed over thrombus generated in response to IEL removal. No neointimal proliferation was seen in the remaining 7 animals from the dexamethasone group (Figure 4.3b). Therefore, when fibrous neointimal area was quantified there was a highly significant decrease seen in the animals administered systemic glucocorticoid (Figure 4.4).

Although very little neointimal proliferation was observed in animals treated with dexamethasone, organised lesions that occluded the lumen of injured vessels were still observed in this group. United States trichrome staining indicated that these lesions were very different in composition from the fibrous lesions seen in vehicle-treated controls (Figure 4.5a). They appeared smooth, contained very few or no cells, and did not stain positively for elastin (Figure 4.5b). Whilst lesions in vehicle-treated control arteries stained positively for smooth muscle α-actin, as expected (Figure 4.5c), lesions in animals treated with systemic glucocorticoid did not contain cells expressing this marker (Figure 4.5d). Instead, lesions in the dexamethasone group stained strongly and uniformly for fibrinogen (Figure 4.5f); immunoreactivity for fibrinogen was much less intense in controls (Figure 4.5e). Fibrinogen-rich lesions were seen exclusively in the dexamethasone-treated group, and were observed both over areas of IEL removal, and in sections of arteries where the IEL remained intact.

The fibrinogen-rich lesions observed in dexamethasone-treated mice still acted to block the lumen of injured vessels, and therefore no difference was seen in luminal narrowing between the dexamethasone- and vehicle-treated groups (Figure 4.6).

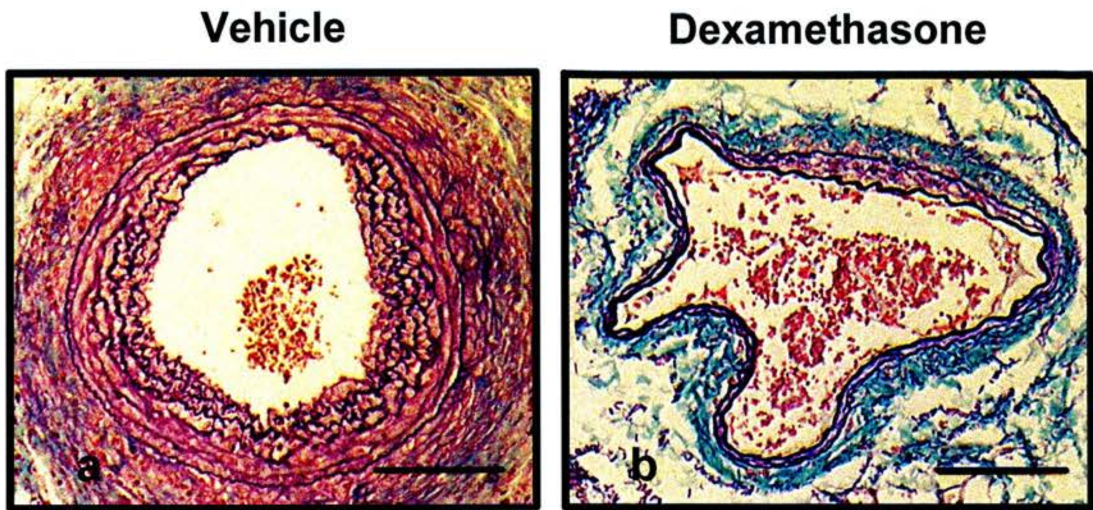


Figure 4.3: Effect of systemic dexamethasone on neointimal proliferation after femoral artery injury

Sections of wire-injured arteries from animals treated with 1mg/kg/day dexamethasone or vehicle for 21 days, stained with the United States trichrome stain. Extensive neointimal lesions developed in the vehicle-treated control group (a), but fibroproliferative lesion formation was inhibited in animals treated with dexamethasone (b). Scale bar = 100 μm.

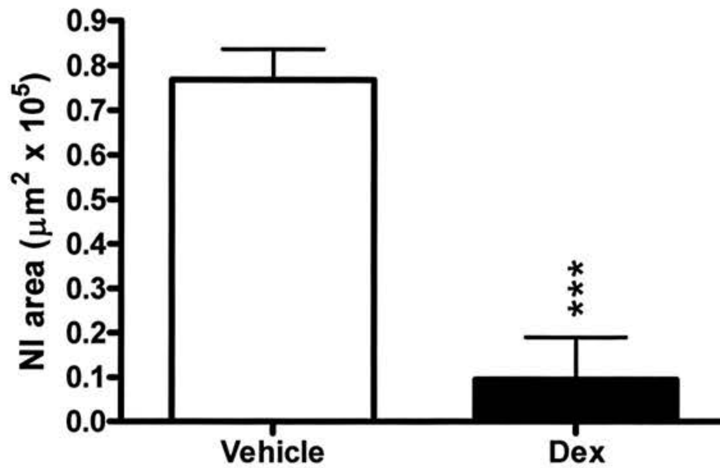


Figure 4.4: Neointimal area in animals treated with dexamethasone or vehicle after femoral artery injury

The area of fibrous neointimal lesions was quantified by image analysis in animals treated with 1mg/kg/day dexamethasone (Dex) or vehicle. Dexamethasone administration caused a significant decrease in neointimal area 21 days after wire-induced femoral artery injury. Data are mean ± S.E.M, n= 8 per group. Analysed by unpaired t test: *** $p<0.001$ vs. vehicle.

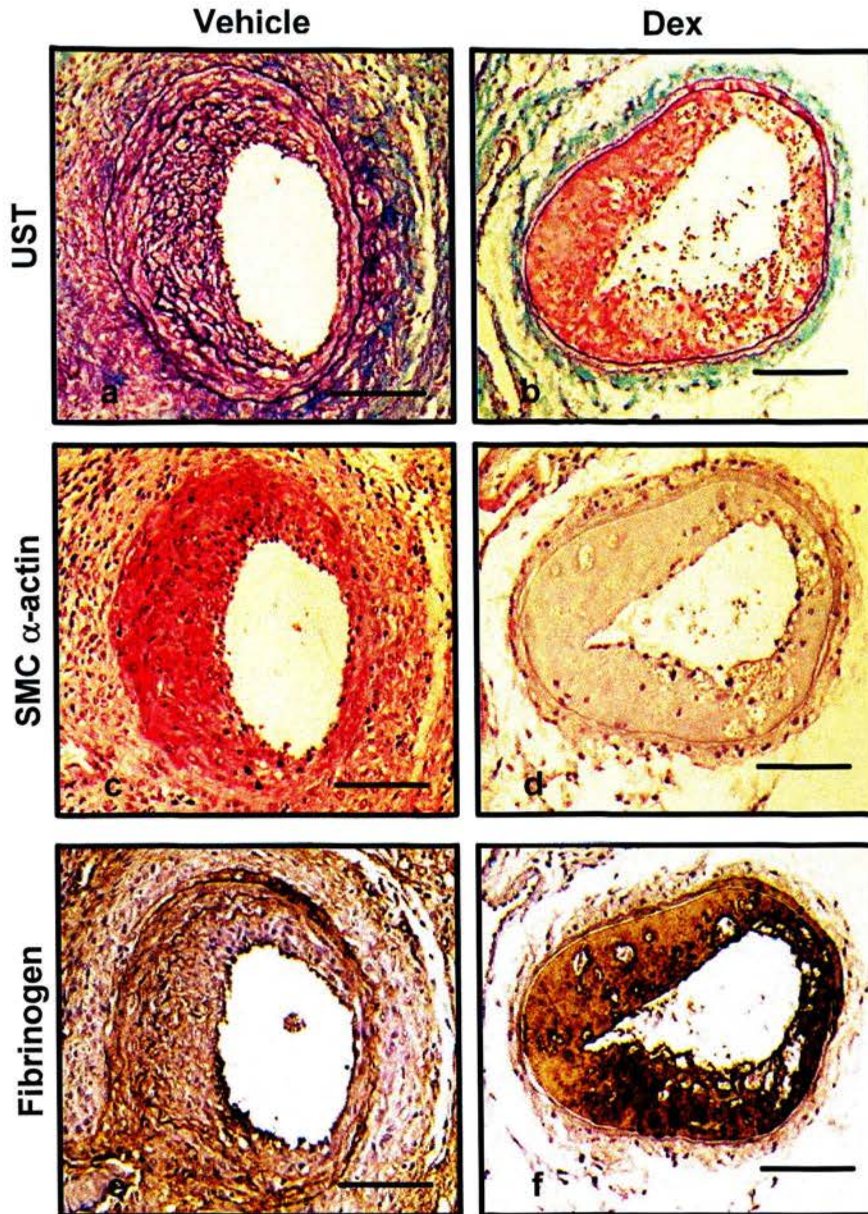


Figure 4.5: Composition of lesions formed after femoral artery injury in animals treated with systemic glucocorticoid

Although administration of 1mg/kg/day dexamethasone (Dex) for 21 days after wire-induced femoral artery injury reduced fibrous neointimal proliferation in mice, lesions were still observed in the lumen of arteries from animals in this group (b,d,f). These lesions differed in composition from vehicle-treated controls (a,c,e). Lesions in dexamethasone-treated animals were acellular and elastin-deficient as assessed by United States trichrome staining (a,b), did not contain smooth muscle cells (c,d) and stained strongly for fibrinogen when compared with controls (e,f). Scale bar = 100 μm.

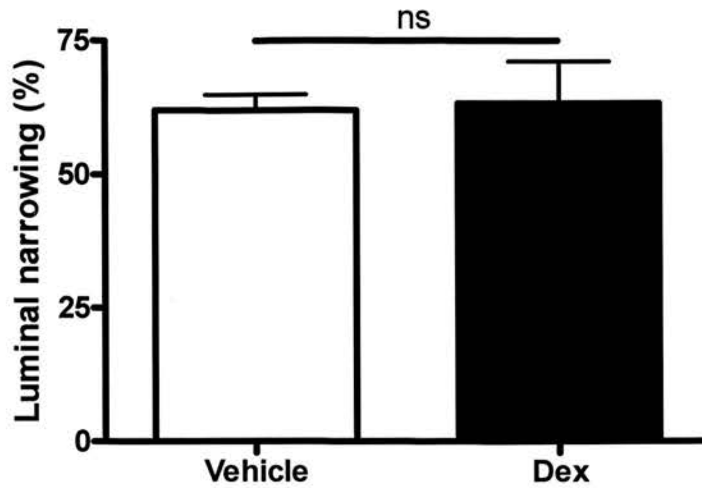


Figure 4.6: Luminal narrowing after femoral artery injury in animals treated with dexamethasone or vehicle

The extent of luminal narrowing after wire-induced vascular injury was determined in animals treated with 1mg/kg/day dexamethasone (Dex) or vehicle. Luminal narrowing was calculated as the area of any lesion present in the vessel lumen, as a percentage of the total area inside the internal elastic lamina. Therefore, this took into account the potential of both fibrous neointima and fibrinogen-rich lesions to occlude the lumen of the vessel. There was no difference in luminal narrowing in mice treated with systemic glucocorticoid when compared with vehicle-treated controls. Data are mean ± S.E.M, n= 8 per group. Analysed by unpaired t test: ns, not significant.

4.3.3 Effect of cortisol pellet implantation on animals

The body weight of mice implanted with cortisol-releasing pellets was compared with those implanted with bilateral vehicle pellets, at the start and the end of the experiment. There were no significant differences in weight between mice implanted with a cortisol pellet, when compared with the control group implanted with bilateral vehicle pellets (Figure 4.7). This confirmed the observation that there were no obvious morphometric differences between treated and untreated mice at the end of the experiment. Similarly, there were no differences seen in fur re-growth or wound healing on the surface of the leg around the operation site.

There were no significant differences in the weights of the adrenals, thymus and spleen (Figure 4.8) or other organs (heart, kidneys, liver; data not shown). However, upon dissection it was observed that connective tissue remodelling was attenuated at the operation site in the leg that had received a cortisol pellet. Furthermore, injured vessels treated with a cortisol pellet were considerably easier to clean of adherent connective tissue and veins when compared with those treated with a vehicle pellet.

When plasma cortisol was assessed in mice implanted with a unilateral cortisol pellet, levels were below the limit of detection in 4/ 6 mice (below the lowest concentration on the standard curve). The mean plasma cortisol concentration was 22.22nmol/l; 95% Cl (4.97, 39.47).

4.3.4 Influence of local glucocorticoid administration on neointimal proliferation

In arteries with an adjacent vehicle pellet, extensive fibrous neointimal lesions developed in 8/ 9 vessels (Figure 4.9a). (In the remaining animal, an exclusively thrombotic response was observed). In two cases, neointimal proliferation occurred over areas of IEL stripping and thrombosis but the remaining six neointimal lesions developed in arteries where the vessel wall remained intact.

In contrast, a very small neointima developed in 5/ 9 arteries treated with a cortisol pellet (Figure 4.9b). In the remaining 4 vessels, no neointimal proliferation was observed, and only a moderate thrombotic response to injury was seen. IEL stripping was seen in 3/ 9 arteries in this group but this was always associated with thrombosis only. All neointimal lesions developed over an intact artery wall.

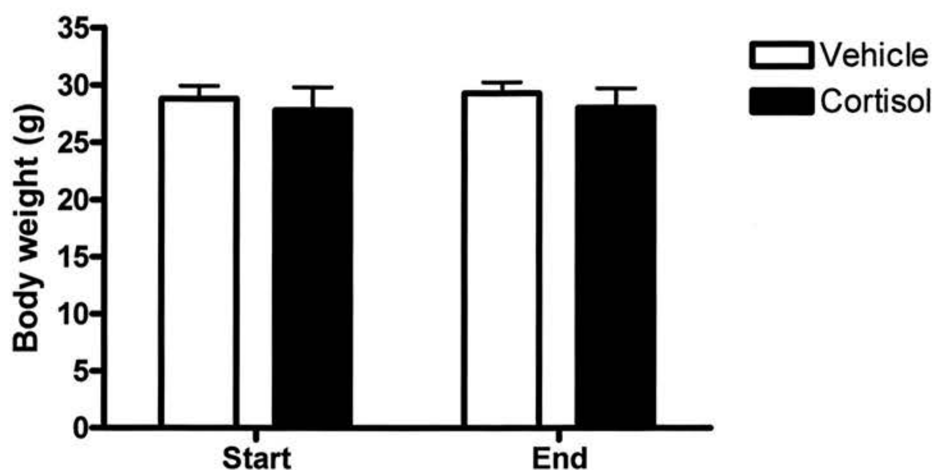


Figure 4.7: Influence of cortisol-releasing pellet on weight change over 21 days

Following bilateral wire-induced femoral artery injury, mice received either bilateral vehicle pellets (vehicle), or a unilateral cortisol pellet and contra-lateral vehicle control (cortisol) for 21 days. The weights of animals at the start and the end of the experiment were compared. There were no significant differences between the groups. Data are mean ± S.E.M, n= 8 for vehicle, n= 9 for cortisol. Analysed by one way ANOVA.

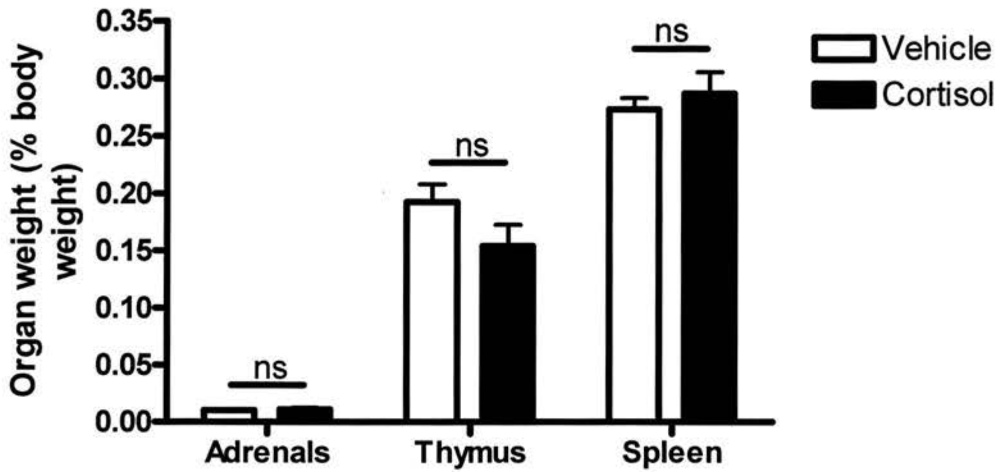


Figure 4.8: Effect of local cortisol administration on organ weights

Following bilateral wire-induced femoral artery injury, mice received either bilateral vehicle pellets (vehicle), or a unilateral cortisol pellet and contra-lateral vehicle control (cortisol). The weight of the adrenal glands, thymus and spleen were expressed as a percentage of total body weight in each animal at the end of the experiment. There was no significant difference in any organ weight between the groups. Data are mean ± S.E.M, n= 8 for all organs in vehicle group; n= 9 for thymus and spleen, n= 6 for adrenals in cortisol group (due to loss of samples). Analysed by unpaired t test: ns, not significant.

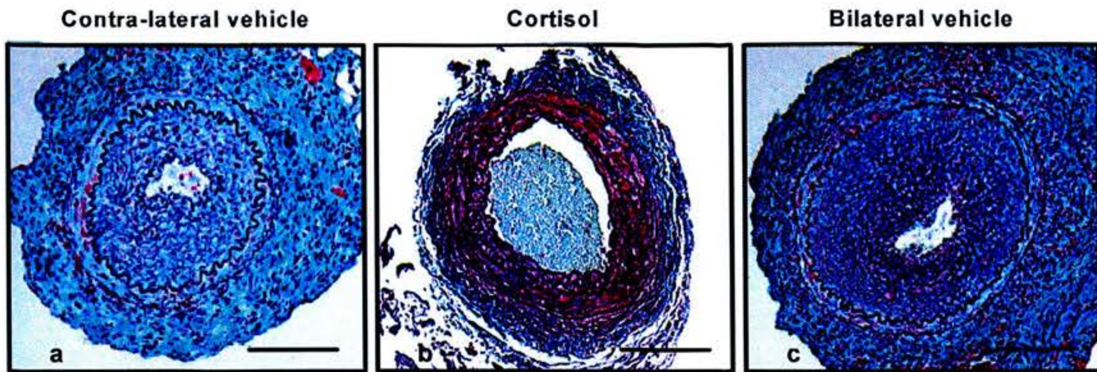


Figure 4.9: Action of local cortisol administration on neointimal proliferation after femoral artery injury

Sections of wire-injured arteries from animals implanted with a contra-lateral vehicle control pellet (a) and unilateral cortisol pellet (b), and a separate group of mice implanted with bilateral vehicle pellets (c). Extensive neointimal lesions developed in contra-lateral vehicle control arteries (a), but neointimal proliferation was dramatically inhibited when a cortisol pellet was placed next to the artery (b). Neointimal proliferation also occurred in animals implanted with bilateral vehicle pellets (c), 21 days after injury. Scale bar = 100 μm.

Quantification of neointimal lesion area indicated that cortisol release from an implanted pellet caused a significant decrease in neointimal proliferation after vascular injury, compared with contra-lateral controls (Figure 4.10). Neointimal lesions also developed in wire-injured arteries from the group of animals that received bilateral vehicle pellets (Figure 4.9c); there was no difference in the size of these lesions when compared with contra-lateral vehicle controls (Figure 4.10). Since no solid, fibrinogen-rich lesions were observed, cortisol treatment also decreased luminal narrowing, compared to contra-lateral controls (Figure 4.11).

In the group of mice which underwent bilateral pellet implantation without femoral artery manipulation, no vessel remodelling or neointimal proliferation was seen in any of the 6 arteries analysed (data not shown).

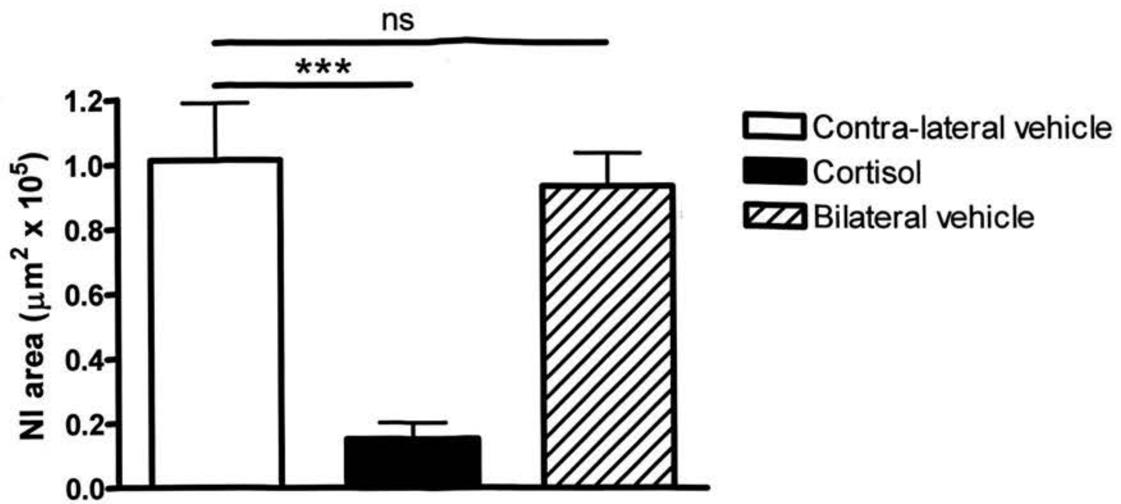


Figure 4.10: Decreased neointimal lesion development in arteries treated with cortisol-releasing pellets

Neointimal area was quantified using an image analysis system, in animals implanted with a unilateral cortisol pellet (cortisol) and contra-lateral vehicle control (contra-lateral vehicle). Neointimal area was also quantified in a separate group of mice implanted with bilateral vehicle pellets (bilateral vehicle). The presence of a cortisol pellet caused a significant decrease in neointimal area compared with contra-lateral vehicle controls, 21 days after wire-induced femoral artery injury. There was no difference in neointimal area between contra-lateral vehicle controls and bilateral vehicle controls. Data are mean ± S.E.M, n= 9 for contra-lateral vehicle, n= 9 for cortisol, n= 8 for bilateral vehicle. Analysed by one way ANOVA with Tukey's post hoc test: *** $p<0.001$ vs. contra-lateral vehicle; ns, not significant.

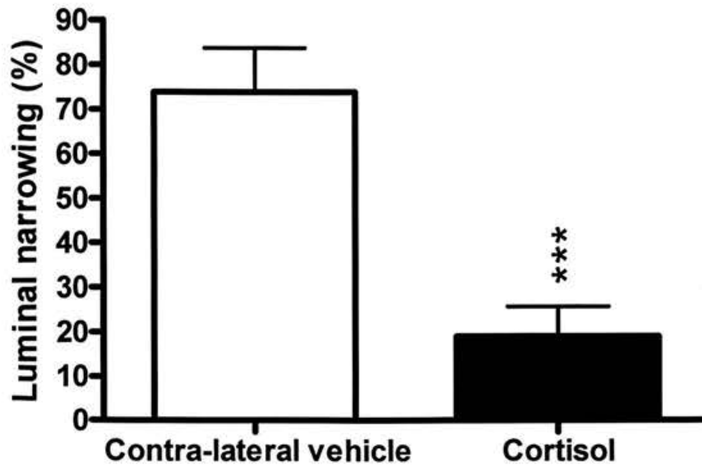


Figure 4.11: Reduced luminal narrowing in arteries treated with a cortisol-releasing pellet

The extent of luminal narrowing after wire-induced vascular injury was determined in animals implanted with a unilateral cortisol pellet and contra-lateral vehicle control. Luminal narrowing was calculated as the area of any lesion present in the vessel lumen, as a percentage of the total area inside the internal elastic lamina. There was a significant decrease in luminal narrowing in arteries treated with a cortisol pellet when compared with contra-lateral vehicle controls. Data are mean ± S.E.M, n= 9 per group. Analysed by unpaired test: *** p<0.001 vs. vehicle.

4.4 Discussion

These studies demonstrated that glucocorticoid administration inhibits neointimal proliferation following wire-induced intra-luminal injury of the mouse femoral artery. Whilst systemic administration of 1mg/kg dexamethasone virtually abolished the formation of smooth muscle-rich fibrous neointimal lesions, organised fibrinogen-rich lesions formed in their place. This resulted in a similar extent of luminal narrowing in mice receiving systemic glucocorticoids and in vehicle-treated controls. In contrast, administration of cortisol from an implanted pellet significantly decreased smooth muscle-rich fibrous lesion formation, without the formation of alternative fibrinogen-rich lesions. As a consequence, local cortisol administration reduced neointimal proliferation and luminal narrowing after vascular injury.

Effects of increased systemic glucocorticoid

Systemic dexamethasone was administered sub-cutaneously at a pharmacological dose of 1mg/kg/day, which was chosen as it inhibited neointimal lesion formation in previous studies (Van Put *et al.* 1995; Poon *et al.* 2001). Inhibition of weight gain, impaired wound healing and a decrease in weight of the adrenal glands, thymus and spleen were observed in animals treated with this dose of dexamethasone, indicating that it had significant systemic side-effects. Such side-effects are well recognised in clinical practice, where prolonged glucocorticoid therapy can lead to altered body fat composition, muscle atrophy and impaired wound healing, as well as impaired glucose tolerance, hypertension and increased plasma lipid levels. In addition, adverse effects associated with systemic glucocorticoid administration have previously been shown in the mouse (Pires *et al.* 2005), where the authors reported weight loss, impaired wound healing and a decrease in fur quality and daily activity. In the current experiments, the observed side effects confirmed that daily sub-cutaneous injections of 1mg/kg dexamethasone were pharmacologically active in the animals.

It was hypothesised that glucocorticoid administration would decrease neointimal proliferation after vascular injury. Therefore, arteries were analysed after 21 days, as neointimal lesion area was shown to be maximal at this time during development of the model (chapter 3). The dramatic reduction in fibrous neointimal lesion formation observed in these studies with systemic dexamethasone treatment is consistent with

previous reports in a variety of animal models of neointimal proliferation, such as the rabbit (Van Put *et al.* 1995; Petrik *et al.* 1998) and the mouse (Pires *et al.* 2005; Schepers *et al.* 2006).

The formation of alternative lesions that lacked smooth muscle cells but were rich in fibrinogen, exclusively in animals treated with systemic glucocorticoid, was unexpected. Since fibrinogen is a principle protein involved in the clotting of blood, it is likely that these lesions formed as the result of thrombosis in response to intra-vascular injury. Therefore, although systemic dexamethasone reduced the formation of smooth muscle-rich neointimal lesions after injury, the formation of thrombotic lesions occurred instead. This led to the same end point, and luminal narrowing was similar between dexamethasone-treated animals and vehicle-treated controls. Differences in lesion composition between dexamethasone- and vehicle-treated animals were not due to the extent of injury induced, as this was the same in both groups. During this experiment it was observed that wire-induced injury caused severe damage to the artery wall, with breaks and stripping of the IEL observed more frequently than during the model development. Exposure of the underlying media induced the formation of thrombus in both groups. In vehicle-treated mice, thrombus was only observed in sections of artery with wall damage, normally near to the site of wire insertion. However, fibrous neointimal lesions also developed in this group. In contrast, mice treated with dexamethasone exhibited an exclusively thrombotic response to injury. It should be noted that only wire-injured femoral arteries were studied in this experiment; therefore the possibility that dexamethasone may have acted to induce a general increase in thrombosis in all areas of the vasculature cannot be excluded. Increased systemic glucocorticoids might have promoted the formation of thrombotic lesions independently from any injury caused to the artery wall. To further investigate this possibility, sections of uninjured arteries from any part of the vasculature could be studied for the presence of thrombotic lesions in animals treated with the same dose of dexamethasone as used in the current experiments.

Action of locally-released cortisol

Implanted cortisol-releasing pellets were used to deliver glucocorticoid locally to the vessel wall, and several observations indicated that this was achieved. No systemic

side effects, such as significant changes in body weight or organ weights, were seen in mice implanted with cortisol-releasing pellets. Cortisol-releasing pellets reduced tissue remodelling around the operation site after vascular injury, in comparison with the contra-lateral leg of animals. Furthermore, the fact that there was no difference in neointimal lesion size between contra-lateral vehicle control arteries and those from different animals implanted with bilateral vehicle pellets, demonstrates that cortisol only acted to reduce neointimal proliferation within the artery of the leg in which it was released. Finally, measurement of plasma cortisol in mice confirmed that levels of circulating cortisol after pellet implantation were undetectable or very low.

The current experiments were consistent with a previous study using dexamethasone-eluting cuffs to investigate the effect of locally delivered glucocorticoid on neointimal proliferation (Pires *et al.* 2005). In this study, no changes in body weight or general well-being were observed in mice, supporting the idea that glucocorticoids released in the leg do not cause systemic side-effects. However, in contrast to the current experiments, impaired wound healing was seen in the skin at the operation site in 2/ 6 mice at the highest concentration used (20% w/w) (Pires *et al.* 2005). This discrepancy may be explained by the difference in steroids used between studies; dexamethasone may have effects on wound healing at this concentration, while cortisol may not since it is more rapidly inactivated by 11β-HSD2 (Best *et al.* 1997). However, since it is unlikely that levels of 11β-HSD2 are high enough in the skin to cause this difference, it is more probable that Pires *et al* (2005) achieved a higher concentration of glucocorticoid administration.

Comparison of glucocorticoids applied via alternative administration routes

In the current studies two different glucocorticoids were administered: dexamethasone and cortisol. Dexamethasone was administered systemically to reproduce previous animal studies investigating the effect of glucocorticoids on the vascular response to injury (Villa *et al.* 1994; Van Put *et al.* 1995; Guzman *et al.* 1996; Petrik *et al.* 1998; Poon *et al.* 2001; Nagasaki *et al.* 2004; Pires *et al.* 2005). Cortisol, rather than dexamethasone, was used for local delivery as it is more readily metabolised by 11β-HSD2 (Best *et al.* 1997) and so has a shorter half-life. Therefore, cortisol activity was more likely to be restricted to immediately adjacent tissues, and less likely to reach the systemic circulation. Cortisol was chosen over corticosterone,

the glucocorticoid endogenous to rodents, so that its plasma levels could be measured and used as an indicator of systemic glucocorticoid levels achieved by pellet implantation.

The use of two different steroids prevents an unequivocal conclusion that differences seen with systemically-applied and locally-applied glucocorticoids were due simply to the route of administration. Firstly, cortisol and dexamethasone have different actions (the former being a selective GR agonist, and the latter able to bind and activate both GR and MR), and different potencies at GR. Therefore, the effect of cortisol on the vascular response to injury may have been due to MR activation (the influence of MR on neointimal proliferation is discussed further in chapter 5.4). It is also possible that dexamethasone had a higher potency within the vessel wall than cortisol, as it is relatively resistant to metabolism by 11β-HSD2 (Best *et al.* 1997). The issue of potency is important, as the dose of glucocorticoid reaching the vessel wall was not matched in these studies. Therefore, the difference in action on the vascular response to injury seen using dexamethasone and cortisol could have been due to differences in the dose of glucocorticoid administered, rather than differences between systemic and local effects. Thus, it is possible that systemically-applied dexamethasone at a lower concentration would decrease neointimal proliferation in the absence of increased thrombosis. Similarly, a higher concentration of locally-released cortisol may have increased thrombogenicity in the vessel wall after injury. The fact that wound healing at the site of the operation was inhibited in animals administered systemic dexamethasone, but not in mice implanted with cortisol-releasing pellets, suggests that the extent of local GR activation in the tissues of the leg may have been higher in animals treated with dexamethasone. However, an accurate measurement of GR exposure to glucocorticoids in the vessel wall could not be obtained; indeed it would be very difficult to design an experiment *in vivo* where this would be possible. Subjective observations of connective tissue remodelling and wound healing at the operation site suggest that both steroids had an anti-inflammatory effect in the leg. Hence, what can be concluded is that dexamethasone and cortisol were both active locally in the tissues of the leg, with dexamethasone having additional systemic actions.

Mechanisms of glucocorticoid action

There are several potential mechanisms by which exogenously applied glucocorticoids may have reduced neointimal proliferation in these studies. Firstly, glucocorticoids are potent anti-inflammatory agents. They suppress several inflammatory pathways by blocking the transcriptional activity of NFκB (De *et al.* 1997a; McKay & Cidlowski 1999), and so decrease the release of inflammatory mediators such as cytokines, chemokines and cell adhesion molecules. Inflammation plays a critical role in neointimal proliferation following vascular injury in animals (Miller *et al.* 2001), and elevated systemic markers of inflammation are associated with a higher risk of restenosis in humans (Gaspardone *et al.* 1998; Buffon *et al.* 1999; Walter *et al.* 2001b). Indeed, glucocorticoid administration has been shown to reduce macrophage accumulation (Poon *et al.* 2001) and inhibit the release of several inflammatory cytokines (Ribichini *et al.* 2007) after vascular injury in the rabbit. Stent-mediated glucocorticoid release also reduced the macrophage content of neointimal lesions in the pig (Wang *et al.* 2005). Therefore, glucocorticoids may inhibit neointimal proliferation by suppressing the initial inflammatory response to vascular injury that is crucial to the formation of neointimal lesions.

Glucocorticoids are also anti-proliferative, and decrease proliferation of vascular lesion-derived smooth muscle cells *in vitro* (Longenecker *et al.* 1984; Berk *et al.* 1988; Voisard *et al.* 1994). Results from early studies indicated that high concentrations of steroid are necessary to inhibit proliferation *in vitro*, and a more recent study using concentrations comparable to those used *in vivo* failed to find an anti-proliferative effect (Ribichini *et al.* 2007). However, the authors did report an inhibition of VSMC migration at this concentration, another important process in neointimal lesion formation. Therefore, glucocorticoids may suppress neointimal proliferation through the inhibition of VSMC proliferation and migration. There is also some evidence that glucocorticoids may suppress collagen synthesis (Krane & Amento 1984), which would inhibit the ECM deposition and remodelling involved in neointimal lesion development.

Whilst it is possible that steroids might affect more that one of the aforementioned processes, it is likely that the most important mechanism of glucocorticoid-mediated inhibition of neointimal lesion formation is the suppression of vascular inflammation

after injury (Miller *et al.* 2001; Poon *et al.* 2001). Further experiments would be required to investigate the effect of exogenous glucocorticoids on inflammation after vascular injury in this model; for example by testing their ability to inhibit the adhesion of radiolabelled leukocytes to injured arteries *ex vivo* (Kennedy *et al.* 2000b). Alternatively, injured arteries could be excised at a chosen time-point, cultured *in vitro* with and without glucocorticoids, and cytokine levels in the culture medium analysed (Ribichini *et al.* 2007). To determine if the *in vivo* proliferation of VSMCs is altered in animals treated with glucocorticoid, immunohistochemical staining for PCNA could be carried out. Alternatively, injection of the thymidine analogue 5-bromo-2deoxyuridine (BrdU) would provide a nuclear marker of proliferating cells in sections of injured arteries.

In addition to inhibiting neointimal proliferation, systemic dexamethasone administration also promoted the formation of organized lesions with a thrombotic origin. It has been proposed that neointimal lesions originate as organizing blood clots that cover the luminal surface after arterial injury (Schwartz *et al.* 1992a). In this model, an initial thrombotic stage occurs, in which platelets, fibrin and red blood cells accumulate at the site of vascular injury. This is followed by a cellular recruitment stage, during which the thrombus becomes re-endothelialised and leukocytes infiltrate from the lumen. Finally, during the proliferative stage, VSMCs form a cap on the luminal surface of the lesion and, through ECM secretion and continuing recruitment, this cap progressively thickens. Thus, this model is based on the idea that thrombus plays a fundamental role in neointimal formation, by providing a scaffold into which VSMCs migrate and proliferate. It is possible that dexamethasone inhibited recruitment of inflammatory cells, proliferation and/or migration of smooth muscle cells into the intimal thrombus, resulting in the reduction of fibrous neointimal lesions but the persistence of organizing thrombus generated by vascular injury. However, evidence from development of the model of vascular injury used in the current studies suggests that a thrombotic scaffold is not necessary for neointimal lesion formation (chapter 3). Therefore, it seems unlikely that systemic dexamethasone acted to inhibit lesion formation in this way.

There is also some evidence that glucocorticoids increase the thrombogenicity of blood; in particular by increasing the expression of plasminogen activator inhibitor-1

(PAI-1). PAI-1 is the principle protein involved in inhibiting fibrinolysis and, therefore, an increase in its levels would be expected to impede the breakdown of blood clots. Studies *in vitro* have shown that glucocorticoids cause an increase in PAI-1 release (Fukumoto *et al.* 1992; Reinders *et al.* 1992; Halleux *et al.* 1999; Morange *et al.* 1999), and there is also evidence that dexamethasone can augment cytokine-stimulated PAI-1 release (He *et al.* 2000; Yamamoto *et al.* 2004). Glucocorticoids increase PAI-1 in the rat (van Giezen & Jansen 1992; van Giezen *et al.* 1994), whilst elevated circulating glucocorticoids in patients are associated with increased PAI-1 levels and hyper-coagubility of the blood (Ikkala *et al.* 1985; Patrassi *et al.* 1985; Patrassi *et al.* 1992; Sartori *et al.* 1999; Fatti *et al.* 2000; Sartori *et al.* 2000; Udden *et al.* 2002). It is possible that in animals treated with systemic dexamethasone the resolution of blood clots formed in response to vascular injury was delayed, leading to inhibition of neointimal proliferation of smooth muscle cells and alternative formation of organised thrombotic lesions. To examine whether this mechanism was involved in the observed effects of dexamethasone, separate investigations into the influence of glucocorticoids on the wide range of factors involved in blood clotting, such as PAI-1, could be carried out in the mouse.

Comparing effects of glucocorticoids on the vascular response to injury with other animal studies

Previous animal studies have shown inhibition of neointimal proliferation following glucocorticoid treatment (Villa *et al.* 1994; Van Put *et al.* 1995; Guzman *et al.* 1996; Petrik *et al.* 1998; Strecker *et al.* 1998; Nagasaki *et al.* 2004; Pires *et al.* 2005; Wang *et al.* 2005; Ribichini *et al.* 2007). However, these papers do not report the organised thrombotic lesions seen with systemic dexamethasone treatment in these studies. There may be several reasons for this. Firstly, the arteries in the current experiments were not perfusion-fixed before collection, so any thrombus generated in response to injury would remain in the vessel. In contrast, the majority of previous studies used perfusion fixation under pressure, which is likely to have removed mural thrombi. Secondly, no anti-coagulants were used in the current experiments, in order to avoid effects related to administration of additional drugs, and due to the difficulty associated with giving anti-coagulants to mice while controlling bleeding during operational procedures. Therefore, thrombosis was allowed to take place. However,

an anti-coagulation regime was applied in some previous animal studies, particularly those using rabbits (Van Put *et al.* 1995; Ribichini *et al.* 2007), which may have inhibited the formation of organised thrombotic lesions. A third source of variation between previous studies is the type of vascular injury induced. Some studies used models of perivascular injury (Van Put *et al.* 1995; Pires *et al.* 2005), where thrombosis does not play a major role in the response to injury (Moroi *et al.* 1998). Finally, differences in the type and dose of steroid administered, or the species used may have contributed to variation in the thrombotic response. Therefore, the reason that organised thrombus has not been reported previously may be that the methods used for inducing and analysing neointimal proliferation after vascular injury exclude or remove the formation of thrombus.

In the most comparable study to these experiments (Pires *et al.* 2005), the authors administered dexamethasone both systemically and locally in mice, and studied its effect on cuff-induced neointimal proliferation. Although they found that systemic dexamethasone abolished neointimal lesion formation, as in the current investigation, they did not report formation of organised thrombotic lesions. However, as mentioned above, the cuff-induced model of vascular injury does not induce the generation of thrombus in the artery lumen (Moroi *et al.* 1998), and so any effects of systemic glucocorticoids on thrombosis would not be observed. The intra-luminal method of injury used in the current investigations allows the action of glucocorticoids on thrombosis after vascular injury to be studied, a process that is important to the formation of neointimal lesions. In experiments where different doses of dexamethasone were delivered locally from the cuff to the vessel wall, all doses strongly reduced or abolished neointimal proliferation (Pires *et al.* 2005). However, higher doses also produced a loss of vessel wall integrity and adverse toxic effects on vascular cells, such as medial atrophy, apoptosis and loss of smooth muscle cells. Such effects were not observed with systemic dexamethasone treatment (Pires *et al.* 2005). Therefore, the authors concluded that although systemic glucocorticoid administration may be associated with adverse side effects that can be overcome by local application, this must be carried out in a relatively narrow therapeutic window above which pathophysiological effects are observed (Pires *et al.* 2005). This is in contrast to the current studies, where no effects of locally-released

cortisol on vessel wall integrity or medial atrophy were obvious when compared with contra-lateral controls. Again, this difference may be explained by the different models used to induce vascular injury in the two studies, since wire-induced injury itself causes vessel wall damage, medial atrophy and cellular apoptosis. Consequently, any additional effect of locally-released glucocorticoid on these factors may have been masked.

Clinical significance of observations

In the current studies, systemic dexamethasone treatment failed to decrease luminal narrowing after vascular injury, due to the formation of organised thrombotic lesions. This is interesting, as it may go some way to explaining the disappointing results of early clinical trials using systemic glucocorticoids to treat restenosis (Stone *et al.* 1989; Pepine *et al.* 1990; Rab *et al.* 1991; Lee *et al.* 1999). It is possible that any beneficial effect of steroids on restenosis in these studies was masked by the generation of organised thrombus. The quantification of these thrombotic lesions during angiography could account for the lack of improvement in angiographic outcome. However, it is important to note that although reduced restenosis may have been concealed in these studies, glucocorticoids did not improve the functional end-point of lumen loss, which restricts blood flow to the myocardium. The lack of a reduction in luminal narrowing in the clinical studies could also be explained by the association between systemic glucocorticoid excess and cardiovascular risk. Increased blood pressure, endothelial dysfunction (Mangos *et al.* 2000) and the risk of coronary aneurysms (Rab *et al.* 1991) caused by increases in circulating glucocorticoids could all be detrimental, causing vasospasm or altered wall healing after coronary intervention.

The failure of clinical trials using intravenous single pulse pre-treatment or short term glucocorticoid administration was attributed to non-lasting and insufficient local effects of steroids (Stone *et al.* 1989; Pepine *et al.* 1990; Rab *et al.* 1991; Lee *et al.* 1999). However, prolonged treatment with systemic prednisolone did have a beneficial effect on restenosis (Versaci *et al.* 2002), and the most recent evidence indicates that the use of dexamethasone-eluting stents is associated with an improvement in clinical and angiographic outcomes after coronary intervention (Han *et al.* 2006; Konig *et al.* 2007). Therefore, it seems that prolonged application of

steroids at the vessel wall represents the most promising way to administer glucocorticoids for the treatment restenosis. This concept is supported by the results of experiments using locally-released cortisol reported here.

Conclusions

These studies have indicated that, whilst glucocorticoid administration inhibits neointimal proliferation after vascular injury, systemic administration of dexamethasone is associated with adverse side-effects and the formation of organised thrombus. Local application of cortisol at the vessel wall is more beneficial, as it reduces luminal narrowing after injury and has no associated systemic effects. These results suggest a balance exists between the beneficial actions of glucocorticoids on the vascular response to injury when they are acting locally at the vessel wall, and the adverse effects of increased systemic glucocorticoids on cardiovascular risk factors such as hypertension, obesity, impaired glucose tolerance and increased plasma lipid levels. This supports the concept that local application of glucocorticoids is more favourable than systemic administration in humans. The availability of drug-eluting stents allows the local application of steroids in clinical practice, and avoids the undesirable side-effects of oral steroid treatment mentioned above, which would exacerbate conditions such as diabetes or heart failure. However, there are also advantages of oral therapy over drug-eluting stents, such as low cost and the avoidance of long-term anti-platelet therapy after coronary interventions. In addition, concerns that drug-eluting stents may delay long term healing of the vessel wall and increase the risk of late stent thrombosis should be taken into account in future studies.

The successful application of exogenous steroids at the vessel wall as treatment for neointimal lesion formation raises the possibility that endogenous glucocorticoids may influence the vascular response to injury. Inhibition of systemic 11β-HSD1 activity is currently being developed as a therapeutic strategy to reduce cardiovascular risk factors. However, the role that modulation of endogenous glucocorticoid activity by 11β-HSD1 within the artery wall plays in neointimal lesion development needs further investigation, and this is considered in the next chapter.

Chapter 5

Influence of endogenous glucocorticoids on the vascular response to injury in the mouse

5.1 Introduction

The work described in the previous chapter confirmed that glucocorticoid administration inhibits neointimal lesion formation, and can reduce the vascular response to injury when carried out locally at the vessel wall. This highlights a potential role for endogenous glucocorticoid hormones in the regulation of neointimal proliferation. Glucocorticoid levels are regulated both systemically, by the HPA axis (chapter 1.2.2.1), and at a local tissue level, by the 11β-HSD enzymes (chapter 1.2.3.1). Surprisingly, the effect of endogenous glucocorticoids on neointimal proliferation after vascular injury has not been investigated previously.

Several lines of evidence suggest that endogenous glucocorticoids may act to influence vascular remodelling (Hadoke *et al.* 2006). Increased systemic glucocorticoid concentrations in Cushing's syndrome are associated with a higher risk of developing atherosclerotic lesions (Colao *et al.* 1999; Faggiano *et al.* 2003). Detection of expression (Walker *et al.* 1991; Christy *et al.* 2003) and activity (Small *et al.* 2005) of the 11β-HSD enzymes in the vascular wall suggests that endogenous glucocorticoid regeneration occurs locally within this tissue. Indeed, local generation of endogenous glucocorticoids by 11β-HSD1 has been shown to modulate angiogenesis after myocardial infarction in mice (Small *et al.* 2005). Furthermore, the suggestion that local inflammation in the vessel wall, a critical process in neointimal lesion formation, up-regulates 11β-HSD1 activity (Cai *et al.* 2001) indicates that a negative feedback loop for the control of inflammation via glucocorticoid generation may exist at this site. However, studies in intact vessels (Dover *et al.* 2007), and wire-injured femoral arteries (chapter 3.2.5) have failed to confirm this idea.

Although the effect of 11β-HSD on the formation of neointimal lesions after acute injury has not been investigated, the influence of 11β-HSD1 activity on atherosclerotic vascular lesions has been addressed recently. Selective inhibition of this enzyme was found to prevent the progression of atherosclerosis in ApoE knockout mice (Hermanowski-Vosatka *et al.* 2005). In addition, non-specific inhibition of 11β-HSD activity with carbenoxolone decreased atherosclerotic lesion formation in the hyperlipidemic and hyperphagic Agouti protein over-expressing/

LDL receptor knockout mouse (Nuotio-Antar *et al.* 2007). Therefore, these studies indicate that generation of endogenous glucocorticoids by 11β-HSD1 increases atherosclerotic lesion formation. However, what remains unclear from these studies is the mechanism by which 11β-HSD1 activity influences atherogenesis, and whether it is via systemic actions on factors such as plasma lipid levels or blood pressure (mediated by inhibition of 11β-HSD1 in e.g. liver or adipose tissue), or caused by a local effect on the cells of the vascular wall.

To begin to explore the influence of endogenous glucocorticoids on neointimal proliferation during the vascular response to injury, it was hypothesised that the local generation of these hormones by 11β-HSD1 would inhibit neointimal lesion development in the mouse model of vascular injury. This was based on previous observations that locally-applied exogenous glucocorticoids decrease neointimal lesion formation in this model, which primarily involves the cells of the vessel wall and lumen. With this in mind, the specific aims of this chapter were:

(i) To determine whether endogenous glucocorticoids decrease neointimal lesion formation via local administration of a GR antagonist to oppose this effect.

(ii) To assess if the abolition of 11β-HSD1 activity increases neointimal proliferation using 11β-HSD1$^{-/-}$ mice, and mice administered an 11β-HSD1 inhibitor.

5.2 Methods

5.2.1 Animals

Male C57B16 (Harlan Olac, U.K.) and 11β-HSD1$^{-/-}$ (bred in-house at the Biomedical Research Facility, Little France, Edinburgh, U.K.) mice weighing 25-35 grams were used in all studies. Genetic inactivation of 11β-HSD1 has been described previously on an MF-1/129 background (Kotelevtsev et al, 1997); for the current experiments mice were backcrossed onto a C57Bl6 background over more than 10 generations (Morton et al, 2004). To allow collection of trunk blood, animals were killed by decapitation, according to Home Office guidelines.

5.2.2 Femoral artery injury

In experiments investigating the influence of the GR antagonist RU38486 on the vascular response to injury, wire-induced intra-luminal vascular injury (chapter 2.3.1) was carried out both in the left and the right femoral arteries. In studies assessing the impact of endogenous glucocorticoid regeneration, 11β-HSD1$^{-/-}$ mice and C57Bl6 controls (n= 8 per group) underwent wire-induced injury of the left femoral artery, and ligation-induced injury of the right femoral artery (chapter 2.3.2). Similarly, animals treated with 11β-HSD1 inhibitor or vehicle underwent wire-induced injury of the left femoral artery, and ligation-induced injury of the right femoral artery.

5.2.3 Administration of drugs

5.2.3.1 Locally-released glucocorticoid receptor antagonist

To assess the action of GR antagonism on neointimal proliferation, silastic pellets (chapter 2.4.2) were placed next to the femoral artery after wire-induced injury. Pellets were inserted into a pocket created at the operation site and kept in place when the skin incision was closed with sutures at the end of the operation. During bilateral, wire-induced injury a pellet containing RU38486 was implanted in one leg, and a vehicle pellet (silastic elastomer only) was implanted in the other leg (n= 8). RU38486 or vehicle pellets were implanted randomly in either the right or the left leg. Each 10 mg pellet contained 3.3 mg RU38486 (33% w/w). Results were also compared with the additional control group previously described in chapter 4, in

which animals underwent bilateral femoral artery injury, and vehicle pellets were placed in both legs (n= 8).

5.2.3.2 Systemically-administered 11β-HSD1 inhibitor

To investigate the effect of systemic pharmacological inhibition of 11β-HSD1 on neointimal proliferation, mice were dosed with 30mg/kg of a selective inhibitor (compound 815 (Hermanowski-Vosatka *et al.* 2005)) twice daily by oral gavage (chapter 2.4.3). A group of control animals received vehicle alone (5% cyclodextrin); n= 7 per group. Gavaging was performed by staff at the Biomedical Research Facility, Little France, Edinburgh, U.K. Loading doses were given one day before surgery, and dosing was then continued in the morning and afternoon for every day of the experiment. Body weight was recorded each day.

5.2.4 Tissue collection

Tissues from the RU38486 experiment were collected 21 days following surgery, as this experiment was carried out in parallel with experiments using cortisol-releasing pellets (chapter 4). Tissues were collected 28 days after surgery from 11β-HSD1$^{-/-}$ mice to ensure that advanced neointimal lesions had developed which could be quantified. In the experiment in which animals were treated with 11β-HSD1 inhibitor, tissues were collected after 14 days so that an effect on neointimal lesion size in either direction could be discerned. However, thrombotic involvement and the small and cellular nature of lesions at this time-point made interpretation of sections and accurate measurement of lesion size difficult.

At the end of all experiments mice were weighed for a final time, and then trunk blood was collected into heparinised tubes on wet ice. Blood samples were centrifuged at 3000 rpm for 5 minutes, and the plasma (supernatant) was removed into fresh eppendorf tubes. Organs (adrenals, thymus, spleen, kidneys, heart and liver) were removed, cleaned of connective tissue and weighed. Plasma and organs were then snap frozen on dry ice and stored at -80°C.

Left and right femoral arteries were cleaned of connective tissue and veins, and excised from the bifurcation of the iliac artery to the branch with the popliteal artery. Femoral arteries were immediately placed in 10% neutral buffered formalin, fixed for up to 24 hours and then stored in 70% ethanol if necessary. The arteries were

dehydrated in a graded alcohol series, embedded in paraffin and cut into transverse sections: sets of eight 4 μm serial sections were taken at 100 μm intervals along the entire arterial segment.

5.2.5 Measurement of neointimal proliferation

To assess the extent of neointimal proliferation in these experiments, transverse sections every 100 μm along injured arteries were stained with the United States trichrome histological stain (chapter 2.6.2). The area (μm^2) of neointima in each section was measured using a light microscope coupled to a colour camera and image analysis system (chapter 2.7). The section with the largest area of neointima was chosen to represent each arterial sample. All analyses were performed with the investigator blinded as to which group the sample belonged to.

5.2.6 Plasma corticosterone levels

Corticosterone levels in plasma from mice implanted with a unilateral RU38486 pellet and contra-lateral vehicle control, and mice implanted with bilateral vehicle pellets, were analysed using radioimmunoassay (n= 8 per group; chapter 2.11.2).

5.2.7 Genotype analysis by polymerase chain reactions

11β-HSD1$^{-/-}$ mice used in these studies were kindly provided by Dr. M. Holmes. Animals were bred in an established colony (Biomedical Research Facility, Little France, Edinburgh, U.K.) by crossing 11β-HSD1$^{-/-}$ parents. C57Bl6 controls were obtained from Harlan Olac, U.K. PCR were carried out to confirm the genotype of the male offspring used in these studies (chapter 2.12).

5.2.8 Effect of enzyme inhibitor on 11β-HSD1 activity

The effect of systemic administration of an 11β-HSD1 inhibitor on enzyme activity was assessed in liver microsomes (chapter 2.10.4). Activity was measured in the dehydrogenase direction, with conversion of [^{3}H] corticosterone to [^{3}H] 11-dehydrocorticosterone determined by HPLC (chapter 2.10.5).

5.3 Results

5.3.1 Effect of RU38486 pellet implantation on animals

When mice implanted with a unilateral RU38486 pellet were compared with those implanted with bilateral vehicle pellets, there was no difference in body weight between groups at the start or the end of the experiment (Figure 5.1). Similarly, there were no significant differences in the weight of organs known to change in size in response to glucocorticoids (adrenal glands, thymus and spleen; Figure 5.2), or other organs (heart, kidneys and liver; data not shown), between the two groups at the end of the experiment.

Plasma corticosterone levels were not significantly different between mice implanted with an RU38486 pellet and those implanted with bilateral vehicle pellets, despite a trend ($p= 0.16$) towards reduced corticosterone levels in the group receiving RU38486 (Figure 5.3a). Plasma collections were not always performed at the same time of day, due to practical constraints. To take diurnal variation of corticosterone levels into account, plasma samples collected in the morning were compared. This sub-division demonstrated that it was at this time of the day that a trend ($p= 0.081$) towards lower corticosterone levels occurred in mice implanted with an RU38486 pellet (Figure 5.3b). In contrast, this effect of RU38486 was not seen in plasma samples collected in the afternoon (Figure 5.3c, $p= 0.45$).

5.3.2 Influence of GR antagonism on neointimal proliferation

Femoral arteries were analysed from the group of mice ($n= 8$) receiving an RU38486 pellet and a contra-lateral vehicle pellet following bilateral wire-induced injury. Some damage to the artery wall was caused by intra-luminal wire injury in this experiment, which took the form of breaks or stripping of the IEL. This was usually located near to the branch of the popliteal artery, where the wire is inserted. However, the damage was not sufficient to cause severe or occlusive thrombosis, and occurred to a similar extent in arteries treated with RU38486 and vehicle pellets, allowing the two groups to be compared.

Extensive fibrous neointimal lesions developed in arteries treated with RU38486 and in contra-lateral vehicle controls (Figure 5.4a and b). Neointimal lesions also

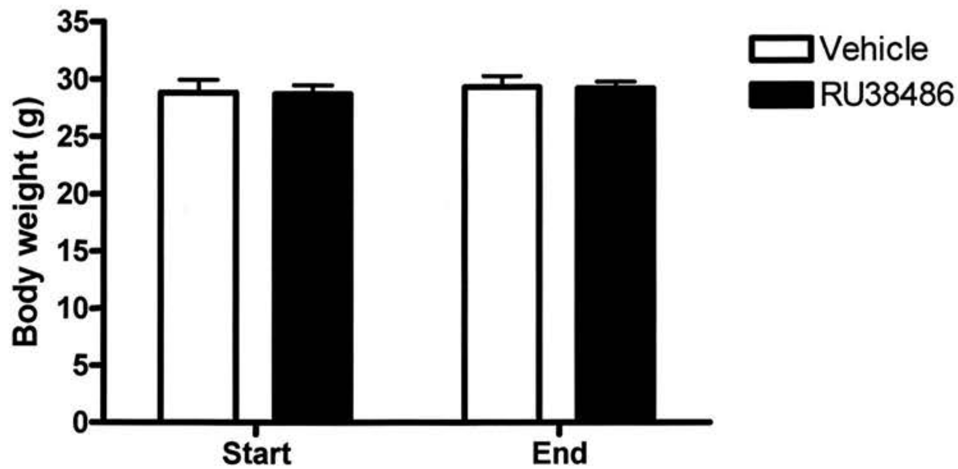


Figure 5.1: Effect of RU38486-releasing pellet on weight change over 21 days after femoral artery injury

Following bilateral, wire-induced femoral artery injury, mice received either bilateral vehicle pellets (vehicle), or a unilateral RU38486 pellet plus contra-lateral vehicle control (RU38486). The weights of animals at the start and the end of the experiment were compared. There was no significant difference in weight between the groups at either time-point. Data are mean ± S.E.M, n= 8 per group. Analysed by one way ANOVA.

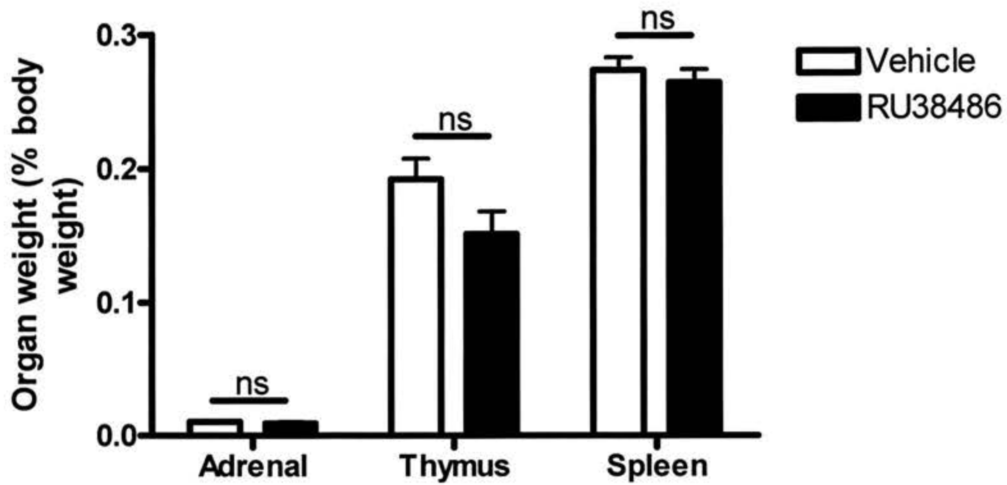


Figure 5.2: Influence of RU38486 administration on organ weights

Following bilateral, wire-induced femoral artery injury, mice received either bilateral vehicle pellets (vehicle), or a unilateral RU38486 pellet and contra-lateral vehicle control (RU38486). The weight of the adrenal glands, thymus and spleen were calculated as a percentage of total body weight in each animal at the end of the experiment. Despite a trend (p= 0.091) towards decreased thymus weight in animals treated with RU38486, there was no significant difference in any organ weight between the groups. Data are mean ± S.E.M, n= 8 per group. Analysed by unpaired t test: ns, not significant.

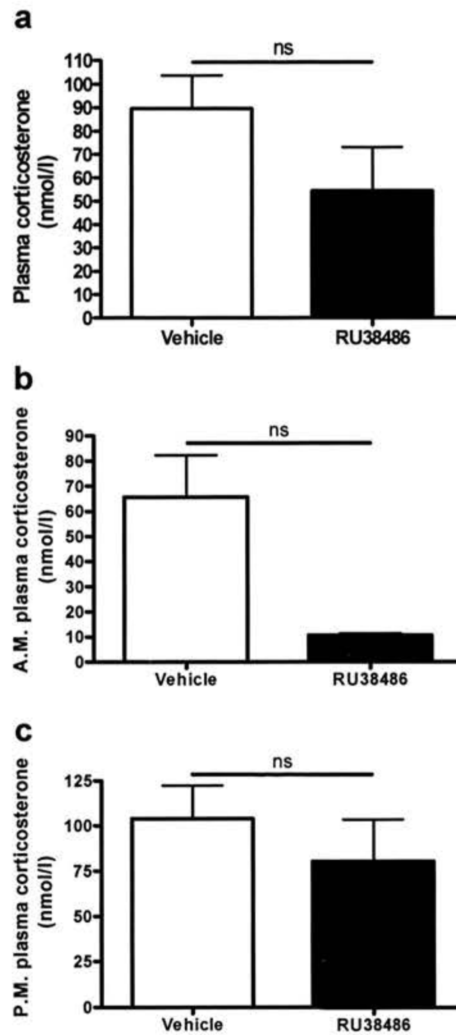


Figure 5.3: Plasma corticosterone levels in mice implanted with an RU38486-releasing pellet

Following bilateral, wire-induced femoral artery injury, mice received either bilateral vehicle pellets (vehicle), or a unilateral RU38486 pellet and contra-lateral vehicle control (RU38486) for 21 days. Plasma corticosterone levels were measured by radioimmunoassay at the end of the experiment. There was no difference (p= 0.16) in corticosterone levels when all mice in each group were compared (a). However, samples collected in the morning showed a trend (p=0.081) towards lower corticosterone in animals treated with RU38486 (b), while there was no difference (p= 0.45) in samples collected in the afternoon (c). Data are mean ± S.E.M, n= 8 per group for (a), n= 3 for (b) and n= 5 for (c). Analysed by unpaired t test (with Welch's correction in (b)): ns, not significant.

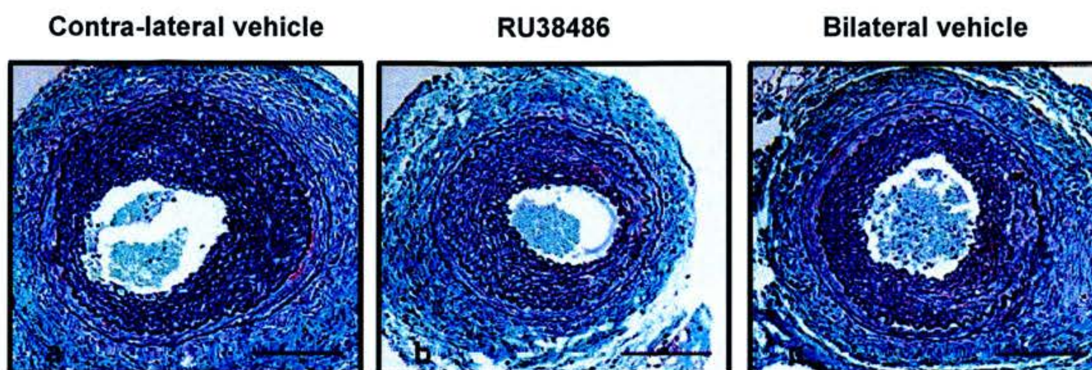


Figure 5.4: Influence of the GR antagonist RU38486 on neointimal proliferation in the mouse femoral artery

Transverse sections of wire-injured femoral arteries stained with the United States trichrome stain from mice implanted with a contra-lateral vehicle control pellet (a) and unilateral RU38486 pellet (b), and from a separate group of mice with bilateral vehicle pellets (c). Extensive neointimal lesions developed in all groups, 21 days after injury. Scale bar = 100 μm.

developed in wire-injured arteries from the group of animals that received bilateral vehicle pellets (Figure 5.4c). When neointimal area was quantified, a trend towards increased lesion size in arteries treated with RU38486 was not significant when compared with contra-lateral vehicle controls (Figure 5.5, $p > 0.05$). There was also no difference in neointimal proliferation between contra-lateral vehicle control arteries, and those from separate mice with bilateral vehicle pellets (Figure 5.5, $p > 0.05$).

The failure of the trend towards increased neointimal lesion area in arteries treated with RU38486 to reach significance suggested that the experiment may have been under-powered. This was addressed by calculating power using the data generated and appropriate software (GraphPad StatMate v.2.00). Calculations demonstrated that the experiment would detect the observed difference (0.24 $\mu m^2 \times 10^5$) with only 30% power. However, to detect this difference with 90% power would have required an increase in numbers to n= 60 per group; this was highly impractical due to practical and time constraints. Furthermore, if a statistically significant result was documented in this way, the magnitude of difference in means is of dubious biological significance, and so arguably it would not be an ethically justified experiment.

5.3.3 Neointimal proliferation in 11β-HSD1$^{-/-}$ mice

Genotype analysis by PCR confirmed that mice used in these studies were homozygous null for 11β-HSD1 (Figure 5.6). 11β-HSD1$^{-/-}$ and C57Bl6 control mice underwent wire-induced injury of the left femoral artery, and ligation-induced injury of the right femoral artery (n= 8 per group). Again, some damage to the artery wall was caused by intra-luminal wire injury in both groups; however, the damage was not sufficient to prevent the growth of fibrous neointimal lesions and occurred to a similar extent in both groups. In addition, breaks in the IEL were caused by ligation-induced injury in 2/ 16 animals.

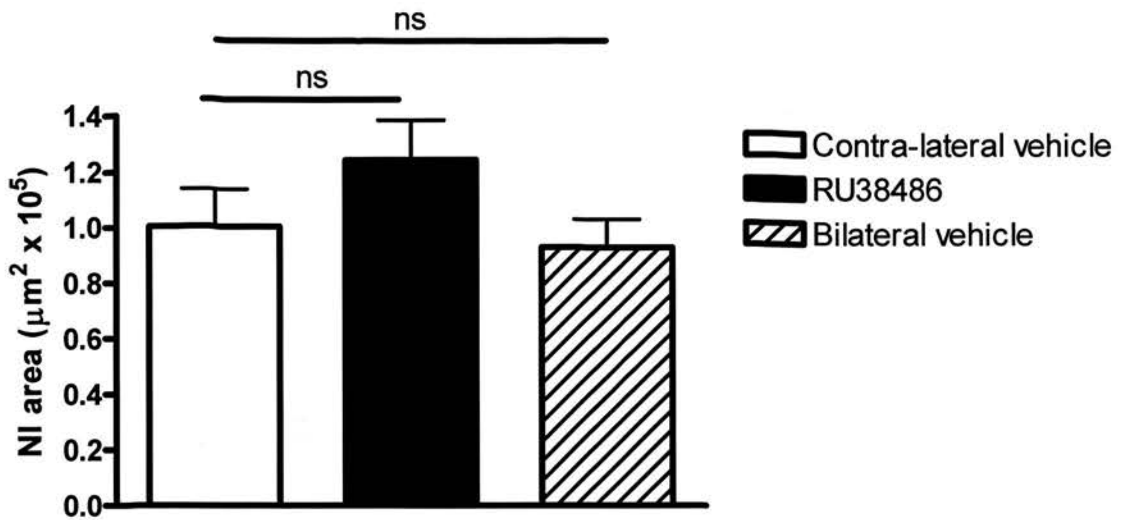


Figure 5.5: Influence of local GR antagonism with RU38486 on neointimal lesion development following femoral artery injury

Neointimal area was quantified by image analysis, in wire-injured arteries from mice treated with a vehicle control pellet in one leg (contra-lateral vehicle) and an RU38486 pellet in the other leg (RU38486). Neointimal area was also quantified in a separate group of mice implanted with bilateral vehicle pellets (bilateral vehicle). The presence of an RU38486 pellet caused a trend towards increased neointimal area compared with contra-lateral vehicle controls that did not reach significance, 21 days after femoral artery injury. There was also no difference in neointimal area between contra-lateral vehicle control arteries, and those from separate mice implanted with bilateral vehicle pellets. Data are mean ± S.E.M, n= 8 per group. Analysed by one way ANOVA: ns, not significant.

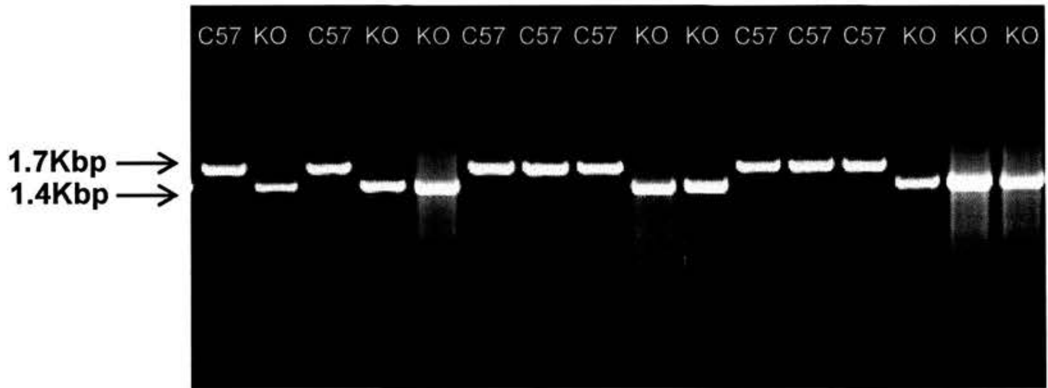


Figure 5.6: Genotyping of 11β-HSD1$^{-/-}$ and C57Bl6 mice by polymerase chain reactions

Genomic DNA isolated from C57Bl6 (C57) and 11β-HSD1$^{-/-}$ (KO) mice was used in polymerase chain reactions with specific primers to detect either the 11β-HSD1 wild type allele (1.7Kbp) or the transgenic 11β-HSD1 allele with integrated targeting vector (1.4Kbp). Gel electrophoresis confirmed that only transgenic alleles were detected in the group of 11β-HSD1$^{-/-}$ mice, confirming their genotype as homozygous null for 11β-HSD1.

Wire-induced femoral artery injury induced the development of fibrous neointimal lesions in 11β-HSD1$^{-/-}$ mice and C57Bl6 controls (Figures 5.7a and b). A trend towards increased neointimal lesion area 28 days after surgery in 11β-HSD1$^{-/-}$ mice did not reach significance (Figure 5.8a, p= 0.26).

Ligation-induced femoral artery injury also produced the development of fibrous neointimal lesions in 11β-HSD1$^{-/-}$ mice and C57Bl6 controls (Figures 5.7c and d), although these lesions were smaller than those induced by wire injury (Figure 5.8). There was no significant difference in neointimal lesion area induced by ligation between 11β-HSD1$^{-/-}$ mice and controls (Figure 5.8b, p= 0.77).

Again, the trend towards increased neointimal lesion area in 11β-HSD1$^{-/-}$ mice after wire-induced injury was investigated further by carrying out power calculations. This demonstrated that the experiment would detect the difference in means between 11β-HSD1$^{-/-}$ and wild type mice (0.21 μm^2 x 10^5) with only 20% power. In order to detect this difference with adequate power (90%) the sample size would have to be increased to n= 70 per group. Due to practical and time constraints it was not feasible to increase group size by this extent and, as for experiments with RU38486, the biological relevance of the magnitude of difference in means is questionable.

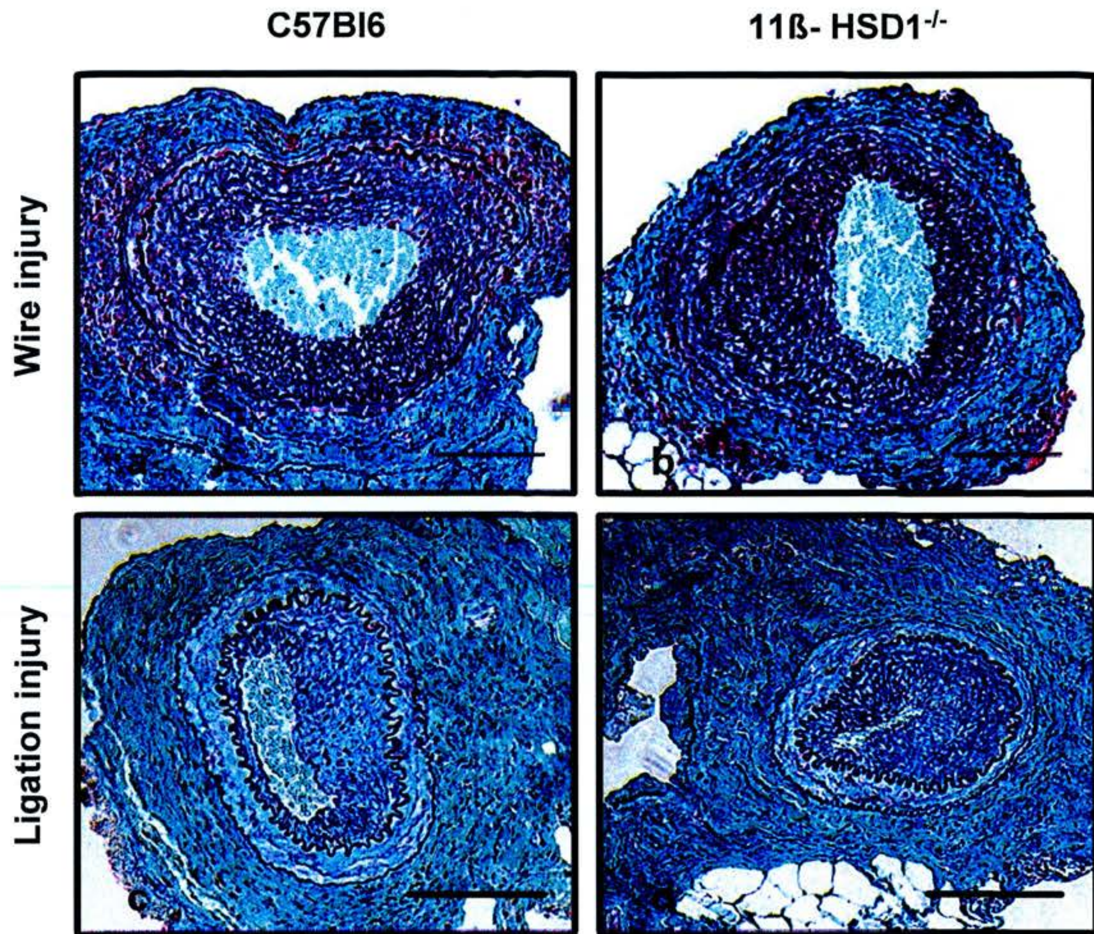


Figure 5.7: Neointimal proliferation after wire- or ligation-induced vascular injury in 11β-HSD1$^{-/-}$ and C57Bl6 mice

Transverse sections of femoral arteries stained with the United States trichrome stain. Fibrous neointimal lesions developed 28 days after wire-induced injury in C57Bl6 (a) and 11β-HSD1$^{-/-}$ mice (b). Neointimal proliferation also occurred in the femoral artery 28 days after ligation of the popliteal artery in C57Bl6 (c) and 11β-HSD1$^{-/-}$ mice (d). Scale bar = 100 μm.

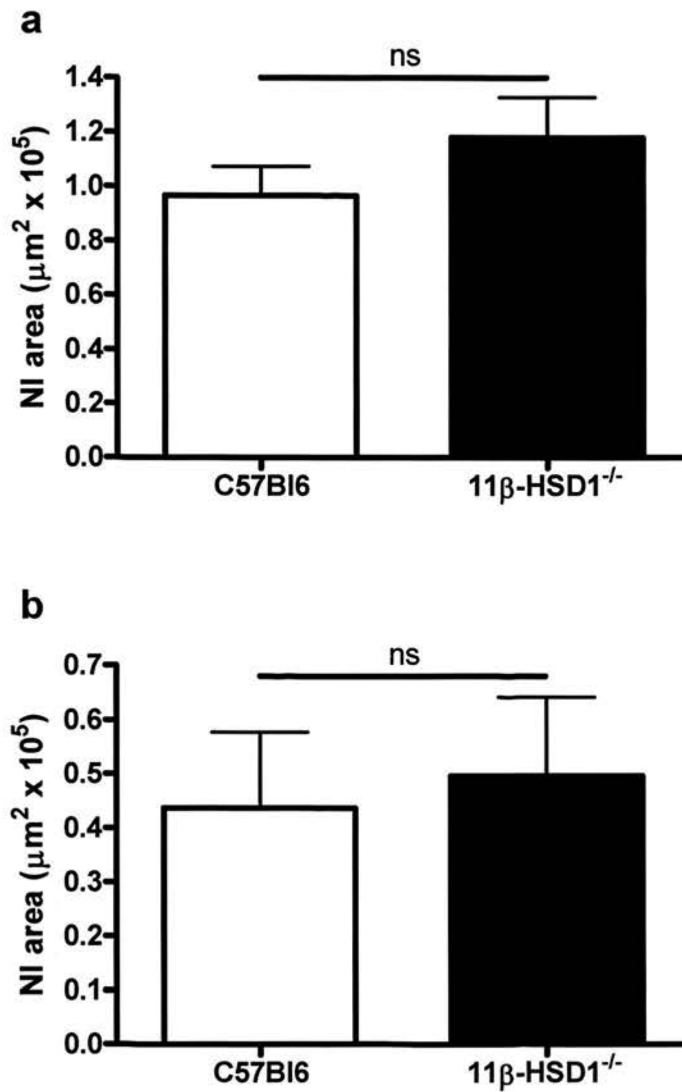


Figure 5.8: Comparison of neointimal lesion area in C57Bl6 and 11β-HSD1$^{-/-}$ mice

Neointimal area was quantified by image analysis in arteries from C57Bl6 and 11β-HSD1$^{-/-}$ mice 28 days after wire-induced (a) or ligation-induced injury (b). Although there was a trend ($p= 0.26$) towards increased lesion area in 11β-HSD1$^{-/-}$ mice after wire-induced injury, there was no significant difference in the size of neointimal lesions induced by either type of injury between the groups ($p= 0.77$ for ligation injury). Wire injury caused the growth of larger neointimal lesions than ligation injury. Data are mean ± S.E.M, n= 8 per group. Analysed by unpaired t test: ns, not significant.

5.3.4 Effects of 11β-HSD1 inhibitor administration on mice

As an extension of studies in 11β-HSD1$^{-/-}$ mice, a group of animals were given a selective 11β-HSD1 inhibitor. In these preliminary experiments, systemic administration of 11β-HSD1 inhibitor or vehicle by oral gavage twice a day had obvious adverse effects on the animals. Mice in both groups became smaller and thinner during the study, and were less active. There was no difference in weight between the groups at the start of the experiment (Figure 5.9). A comparison of body weight during the experiment showed that mice in both groups lost weight initially after surgery, but then failed to re-gain weight over the course of the experiment. There was no difference in weight change between animals administered vehicle and those given 11β-HSD1 inhibitor (Figure 5.9). There were no differences in the weight of the adrenal glands, thymus and spleen (Figure 5.10) or other organs (data not shown) after treatment with the 11β-HSD1 inhibitor.

To assess the action of the 11β-HSD1 inhibitor on enzyme activity, the conversion of [^{3}H] corticosterone to [^{3}H] 11-dehydrocorticosterone was measured in a preparation of liver microsomes. There was no significant difference in dehydrogenase reaction velocity between animals treated with 11β-HSD1 inhibitor and those treated with vehicle alone (Figure 5.11).

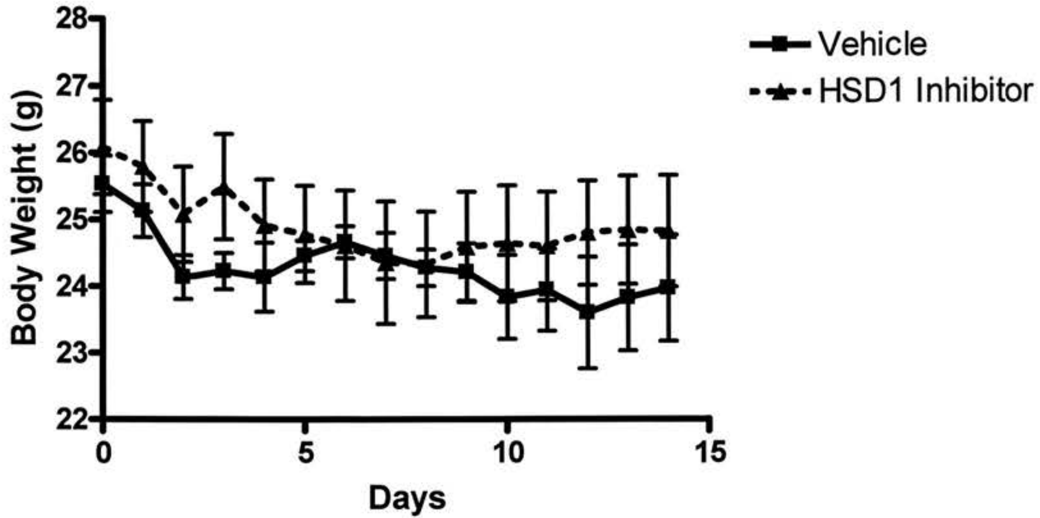


Figure 5.9: Weight change in animals during administration of 11β-HSD1 inhibitor by oral gavage

Mice received 30mg/kg 11β-HSD1 inhibitor or vehicle by oral gavage, twice a day for 14 days after bilateral femoral artery injury. There was no difference in weight change between animals administered 11β-HSD1 inhibitor and those that received vehicle. However, both groups of animals showed a significant failure to gain weight over the duration of the study ($p < 0.001$). Data are mean ± S.E.M, n= 7 per group. Analysed by two way ANOVA.

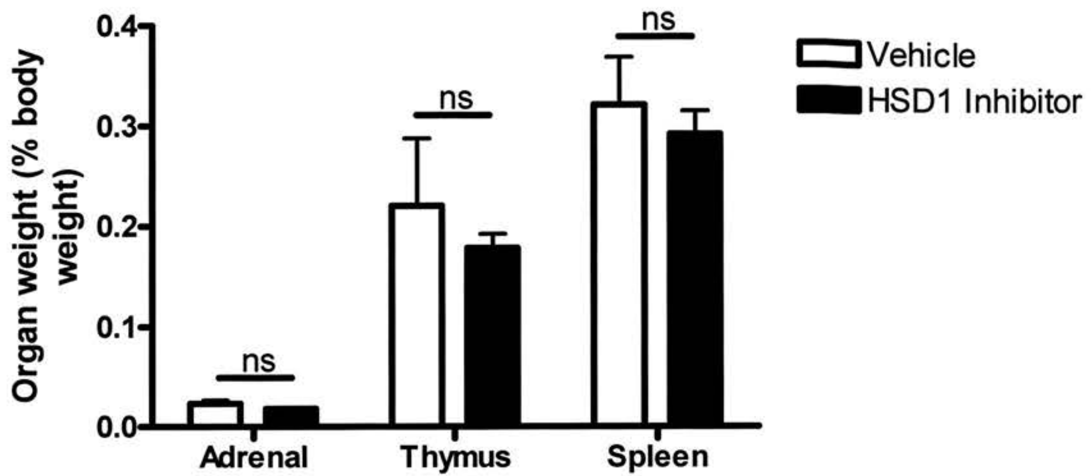


Figure 5.10: Effect of 11β-HSD1 inhibitor on organ weights

Following bilateral femoral artery injury, mice received either 30mg/kg 11β-HSD1 inhibitor or vehicle by oral gavage for 14 days. The weight of the adrenal glands, thymus and spleen were calculated as a percentage of total body weight in each animal at the end of the experiment. There was no significant difference in adrenal ($p= 0.22$), thymus ($p= 0.55$) or spleen ($p= 0.61$) weights between the groups. Data are mean ± S.E.M, $n= 7$ per group. Analysed by unpaired t test: ns, not significant.

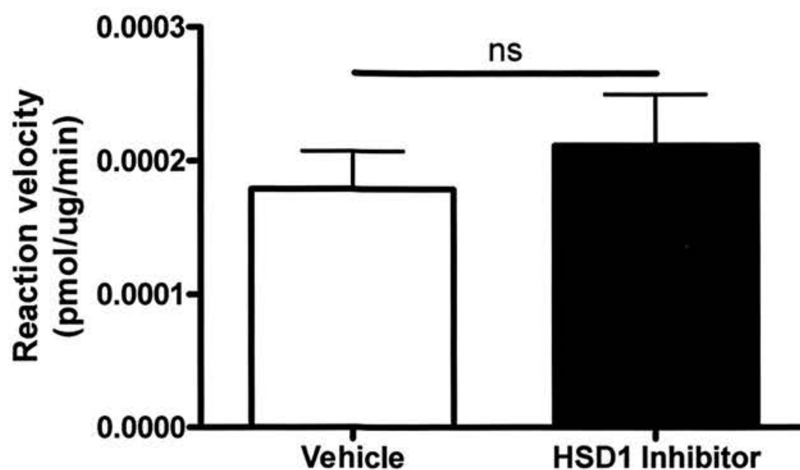


Figure 5.11: Enzyme activity after administration of 11β-HSD1 inhibitor

Mice received 30mg/kg 11β-HSD1 inhibitor or vehicle twice daily by oral gavage, for 14 days after bilateral femoral artery injury. 11β-HSD1 activity was measured in the dehydrogenase direction in liver microsome preparations. There was no significant difference in enzyme activity between mice administered 11β-HSD1 inhibitor and those given vehicle alone ($p= 0.50$). Data are mean ± S.E.M, $n= 7$ per group. Analysed by unpaired t test: ns, not significant.

5.3.5 Influence of 11β-HSD1$^{-/-}$ inhibition on neointimal proliferation

Mice treated with 11β-HSD1 inhibitor or vehicle underwent wire-induced injury of the left femoral artery, and ligation-induced injury of the right femoral artery (n= 7 per group). In both groups a similar extent of damage to the artery wall was caused by intra-luminal wire injury. In addition, breaks in the IEL were caused by ligation-induced injury in 4/ 14 animals.

Wire-induced femoral artery injury stimulated the development of small cellular neointimal lesions in mice treated with 11β-HSD1 inhibitor and in vehicle-treated controls (Figures 5.12a and b). Fourteen days after injury there was a trend (p= 0.15) towards a decrease in neointimal lesion area after wire-induced injury in mice treated with enzyme inhibitor when compared with vehicle-treated controls (Figure 5.13a) but this did not reach significance.

Ligation of the popliteal artery produced small neointimal lesions in mice treated with 11β-HSD1 inhibitor and vehicle treated controls (Figures 5.12c and d); these lesions were smaller than those induced by wire injury (Figure 5.13). Again, there was a trend (p= 0.13) towards a decrease in neointimal lesion area induced by ligation in mice treated with enzyme inhibitor when compared to vehicle-treated controls (Figure 5.13b) which did not reach significance.

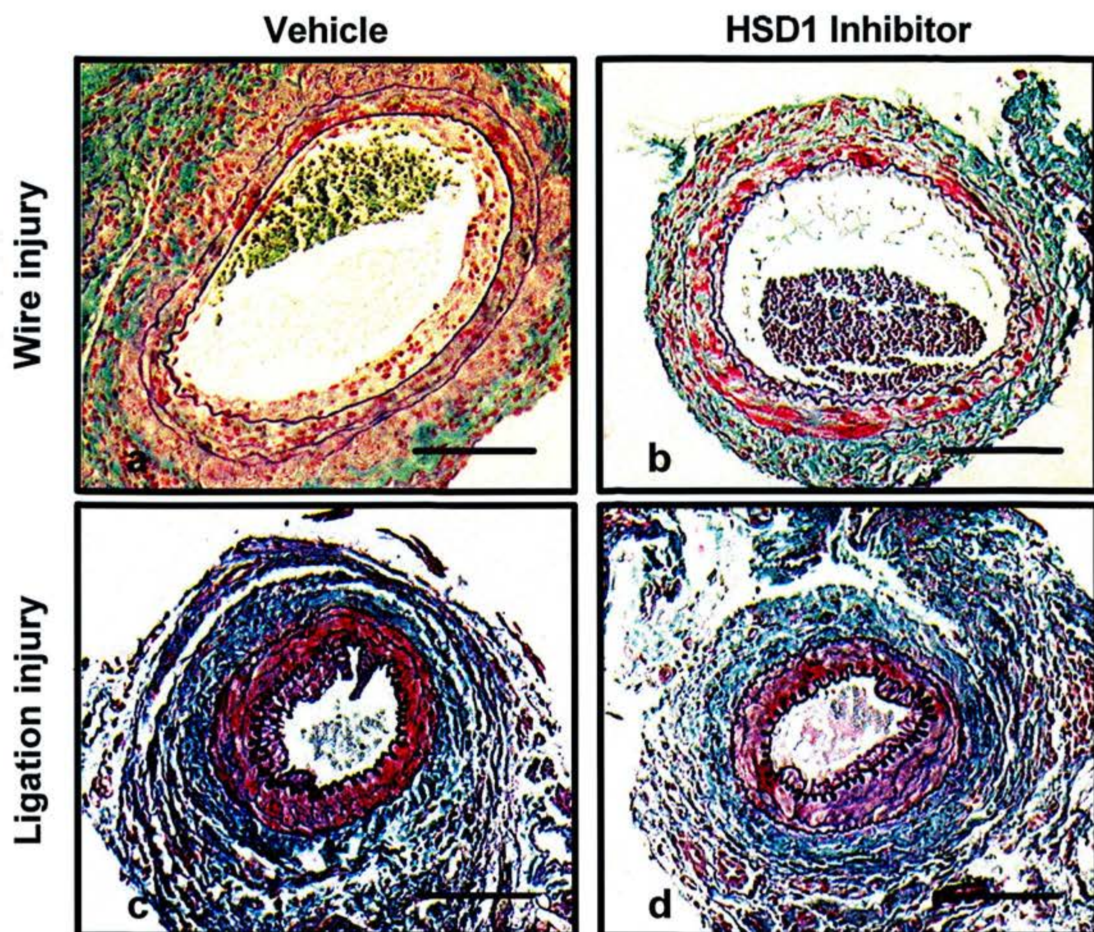


Figure 5.12: Neointimal proliferation after wire- or ligation-induced vascular injury in mice administered systemic 11β-HSD1 inhibitor or vehicle

Transverse sections of femoral arteries stained with the United States trichrome stain. Small cellular neointimal lesions developed 14 days after wire-induced injury in vehicle controls (a) and mice treated with 11β-HSD1 inhibitor (b). Neointimal proliferation also occurred in the femoral artery 14 days after ligation of the popliteal artery in vehicle controls (c) and mice treated with 11β-HSD1 inhibitor (d). Scale bar = 100 μm.

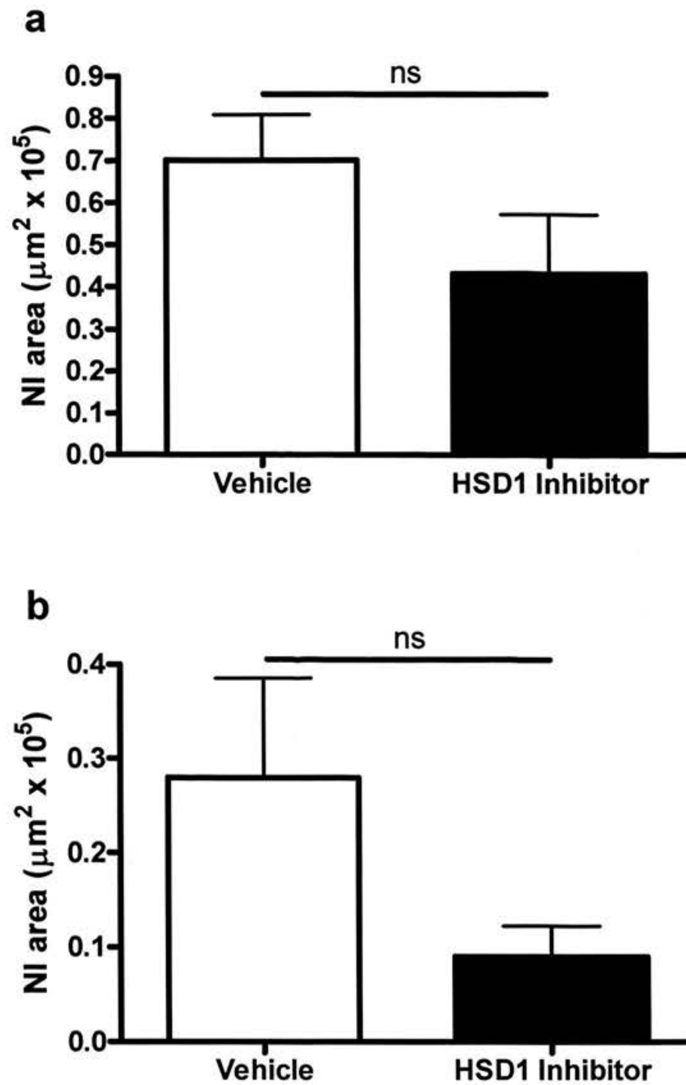


Figure 5.13: Effect of 11β-HSD1 inhibition on neointimal lesion area in the mouse femoral artery

Neointimal area was quantified by image analysis in wire-injured arteries from vehicle controls and mice treated with 11β-HSD1 inhibitor (a), and after ligation-induced injury in vehicle controls and mice treated with 11β-HSD1 inhibitor (b). After 14 days, there was no significant difference in the size of neointimal lesions induced by either type of injury between the groups, although there was a trend ($p= 0.15$ for wire injury, $p= 0.13$ for ligation injury) towards decreased lesion area in mice treated with enzyme inhibitor. Wire injury caused the growth of larger neointimal lesions than ligation injury. Data are mean ± S.E.M, $n= 7$ per group. Analysed by unpaired t test: ns, not significant.

5.4 Discussion

The experiments described in this chapter suggested that endogenous glucocorticoid action has only a small, if any, effect on neointimal proliferation after intra-luminal injury of the mouse femoral artery. However, there were trends for effects on lesion size which, if confirmed in further experiments, might imply a role for endogenous glucocorticoids which was beyond the limit of detection for these studies. There are also a number of methodological concerns to address.

Effects of antagonising endogenous glucocorticoid action

The GR antagonist RU38486 was applied via release from silastic pellets at a concentration of 33% w/w. This approach has been used previously to successfully alter angiogenesis in sub-cutaneous sponges (Small *et al.* 2005), indicating that biologically active concentrations of the drug are released from the pellet and do not exert systemic effects. The time-scale of the current experiment was 21 days; silastic pellets have been shown to release steroids at a constant rate *in vivo* for at least three weeks (Soro *et al.* 1997; Small *et al.* 2005). In the current study, pellets were placed next to the vessel wall, with the aim of administering RU38486 locally, thus avoiding systemic side effects. The observation that there was no difference in body weight or organ weights between animals implanted with a unilateral RU38486 pellet when compared to those implanted with bilateral vehicle pellets, indicates that RU38486 was being administered locally at the vessel wall without having systemic actions in the animals. This is supported by the fact that there was no difference in neointimal lesion area between contra-lateral vehicle control arteries and injured arteries from separate mice implanted with bilateral vehicle pellets, confirming that RU38486 did not have any systemic effects on neointimal proliferation.

If RU38486 were to act systemically, it would be expected to activate the HPA axis via central antagonism of GR and so increase corticosterone secretion. Indeed, clinical use of RU38486 increases plasma glucocorticoids in this way (Byrne *et al.* 2004), and RU38486 has also been shown to increase plasma corticosterone in animal studies (Wade *et al.* 1988). The fact that there was no significant difference in plasma corticosterone levels between the groups in the current experiment indicates that RU38486 did not have central actions on feedback to the HPA axis. However, morning corticosterone measurements in mice treated with RU38486 showed a trend

towards a *decrease*, suggesting some systemic effects. This decrease in corticosterone may be explained by reports that RU38486 inhibits steroidogenic enzymes in the adrenal gland to reduce steroid production (Albertson *et al.* 1994), or by a study showing that RU38486 may impair plasma corticosterone rises in response to repeated stress (Moldow *et al.* 2005). Alternatively, the trend towards decreased corticosterone may have been caused by local actions of RU38486, via antagonism of GR-mediated inflammatory cell signalling within the artery wall and, hence, inhibition of cytokine-mediated neuroendocrine activation of the HPA axis (reviewed in (Eskandari *et al.* 2003)) and attenuation of the stress response. Interestingly, the use of RU38486-releasing silastic pellets in mice significantly decreased plasma corticosterone in previous studies on angiogenesis (Small *et al.* unpublished observations).

In the models used in this chapter, neointimal proliferation occurs in response to an acute, localised injury to the vessel wall. It was proposed, therefore, that endogenous glucocorticoid activity within the artery wall would inhibit neointimal proliferation by reducing inflammation, proliferation and migration. Thus, local GR antagonism would be expected to increase neointimal lesion size. The demonstration that RU38486 produced a non-significant trend towards increased lesion size suggests that if endogenous glucocorticoids do play a role during this response, it is not extensive. However, the small increase seen may become physiologically significant in combination with other risk factors (such as hyperlipidemia or hypertension). A major limitation of the study was the inability to confirm that RU38486 was actually antagonising GR within the vascular wall. Although the trend towards decreased morning corticosterone levels (see above) indicates that RU38486 was active, this was not significant and it is not clear whether it was due to systemic or local effects of the antagonist. In the absence of an effect on lesion development, it is difficult to suggest appropriate positive controls for local administration and it is worth emphasising that use of this type of administration has been successful previously (Small *et al.* 2005).

RU38486 is also a progesterone receptor antagonist (Yamamoto *et al.* 1994). Progesterone attenuates the inhibitory effect of estrogens on neointimal proliferation of smooth muscle cells in the rabbit (Hanke *et al.* 1996) and the rat (Levine *et al.*

1996). However, it is unlikely that antagonism of progesterone receptors had any effects in the current study as male mice were used, which have only very low progesterone levels.

The current experiment investigated GR-mediated actions of endogenous glucocorticoids, and did not assess the possible influence of these hormones on neointimal proliferation via MR. Expression of MR has been detected in blood vessels (Takeda *et al.* 1997), localised to vascular smooth muscle and endothelial cells (Lombes *et al.* 1992). Its natural ligand, aldosterone, can be synthesised by vascular smooth muscle and endothelial cells (Hatakeyama *et al.* 1994; Takeda *et al.* 1996), and is known to be a positive regulator of vascular remodelling. Aldosterone enhances angiotensin II-induced VSMC proliferation (Ullian *et al.* 1993; Hatakeyama *et al.* 1994; Xiao *et al.* 2004) and has also been shown to increase vascular inflammation in the rat coronary artery (Rocha *et al.* 2002). In line with this, administration of aldosterone increased neointimal area 28 days after angioplasty of the pig coronary artery (Ward *et al.* 2001), whilst high plasma aldosterone levels in patients correlate with restenosis 6 months after coronary stent implantation (Amano *et al.* 2006). The MR antagonist eplerenone reduced neointimal formation after angioplasty (Ward *et al.* 2001) and stenting (Wakabayashi *et al.* 2006) in pig coronary arteries. Therefore, an alternative possibility is that endogenous glucocorticoids act through MR to influence neointimal proliferation. If so, it is likely that they would increase neointimal lesion formation, and that antagonism of MR would decrease lesion area. To further investigate this hypothesis, separate experiments administering eplerenone after wire-induced femoral artery injury in the mouse could be carried out.

Attenuation of endogenous glucocorticoid regeneration by transgenic deletion of 11β-HSD1

11β-HSD1$^{-/-}$ mice were studied to determine whether the abolition of endogenous glucocorticoid generation by 11β-HSD1 increases neointimal proliferation. It has been shown previously that 11β-HSD1$^{-/-}$ mice lack reductase activity *in vivo,* and fail to convert inert 11-dehydrocorticosterone to corticosterone (Kotelevtsev *et al.* 1997). These mice are resistant to the development of metabolic syndrome; they resist hyperglycemia induced by obesity or stress (Kotelevtsev *et al.* 1997), have a

cardio-protective lipid profile (Morton *et al.* 2001) and do not gain weight on a high fat diet (Morton *et al.* 2004).

A role for 11β-HSD1 in atherosclerotic lesion development has been indicated by the demonstration that a selective inhibitor of this enzyme decreases aortic lipid incorporation in atherosclerotic ($ApoE^{-/-}$) mice (Hermanowski-Vosatka *et al.* 2005). This work suggested that the observed decrease in atherogenesis was not solely the result of changes in plasma lipids, and implied a direct effect of enzyme inhibition on the arterial wall. However, it is unclear how 11β-HSD1 inhibition within the vasculature would protect against lesion development and, indeed, this hypothesis directly opposes the one proposed in the present studies. The trend towards increased neointimal lesion size in 11β-$HSD1^{-/-}$ mice observed in the current investigations was more consistent with the proposal that reduction of glucocorticoid action in the vessel wall would promote lesion development. It is possible that the time-point chosen to analyse lesion area in 11β-$HSD1^{-/-}$ mice (28 days after injury) was too late to observe any significant difference from C57Bl6 controls. However, since the goal of pharmacological manipulations is to reduce chronic arterial occlusion, the clinical benefit of a treatment that reduced lesion size early during development, but had no effect on final outcome, would be questionable. It is notable, however, that the data obtained in 11β-$HSD1^{-/-}$ mice are broadly consistent to those obtained with those for RU38486 and suggest, at best, a limited role for endogenous glucocorticoids in the regulation of neointimal lesion size.

Given the demonstrable ability of glucocorticoids to inhibit lesion development (chapter 4), and the observation that endogenous glucocorticoids regulate angiogenic remodelling of blood vessels (Small *et al.* 2005), it seems surprising that glucocorticoids do not influence neointimal lesion development. There are several possible explanations for the lack of significant effect of 11β-HSD1 deletion on neointimal proliferation in the current experiments. Although glucocorticoids inhibit proliferation of VSMCs *in vitro,* they also have actions that may increase the proliferation of these cells. For example, dexamethasone can cause up-regulation of endothelin-1 expression (Morin *et al.* 1998), a positive regulator of cell growth, and cortisol decreases the activity of NO (Mangos *et al.* 2000), a potent inhibitor of cellular proliferation. Dexamethasone and hydrocortisone have also been shown to

increase ACE activity in vascular cells (Mendelsohn *et al.* 1982; Fishel *et al.* 1995), which would enhance local generation of angiotensin II, and so stimulate VSMC proliferation. Therefore, although glucocorticoids inhibit proliferation *in vitro*, it is possible that in an *in vivo* setting they also act to stimulate this process. Thus, inhibiting their regeneration by transgenic deletion of 11β-HSD1 would decrease neointimal lesion area by this mechanism, counteracting any increase caused by reduced anti-inflammatory effects of endogenous glucocorticoids.

Currently, the influence of vascular 11β-HSD1 on the regulation of intra-vascular glucocorticoid levels *in vivo* is not clear. It is possible that, although pharmacological concentrations of glucocorticoids can influence neointimal proliferation, changes in endogenous glucocorticoid regeneration by 11β-HSD1 within the vascular wall are not sufficient to alter neointimal lesion development. Since previous experiments have shown that local application of glucocorticoids at the vessel wall is most beneficial for the reduction of neointimal proliferation (chapter 4), focussing on the function of 11β-HSD1 in the vasculature is an important next step. To dissect the role of local regeneration of endogenous glucocorticoids by 11β-HSD1 within the arterial wall, an enzyme inhibitor could be applied at this site via release from an implanted pellet. However, difficulties may be encountered in confirming inhibition of enzyme activity using this method. In the future, further development of an *in vitro* model of neointimal proliferation would be useful for this purpose. Transgenic models of tissue-specific 11β-HSD1 deletion in vascular smooth muscle or inflammatory cells would also be very interesting to use in this model.

Pharmacological inhibition of glucocorticoid regeneration by 11β-HSD1

As an extension of studies in 11β-HSD1$^{-/-}$ mice, an investigation into the effect of selective 11β-HSD1 inhibition on neointimal lesion formation was attempted. The inhibitor of 11β-HSD1 used in the current experiments has been used successfully to selectively inhibit 11β-HSD1 in mice (Hermanowski-Vosatka *et al.* 2005). The authors administered the inhibitor at a dose of 30mg/kg for 9 days by oral gavage, which lowered fasting glucose and improved insulin resistance in a mouse model of type 2 diabetes. Therefore, this dose and route of administration was chosen for use in the current studies. Hermanwoski-Vosatka *et al* (2005) also administered the inhibitor at 0.01% in a Western-style diet to ApoE knock out mice for 8 weeks, to

prevent the progression of atherosclerosis. The current experiments had a shorter time scale, and so administration of the inhibitor by this route may not have achieved an active pharmacological dose. Furthermore, this method of administration would have required separate housing of mice, daily preparation of diet and regulation of the amount eaten by each animal. For these practical reasons, this method of drug delivery was not used.

After 14 days of dosing, mice in both groups had lost weight, indicating that oral gavaging was not well tolerated by the animals. When 11β-HSD1 activity was measured in the liver, no difference was detected between mice administered inhibitor and those given vehicle alone. The fact that mice reacted badly to gavaging may suggest that drug administration by this route was not efficient in achieving an active dose in the animals. Alternatively, any inhibition of 11β-HSD1 may have been negated by an increased stress response in animals to twice-daily oral administration of the drug, via up-regulation of 11β-HSD1 activity induced by excess glucocorticoid levels. However, methodological reasons may also account for the lack of effect seen on enzyme activity. It is possible that the drug appeared to have no effect on liver 11β-HSD1 activity due to the timing of tissue collection at the end of the experiment, which was not carried out uniformly after drug administration in all animals. Hermanowski-Vosatka *et al* (2005) demonstrated the inhibition of 11β-HSD1 activity, and studied the pharmacodynamics of the inhibitor, in a group of animals separate from the disease models used in their study. The authors found that inhibition of 11β-HSD1 activity in the liver had nearly disappeared 6 hours after oral administration (Hermanowski-Vosatka *et al.* 2005). Therefore, enzyme inhibition may have been successful over the course of the current experiment, despite not being demonstrated in liver preparations. Furthermore, the fact that there was no difference in liver enzyme activity does not exclude the possibility that there were differences in 11β-HSD1 activity in other tissues, such as the vessel wall. The demonstration of an effect of the inhibitor on enzyme activity would necessitate the design of separate experiments, to recapitulate Hermanowski-Vosatka *et al* (2005). This would involve administration of the inhibitor to separate mice and the measurement of enzyme activity in different tissues at defined time-points after drug administration.

Whilst there was no significant difference in neointimal proliferation between mice treated with 11β-HSD1 inhibitor and vehicle controls, there was an apparent trend towards a decrease in lesion size. This result was contrary both to the hypothesis that inhibition of endogenous glucocorticoid regeneration would increase neointimal proliferation, and to the trend towards increased lesion size in 11β-HSD1$^{-/-}$ mice. These results are particularly interesting, given the ability of the 11β-HSD1 inhibitor to reduce atherosclerosis (Hermanowski-Vosatka *et al.* 2005) and may suggest enzyme-independent actions. It is notable that reduced atheroma in mice receiving the 11β-HSD1 inhibitor was attributed to a decrease in vascular expression of the inflammatory chemokine MCP-1 (Hermanowski-Vosatka *et al.* 2005). This process may be less significant in models of mechanical injury involving endothelial cell removal and also in the absence of a Western-style diet, which is itself an inflammatory stimulus.

Interpretation of the results in current experiments using the 11β-HSD1 inhibitor is made difficult by the fact that there were concerns over the efficiency of drug administration, the health of the mice during the experiment and the fact that no difference in enzyme activity was seen between the groups. Additionally, arteries were collected at an earlier time-point (14 days) during this study than in previous experiments. This was designed to allow detection of either an increase in lesion size, in line with our own hypothesis, or a reduction in lesion size, consistent with Hermanowski-Vosatka *et al* (2005). Furthermore, the method of drug administration by twice daily gavage, and the adverse reaction of mice to gavaging, limited the time-course of the study. However, analysis of arteries at 14 days involved the measurement of small and cellular neointimal lesions, meaning that lesion quantification may have been more unreliable and less sensitive than at later time-points. Overall, it appears likely that this study did not work as intended, due primarily to a lack of detectable reduction in 11β-HSD1 activity. Therefore, the absence of a significant effect in 11β-HSD1$^{-/-}$ mice on neointimal proliferation gives a more reliable indication that this enzyme does not play a major role in the vascular response to injury.

Conclusions

The experiments described in this chapter were designed to explore a novel area; the potential influence of endogenous glucocorticoids and their metabolism by 11β-HSD1 on the vascular response to injury. Initial observations have not shown that endogenous glucocorticoids significantly influence neointimal proliferation in response to wire-induced vascular injury in the mouse. In addition, glucocorticoid metabolism by 11β-HSD1 has not been shown to play a major role in the development of neointimal lesions after wire-induced injury of the femoral artery or ligation of the popliteal artery. With more time, and limitless resources, it would be interesting to pursue these preliminary results in more detail. The extension of this work in future studies may confirm a more subtle role for endogenous glucocorticoids during neointimal proliferation. Overall the current results indicate that, whilst manipulation of endogenous glucocorticoid action may not represent a therapeutic target for the treatment of restenosis, use of 11β-HSD1 inhibitors for treatment of the metabolic syndrome should not exacerbate neointimal lesion formation after PCI in these patients.

Chapter 6

General discussion

Cardiovascular disease, and in particular coronary heart disease, represents a major health burden in the U.K. Therefore, it is highly important to study the processes involved during the vascular response to injury, which are relevant to the development of both atherosclerotic and restenotic lesions. The work described in this thesis used a model of neointimal proliferation to assess the role of glucocorticoid hormones in regulating the vascular response to injury. The potential of glucocorticoid administration for the inhibition of atherosclerotic and neointimal lesions has been demonstrated in several animal models. Their potential as anti-restenotics has also been addressed in humans, with recent trials focussing on local application of glucocorticoids at the vessel wall via stent-mediated delivery. However, the action of glucocorticoids on the vascular response to injury is hard to predict, since there is evidence to suggest that endogenous glucocorticoids and tissue 11β-HSD activity contribute to the development of several cardiovascular risk factors, such as central obesity, hypertension, insulin resistance and dyslipidemia. Furthermore, whilst glucocorticoids can influence certain aspects of vascular structure and function (Hadoke *et al.* 2006), the role of endogenous glucocorticoids and 11β-HSD1 in modulating the neointimal response to vascular injury has not been investigated. Therefore, the overall objective of these studies was to advance the understanding of how glucocorticoids influence neointimal lesion formation, using a mouse model of intra-luminal vascular injury.

6.1 Models of neointimal proliferation in the mouse

Undertaking this project was dependent on introduction of an *in vivo* model of neointimal proliferation, to allow the effects of glucocorticoids on artery structure and function after injury to be assessed. A mouse model was chosen since use of this species allows study of the mechanisms of neointimal proliferation at a molecular level, through the use of genetically-modified animals. A well-characterised model of intra-luminal, wire-induced injury (Sata *et al.* 2000), which induced stretching and denudation of the femoral artery followed by time-dependent development of smooth muscle-rich neointimal lesions, was successfully introduced. Characteristics of the model, including thrombus formation, medial cell loss, and the vascular and inflammatory cell types involved in lesion development, were consistent with previous reports (Roque *et al.* 2000; Sata *et al.* 2000). However, the effect of wire

injury and lesion growth on endothelial cell function could not be assessed, as injury completely abolished contractile responses in the femoral artery, probably as a consequence of medial smooth muscle cell loss.

The demonstration that large neointimal lesions developed in sham-operated mice was unexpected, and led to a series of experiments designed to assess the effect of each stage of the operation on lesion growth. This approach demonstrated that placement of ligatures during surgery alone could induce neointimal proliferation. Temporary ligatures occasionally produced limited, focal lesion growth, but this was inconsistent. In contrast, the permanent proximal ligature on the popliteal artery consistently induced lesion growth in the femoral artery, although these were less occlusive than lesions produced by wire injury and did not extend as far along the artery. This finding was interesting, as it had not been reported previously by the groups who had developed models of wire injury in the femoral artery. It also gave added insight into the processes that contribute to neointimal formation after wire injury, an observation that was exploited to provide a complementary model of neointimal lesion growth: simply tying off the popliteal artery, close to its branch point with the femoral artery can induce neointimal proliferation in the femoral artery. This model does not involve endothelial cell denudation, and does not cause such severe stretch damage to the media as the model of wire-induced injury. However, it is likely to include more adventitial remodelling than intra-luminal models of injury, in response to the presence of the ligature around the artery, and involve changes in blood flow and shear stress, caused by constriction of the artery.

Therefore, the first aim of the project (to introduce an *in vivo* model of wire injury and neointimal proliferation) was achieved, and in addition a second model of lesion growth was developed. This allowed the use of these two complementary models of neointimal lesion development in each leg of the same animal, enabling extension of studies investigating the influence of 11β-HSD1 on the response to injury. In the future, the less severe model of injury to medial smooth muscle cells may allow the study of vascular function after neointimal lesion growth.

An additional aim during the model development was to introduce an *in vitro* model of neointimal proliferation. However, an attempt at recapitulating the method of

Guerin *et al* (2004) was not successful in inducing neointimal lesion growth in cultured femoral arteries. This may have been due to problems such as species differences, severity of vascular injury and culture conditions. It was decided that addressing these problems would entail a separate project, and that the current project should focus on use of the *in vivo* models that had already been developed successfully.

It has been proposed that local inflammation in the vessel wall may up-regulate 11β-HSD1 activity (Cai *et al.* 2001). The femoral artery injury model allowed investigation of the effect of vascular inflammation and VSMC proliferation induced by artery injury, and stimulation of injured arteries with an inflammatory cytokine, on 11β-HSD1 activity. However, enzyme activity was not altered by injury or IL-1β, consistent with previous results in intact vessels (Dover *et al.* 2007). Therefore, the up-regulation of endogenous glucocorticoid regeneration by 11β-HSD1 is unlikely to play a significant role in this setting.

6.2 Effects of glucocorticoid administration on the vascular response to injury

The *in vivo* mouse model of neointimal proliferation allowed investigation of the effects of exogenously-applied glucocorticoids on the vascular response to injury. Since previous animal studies have consistently shown that glucocorticoids inhibit neointimal proliferation, this experiment was designed to act as a positive control for glucocorticoid action, with the hypothesis that these hormones would also decrease lesion growth in this model. In addition, since human clinical trials have raised the possibility that there is a difference in the effect of glucocorticoids on the vascular response to injury depending on their route of application, the effects of systemically-applied and locally-applied glucocorticoids were compared in the mouse.

The effects of systemically-applied glucocorticoids on the vascular response to injury were more complex than initially expected, due to their actions on two different aspects of this process. Systemically-applied dexamethasone did inhibit smooth muscle cell proliferation and fibrous neointimal lesion growth after vascular injury, in line with previous reports in animal models. However, it also promoted the

formation of organised acellular lesions with a thrombotic origin, which occluded the lumen of injured vessels, leading to a similar extent of luminal narrowing as seen in vehicle-treated controls. In contrast, the release of cortisol locally at the vessel wall significantly decreased neointimal lesion growth, with no formation of organised thrombotic lesions. Therefore, this caused significant protection from luminal narrowing after wire-induced injury, in comparison to contra-lateral vehicle controls. In conclusion, this set of experiments provided proof of concept that glucocorticoid administration inhibits neointimal lesion growth in a mouse model of intra-luminal injury. In addition, they highlighted a potential difference in the effect of glucocorticoids depending on their route of application, although there may also have been differences in the action of the two glucocorticoids applied (discussed further below). With these caveats, the data support the idea that local release at the vessel wall reduces neointimal lesion growth, without causing adverse changes in systemic cardiovascular risk factors, i.e. increased thrombosis in response to vascular injury.

6.3 Influence of endogenous glucocorticoids and 11β-HSD1 on neointimal proliferation

Following the demonstration that locally-applied glucocorticoids inhibit neointimal lesion formation, the next aim of the project was to investigate the influence of endogenous glucocorticoids within the vessel wall. This was carried out by local application of the GR antagonist RU38486, via release from an implanted pellet. It was hypothesised that endogenous glucocorticoids acting at the vessel wall would also decrease neointimal proliferation after wire-induced vascular injury and, therefore, that antagonism of GR would increase lesion area. However, despite a trend towards increased lesion area, no significant difference was found between arteries treated with RU38486 and contra-lateral controls. In conclusion, the role of endogenous glucocorticoid action via GR-mediated signalling at the vessel wall was addressed, and the experiments carried out did not indicate that these hormones have a significant effect on neointimal lesion formation after artery injury.

The influence of endogenous glucocorticoid generation by 11β-HSD1 on neointimal proliferation was also investigated using 11β-HSD1$^{-/-}$ mice, and a selective 11β-HSD1 inhibitor. Previous studies have indicated that inhibition of 11β-HSD1 activity causes a decrease in atherosclerotic lesion formation (Hermanowski-Vosatka

et al. 2005; Nuotio-Antar *et al.* 2007); this may have been mediated by effects of enzyme inhibition at the vessel wall, or on systemic factors such as plasma lipid levels or blood pressure. However, in the current studies the model of vascular injury used involves a response to acute, focal injury of the vessel wall. Therefore, it was hypothesised that abolishing 11β-HSD1 activity would increase neointimal lesion area in this model, via the associated decrease in local active glucocorticoid generation. However, no significant changes in neointimal proliferation were seen in 11β-HSD1$^{-/-}$ animals or those administered an 11β-HSD1 inhibitor. In conclusion, these experiments did not indicate that the generation of endogenous glucocorticoids by 11β-HSD1 significantly influences the neointimal response in a model of acute injury to the vessel wall.

6.4 Potential clinical significance of results

Studies assessing the actions of exogenously-applied glucocorticoids on the vascular response to injury have implications for the clinical use of steroids in humans for the treatment of restenosis. Firstly, the observation that systemic pharmacological doses of glucocorticoid increased thrombosis after injury may provide an explanation for the failure of early clinical trials using systemic steroid administration to treat restenosis: an increase in thrombus formation after angioplasty or stenting may have disguised any decrease in restenosis caused by glucocorticoids. Alternatively, an increase in thrombosis may have stimulated restenotic lesion formation via the alternative mechanism that involves thrombus acting as a scaffold for smooth muscle cell proliferation and migration (Schwartz *et al.* 1992a). This model of neointimal lesion formation may involve additional pathways that glucocorticoids do not inhibit, such as interactions of inflammatory cells with platelets and fibrin. In addition, this observation provides more evidence to support the concept that increases in circulating glucocorticoids, seen in patients with Cushing's syndrome or those treated with exogenous steroids for inflammatory conditions, can have adverse effects on cardiovascular risk factors.

The decrease in neointimal lesion development and luminal narrowing after intra-luminal injury caused by the release of cortisol at the vessel wall also has clinical implications. This supports the concept that local application of glucocorticoids reduces neointimal proliferation during the vascular response to

injury, and that release of these hormones from coated stents represents the most effective way to deliver glucocorticoids for the treatment of restenosis.

The indication that endogenous glucocorticoids and 11β-HSD1 activity do not significantly influence neointimal proliferation after vascular injury is important with regard to the use of 11β-HSD1 inhibitors for treatment of the metabolic syndrome. Recent evidence suggests that increased tissue 11β-HSD1 activity, and so intracellular glucocorticoid levels, are associated with symptoms of the metabolic syndrome (Wake & Walker 2004; Walker & Andrew 2006). This has led to the proposal that intracellular glucocorticoids may contribute to the aetiology of this condition, and inhibitors of 11β-HSD1 have become an important novel therapeutic target for the treatment of its underlying cardiovascular risk factors (which include central obesity, hypertension, insulin resistance, dyslipidemia and atherosclerosis). However, concerns exist relating to the systemic inhibition of tissue 11β-HSD1 activity. A reduction in inflammatory cell glucocorticoid levels may have pro-inflammatory effects and delay the resolution of acute inflammatory responses (Gilmour *et al.* 2006), potentially leading to the development of chronic inflammatory conditions. In the case of vascular remodelling, pro-angiogenic effects observed in 11β-HSD1$^{-/-}$ mice may be beneficial in some situations, such as wound healing after myocardial infarction (Small *et al.* 2005). However, enhancement of angiogenesis via 11β-HSD1 inhibition would be detrimental in other pathological conditions, such as cancer or diabetic retinopathy. 11β-HSD1 inhibition has been shown to delay the progression of atherosclerosis in mice (Hermanowski-Vosatka *et al.* 2005). Since the results of the current studies suggest that attenuation of 11β-HSD1 activity will not cause a worsening of the restenotic response, the potential use of 11β-HSD1 inhibitors for the treatment of atherosclerosis should not be contraindicated in patients undergoing PCI for the treatment of this condition.

6.5 Future studies

As well as highlighting important aspects regarding the effects of glucocorticoids on the vascular response to injury, the work described in these chapters has also provided a basis for further experiments. The introduction of a mouse model of neointimal proliferation will allow the study of many other potential factors and

pathways in the vascular response to injury. In addition, there are several prospective studies that could extend the work described here.

6.5.1 Systemic vs. local effects of glucocorticoids

Whilst the current studies showed a clear difference in the effect of systemically-applied and locally-applied glucocorticoids on the vascular response to injury, these experiments were carried out using different steroids (dexamethasone systemically and cortisol locally), for the reasons described in chapter 4. This raises the possibility that differences in the action and/ or metabolism of these two steroids may have contributed to the difference in effect observed. To overcome this limitation, experiments could be set up giving systemic doses of cortisol and administering dexamethasone locally from silastic pellets after vascular injury. To allow a direct comparison, the dose of glucocorticoid reaching the vessel wall, and therefore intra-vascular GR exposure, would have to be matched. However, it would be difficult to design experiments where this could be carried out accurately. In addition, it would be interesting to carry out dose-response experiments to determine if, for example, the increase in thrombosis caused by dexamethasone could be avoided with a lower dose of this glucocorticoid. Unfortunately, it was not possible to carry out these further experiments during the time-scale of the current project.

6.5.2 Mechanism of glucocorticoid-mediated inhibition of neointimal proliferation

In the future, it will be important to determine the cellular mechanism by which exogenously-applied glucocorticoids inhibit neointimal proliferation in this model. A clear understanding of whether these hormones target inflammatory cells, inhibit smooth muscle cell proliferation, or both, will aid their development as anti-restenotic drugs. Since it seems most likely that glucocorticoids act by inhibiting the early inflammation after vascular injury (Poon *et al.* 2001) experiments to investigate this hypothesis are an important next step. Quantification of neointimal lesion macrophage content (e.g. via immunohistochemical staining for Mac-2, chapter 2.8.3) in glucocorticoid- and vehicle-treated arteries from the current experiments could be compared. However, these arteries were collected 21 days after injury, when only limited Mac2 expression is observed in advanced neointimal lesions (chapter 3.2.1.8). Testing the ability of glucocorticoids to inhibit the adhesion

of inflammatory cells to the vessel wall at early time-points (e.g. immediately or 2 days) after injury would be more appropriate. It is possible to measure the adhesion of radiolabelled leukocytes to injured arteries *ex vivo* (Kennedy *et al.* 2000b) and, therefore, the influence of glucocorticoids on this process could be investigated. Alternatively, the expression of adhesion factors in the presence and absence of glucocorticoids could be assessed in injured arteries, either by immunohistochemistry or using molecular techniques, such as quantitative PCR or Western blotting, which would allow quantification of expression levels. As well as the adhesion of leukocytes after injury, the release of inflammatory chemokines and cytokines is also important in stimulating neointimal lesion formation. Again, the ability of glucocorticoids to inhibit this process could be assessed, by measuring chemokine and cytokine levels released from injured arteries cultured *in vitro* with or without glucocorticoids.

Glucocorticoids may also directly inhibit the proliferation of VSMCs after injury, leading to the inhibition of neointimal lesion development. Previous *in vitro* studies have used high concentrations of glucocorticoids to demonstrate the inhibition of smooth muscle cell growth, raising questions as to whether they have the same effect *in vivo*. To determine if the *in vivo* proliferation of VSMCs is altered in animals treated with glucocorticoid, sections of arteries from the current experiments could be stained for PCNA (chapter 2.8.4). However, maximal VSMC proliferation does not occur in the advanced lesions studied in these experiments (chapter 3.2.1.8). Alternatively, injection of the thymidine analogue BrdU would provide a nuclear marker of proliferating cells in sections of injured arteries collected at earlier time-points after injury (e.g. 14 days), when most cellular proliferation occurs. Finally, the possibility that glucocorticoids may influence ECM remodelling during the vascular response to injury could be addressed, by measuring the activity of the MMP enzymes using the technique of zymography.

6.5.3 Glucocorticoid receptor vs. mineralocorticoid receptor stimulation by endogenous glucocorticoids

In the current studies a GR antagonist was used to determine the effect of endogenous glucocorticoids on neointimal proliferation. It is possible that endogenous glucocorticoids, and exogenously-applied cortisol, may also act through

MR to influence the development of neointimal lesions. If they do act in this way, previous studies suggest that they would increase neointimal lesion formation, and that antagonism of MR would decrease lesion area (Ward *et al.* 2001; Wakabayashi *et al.* 2006). To investigate this further, a separate project could be carried out using a pharmacological approach to confirm the role of MR in neointimal proliferation in the mouse. Firstly, the MR ligand aldosterone could be administered to animals after vascular injury, to validate the idea that MR agonism increases lesion area. Next, an MR antagonist, such as eplerenone, could be used to confirm the influence of MR on neointimal proliferation.

6.5.4 Tissue-specific study of 11β-HSD1 activity during neointimal lesion development

The studies described here investigated the effect of attenuating whole-body 11β-HSD1 activity on neointimal lesion growth; at present the relative activity of intra-vascular 11β-HSD1 remains unknown. Experiments using exogenously-applied glucocorticoids indicated that glucocorticoids reduce lesion development when they are applied locally at the vessel wall, but that this can be offset by systemic glucocorticoids causing adverse changes in cardiovascular risk factors. It has been suggested that an observed delay in the progression of atherosclerosis after 11β-HSD1 inhibition was mediated by direct effects on the vessel wall, perhaps via decreased expression of MCP-1 (Hermanowski-Vosatka *et al.* 2005). Therefore, it would be interesting to start to determine the role of local 11β-HSD1 activity in the cells of the vessel wall. To do this, several approaches could be taken. Firstly, an inhibitor of 11β-HSD1 could be applied locally at the vessel wall, via release from a sub-cutaneous minipump or implanted pellet. However, difficulties may be encountered in confirming if active drug administration has been successful using this method. Secondly, further development of the *in vitro* model of neointimal proliferation would allow study of glucocorticoid and 11β-HSD1 effects specifically on the cells of the vessel wall. Finally, development of transgenic tissue-specific models of 11β-HSD1 knockout in vascular smooth muscle and macrophages would allow the dissection of this enzyme's role in each of these cells during neointimal proliferation. Alternatively, bone marrow transfer from 11β-HSD1$^{-/-}$ mice into wild type animals would achieve attenuation of 11β-HSD1 activity specifically in

circulating leukocytes. This would also allow further investigation of the mechanisms involved in glucocorticoid-mediated changes in neointimal proliferation, and whether this involves inflammatory cells or VSMCs.

As a result of studies suggesting a role for 11β-HSD1 in atherosclerotic lesion development (Hermanowski-Vosatka *et al.* 2005), 11β-HSD1/ ApoE double knockout mice have been generated recently, to further clarify the role of this enzyme in atherosclerosis. Performing wire-induced vascular injury in vessels from these mice with existing atherosclerotic lesions would be a very interesting experiment to carry out in the future. This would provide a more clinically relevant model of neointimal lesion formation in the setting of existing atherosclerotic disease, and give an indication of the role that 11β-HSD1 plays in vascular remodelling in this context.

6.6 Conclusions

In conclusion, these studies have provided valuable insight into the effects of glucocorticoids on neointimal lesion formation. The role of endogenous glucocorticoids, and their metabolism by 11β-HSD1, during neointimal lesion development was investigated for the first time. In addition, these studies have highlighted that the influence of glucocorticoids on neointimal proliferation depends on a balance between local and systemic actions. The neointimal proliferation of VSMCs in response to inflammation during the vascular response to injury is an important process involved in the formation of both atherosclerotic and restenotic vascular lesions. Therefore, results obtained in the current studies, using a model of neointimal proliferation, have relevance to the development of atherosclerosis and restenosis. For example, the idea that the action of glucocorticoid hormones on vascular remodelling is a balance between local and systemic effects can be extended to investigation of their effects on atherosclerosis. In addition, further clarification of the mechanism of glucocorticoid-mediated inhibition of neointimal lesion formation may shed light on how these hormones influence restenosis in clinical trials. In the future, studies should take into account the ability of intra-vascular glucocorticoids to interact with both GR and MR in cardiovascular tissues, and the contribution of 11β-HSD1 in different cell types during vascular lesion formation.

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Appendix

Publications

Email to editor of Cellular and Molecular Life Sciences

Dear Sir/ Madam,

I am an author on a review published in Cellular and Molecular Life Sciences on January 16th 2006.

Title: "Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function".

Volume: 63. Pages: 565-578.

I would like to include a copy of this article in my PhD thesis, and would be grateful if you could supply me with an email confirming that this is acceptable.

Yours faithfully,

Linsay Macdonald

Response by email

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Best regards,

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Email to editor of Endocrinology

Dear Sir/ Madam,

I am an author on an article published in Endocrinology on September 28th 2006.

Title: "Intravascular glucocorticoid metabolism during inflammation and injury in mice".

Volume: 148(1). Pages: 166-172.

I would like to include a copy of this article in my PhD thesis, and would be grateful if you could supply me with an email confirming that this is acceptable.

Yours faithfully,

Linsay Macdonald

Response by email

Form was submitted by: linsay.macdonald@ed.ac.uk

Original author to publish in a Dissertation.

Journal: Endocrinology
Author Name: A Dover
Title: Intravascular glucocorticoid metabolism during inflammation and injury in mice
Year: 2006
Volume: 148
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Cellular and Molecular Life Sciences

Review

Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function

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Abstract. The ability of glucocorticoids to directly alter arterial function, structure and the inflammatory response to vascular injury may contribute to their well-established link with the development of cardiovascular disease. Recent studies have emphasised the importance of tissue-specific regulation of glucocorticoid availability by the 11β-hydroxysteroid dehydrogenase (11HSD) isozymes, which inter-convert active glucocorticoids and their inactive metabolites. The expression of both type 1 and type 2 11HSDs in the arterial wall suggests that pre-receptor metabolism of glucocorticoids may have a direct impact on vascular physiology. Indeed there is evidence that 11HSDs influence glucocorticoid-mediated changes in vascular contractility, vascular structure, the inflammatory response to injury and the growth of new blood vessels. Hence, inhibition of 11HSD isozymes may provide a novel therapeutic target in vascular disease.

Key words. 11β-Hydroxysteroid dehydrogenase; inflammation; vascular contractility; angiogenesis; cardiovascular disease.

Introduction

There is increasing evidence that direct interaction of glucocorticoids with the vascular wall [1, 2] contributes to their association with increased risk of cardiovascular disease [3, 4]. Certainly, glucocorticoids can interact both with endothelial (EC) and with vascular smooth muscle (VSMC) cells, and furthermore, glucocorticoid-mediated enhancement of vascular contractility has been implicated in the development of hypertension [5]. In addition, glucocorticoids may directly modify new blood vessel formation and vascular lesion development by inhibiting inflammation, proliferation and angiogenic pathways in the arterial wall [6, 7].

Interaction of glucocorticoids with the vasculature is unlikely to be regulated solely by circulating concentrations of these steroids; pre-receptor metabolism within target tissues also has a profound influence on glucocorticoid activity. Such tissue-specific modulation of glucocorticoid activity, regulated by the isozymes of 11β-hydroxysteroid dehydrogenase (11HSD) [8], has a key role, for example, in the development of hypertension, obesity and the metabolic syndrome [9–11]. It is likely that pre-receptor metabolism of glucocorticoids influences steroid action within the vessel wall since both isozymes of 11HSD are expressed in vascular cells [12]. This article reviews the current evidence that vascular 11HSD expression influences glucocorticoid-mediated changes in vascular growth, function, structure and the inflammatory response to vascular injury.

Glucocorticoid signalling in the vascular wall

Glucocorticoids (cortisol in man, corticosterone in rodents) are predominantly synthesised in, and released

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from, the adrenal cortex. Circulating concentrations of these steroids are under the control of the hypothalamic-pituitary-adrenal (HPA) axis, whilst their bioavailability is regulated by interaction with corticosteroid-binding globulin (CBG) and albumin in the plasma. The small proportion of unbound, circulating hormone is able to cross the cell wall and interact with corticosteroid receptors. Classically, glucocorticoids interact with the cytosolic glucocorticoid receptor (GR, or corticosteroid receptor type II). As described below, glucocorticoids may also activate mineralocorticoid receptors (MR, or corticosteroid receptor type I), but this occurs only in a few tissues. GR and MR are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors [13]. Activation of GR results in binding of receptor homodimers to glucocorticoid response elements in target genes, leading to initiation or repression of transcription. There is also increasing evidence that glucocorticoids exert specific, non-genomic actions. Examples exist of rapid glucocorticoid-induced changes to phospholipase A_2 (PLA_2) and phosphoinositide-3-kinase-mediated endothelial nitric-oxide synthase (eNOS) release that are blocked by GR antagonism but not by inhibition of transcription [14, 15]. These non-genomic effects are thought to be mediated by membrane-bound GR [16] (mGR; although the specific signalling pathways associated with these receptors have not been established) or by cytosolic GR (cGR) without requirement for either nuclear translocation or effects on transcription. In the latter case, chaperones or co-chaperones (such as Src) act as signalling components and, therefore, mediators of glucocorticoid-induced effects [17].

Corticosteroid receptors are present in the cells of the vascular wall, supporting the idea that glucocorticoids interact directly with the vasculature. Cytosolic MR and GR have both been demonstrated in freshly isolated vessels [18, 19] and in cultured vascular cells (VSMCs [20, 21] and ECs [22–26]) from a variety of species. The distribution of these receptors may vary with vascular territory, as MR were detected in rabbit aortic and pulmonary VSMCs but not in small arteries [27]. Vascular GR are known to be active as antagonism (with RU38486) blocked dexamethasone-mediated induction of ACE activity in rat aortic ECs [28]. Similarly, activity of MR is demonstrated by their contribution to angiotensin II-induced hypertrophy of VSMCs [29] and aldosterone-induced swelling of ECs [22]. It has not been established whether membrane binding sites for corticosteroids are present, or have a role, in the vascular wall.

The downstream effects of GR activation within the arterial wall, and their influence on cardiovascular risk factors (such as hypertension), are imperfectly understood [5]. Glucocorticoids are essential for maintenance of blood pressure in healthy individuals [1], whilst their ability to increase peripheral vascular resistance in animals devoid of renal mass indicates that a non-renal mechanism contributes to glucocorticoid-induced hypertension [30]. A considerable body of evidence suggests that this non-renal mechanism may involve direct glucocorticoid-mediated alteration of EC and VSMC function [1]. Consequently, regulation of glucocorticoid availability by 11HSDs within the vascular wall may be an important influence on cardiovascular physiology and pathology.

Tissue-specific metabolism of glucocorticoids by 11*β*-hydroxysteroid dehydrogenases

The 11HSDs, microsomal enzymes of the short-chain alcohol dehydrogenase superfamily [8], interconvert active glucocorticoids and their inert 11-keto forms [31]. Two isozymes, 11HSD1 and 11HSD2, have been identified: 11HSD1 is a low-affinity NADP(H)-dependent, predominant reductase *in vivo*. Dehydrogenase activity of this isozyme is generally not seen in intact cells or organs (including liver [32–34], adipose tissue [35], neurons [36] and vascular smooth muscle [37]); early suggestions of 11HSD1 dehydrogenase activity in vascular smooth muscle [38] are probably attributable to 11HSD2 [37]. 11HSD1 dehydrogenase activity observed in some preparations *in vitro* [39] is probably attributable to release of enzyme from damaged or dying cells, with dissociation from hexose-6-phosphate dehydrogenase, which is thought to maintain the high NADPH concentrations required for reductase activity [40]. 11HSD1 has a Km in the micromolar range for both cortisol and corticosterone [41] and is widely expressed in glucocorticoid-target tissues (including liver, lung, adipose tissue, brain, vascular smooth muscle, skeletal muscle, anterior pituitary, gonads and adrenal cortex [8]), where its role is to amplify local glucocorticoid concentrations [42]. Regulatory control of 11HSD1 is complex, with its synthesis and activity influenced by a variety of factors (such as glucocorticoids [43–45], stress [46, 47], sex steroids [48], growth hormone [49], cytokines [50] and peroxisome proliferator-activated receptor agonists [8]) and its activity driven in the reductase direction through local generation of NADPH by hexose-6-phosphate dehydrogenase [51]. Other factors that may drive 11HSD1 activity in the reductase direction include the cellular environment, cofactor availability, redox potential and substrate concentration.

11HSD2, by contrast, is a high-affinity NAD-dependent, exclusive dehydrogenase, which converts active glucocorticoids into inactive 11-ketosteroids and has a Km for cortisol and corticosterone in the nanomolar range. It is found primarily in mineralocorticoid target tissues, such as the kidney, sweat glands, salivary glands and colon 8], where it is constitutively active and serves to protect MR

from illicit occupation by glucocorticoids. Inhibition of 11HSD2 with liquorice or its derivatives results in glucocorticoid-dependent 'apparent' mineralocorticoid excess and hypertension [52]. Similarly, transgenic disruption of 11HSD2 [9] in mice, or congenital deficiency in man [53], recapitulates the major features of the syndrome of apparent mineralocorticoid excess (SAME). The importance of 11HSD2 in SAME was demonstrated by the description of a defect in cortisol metabolism in children with this syndrome [54]; this was later shown to be the result of mutations in the 11HSD2 gene [55, 56]. 11HSD2 is also expressed in tissues which are not classic MR targets, including the lung, lymph nodes, heart, blood vessel wall and placenta) [57–59]. In the placenta 11HSD2 acts to protect the foetus from excessive exposure to maternal glucocorticoids [60, 61], whereas cardiac 11HSD2 activity may have a role in preventing fibrosis resulting from stimulation of MR by glucocorticoids [62].
The influence of 11HSD isozyme activity on cardiovascular physiology and pathophysiology is well recognised (see Krozowski and Chai for review [63]), but details of the role of 11HSDs within the vessel wall have emerged only recently and remain somewhat uncertain.

Intra-vascular glucocorticoid metabolism

Both isozymes of 11HSD are expressed in the blood vessel wall, suggesting that they could influence vascular function by regulating local availability of active glucocorticoids [1, 64]. The cellular distribution of vascular 11HSD1 and 11HSD2 is not completely clear. Our studies using mouse and rat aorta suggest that 11HSD2 is localised to ECs, whereas 11HSD1 is predominantly in the VSMC (fig. 1) [18, 65]. Others, in contrast, have reported activity of both enzymes in the VSMC [37, 50] and also in the EC [66], it should be noted that the latter investigation [66] demonstrated only 11HSD1 in rat VSMC and indicated that 11HSD1 was the predominant isozyme in the endothelium. Direct comparison of studies is often difficult, given the use of arteries from different species and anatomical locations combined with a variety of techniques for detecting 11HSDs. The balance of the literature suggests that cellular distribution of 11HSD isozymes differs in vessels from distinct anatomical locations and that 11HSD activity increases as artery diameter diminishes; in the rat 11HSD, activity was greater in resistance (mesenteric) arteries than in conduit vessels (aorta) [65] and in the mouse 11*β*-reductase activity was higher in iliofemoral arteries than in the aorta [A. R. Dover et al., unpublished data]. These variations in cellular distribution and activity suggest that the role of intravascular glucocorticoid metabolism is not the same in all blood vessels.
There is increasing evidence that interconversion of active and inactive glucocorticoids by vascular cells may influence glucocorticoid-mediated modulation of vascular function, structure, growth and inflammation.

Glucocorticoids, 11HSDs and vascular function

Although it is well established that glucocorticoids contribute to maintenance of vascular tone *in vivo*, the mechanisms have been difficult to establish. A variety of interactions contribute to homeostasis, including glucocorticoid-mediated regulation of cardiac output and fluid and electrolyte balance, with salt and water handling modulated both directly [67] and indirectly by influences on the production of angiotensinogen (liver), arginine vasopressin (AVP; hypothalamus) [68] and atrial natriuretic peptide (ANP; cardiac myocytes) [69]. It is apparent, however, that these cardiac and renal effects cannot account totally for the glucocorticoid-mediated increase in blood pressure, and there is evidence that a component of hypertension arises from enhanced contractility of the vascular wall [70–72]. For example, reversal of adrenocorticotophin-dependent hypertension by administration of l-arginine (the substrate for nitric oxide synthase) suggests that nitric oxide deficiency contributes to the elevation of blood pressure [73, 74].
Glucocorticoid-dependent potentiation of noradrenaline- and angiotensin II-mediated vasoconstriction has been at-

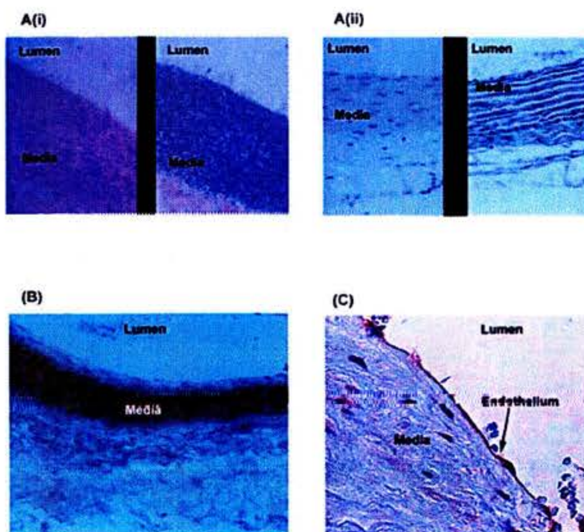

Figure 1. Presence and distribution of 11HSD isozymes in the vascular wall. *In situ* hybridisation (*Ai*) and immunohistochemistry (*Aii*) confirming both expression and activity of 11HSD1 within the rat aortic wall; the enzyme was predominantly localised to medial smooth muscle cells (left-hand panel, sense/control; right hand-panel, antisense/antibody to 11HSD1). Immunohistochemistry demonstrating (*B*) 11HSD1 in rat mesenteric artery smooth muscle and (*C*) 11HSD2 in human intra-renal artery endothelium [unpublished]. Reproduced from [65] with permission. © The Endocrine Society, 1991.

tributed to alterations within the VSMC and the EC (reviewed in Walker and Williams [2] and Ullian [1]). Given that glucocorticoids can act on both MR and GR, the increased contractility observed in many studies may be secondary to increased stimulation of either receptor. Alterations identified within the VSMC (including upregulation of contractile receptors, altered intracellular second messenger activation and modulation of the activity and synthesis of vasoactive substances) result in a direct enhancement of contraction [1]. In contrast, changes in the endothelium can increase contractility in two distinct ways: by increased release of vasoconstrictor compounds (e.g. angiotensin II, endothelin-1 [75, 76]) from the ECs and by impaired endothelium-mediated relaxation. Loss of endothelium-mediated relaxation [77], caused by impaired activity of vasodilators (e.g. prostaglandins, nitric oxide) [78–80], [reduces the ability of the endothelium to modulate contraction.

An alternative mechanism through which glucocorticoids may regulate vascular function is 'foetal programming' of physiological responses [81]. Exposure of the foetus to excess maternal glucocorticoid (either by direct infusion or by inhibition of placental 11HSD2) causes reduced birth weight [82], an outcome associated with increased risk of cardiovascular and metabolic disease in adulthood [83]. Two major causes of low birth weight, maternal dietary restriction and maternal stress, may also be glucocorticoid-dependent [84, 85]. In the ovine foetus, glucocorticoid infusion elevates blood pressure and alters vascular contractility in foetal sheep [86]; this may be significant, as one outcome of foetal programming is elevated blood pressure in adult offspring [87]. However, although enhanced vascular contractility has been demonstrated in rats with programmed hypertension, it is not clear whether this contributes to the elevation of blood pressure [88, 89]. Furthermore, the mechanisms through which pre-natal exposure to excess glucocorticoid programme enhance contractility in adult offspring have not been established.

Influence of 11HSDs on vascular function

In SAME, 11HSD2 deficiency results in sodium retention and severe hypertension, mediated in part by glucocorticoid-dependent activation of MR in the distal nephron [90]. There is, however, a considerable literature to suggest that changes in 11HSD activity within the vascular wall also contribute to elevation of blood pressure. A clear example of this is the demonstration that 11HSD activity is impaired in arteries taken from rat models of hypertension [91–93]. A role for altered vascular function is supported by reports that 11HSD inhibition (with glycyrrhetinic acid) in rats produced an elevation of blood pressure which, whilst mediated by MR activation, was blocked by antagonists of the endothelin-1 system [94, 95]. Moreover, studies of dermal vasoconstriction in patients exposed to liquorice, and in a single individual with SAME [53, 96], demonstrated enhanced cortisol-mediated constriction (fig. 2). The possibility that this is due to changes in glucocorticoid metabolism within the vascular wall, rather than indirect systemic effects of sodium retention, gained further credence with *in vitro* studies which showed that bile acids (e.g. chenodeoxycholic acid), which are endogenous inhibitors of 11HSD [97], pharmacological inhibitors of 11HSD (carbenoxolone, glycyrrhetinic acid) [98, 99] and isozyme selective antisense oligonucleotides [100] alter corticosterone-mediated enhancement of vasoconstriction. Furthermore, 11HSD inhibition (with glycyrrhetinic acid) augmented corticosterone-induced dysfunction in cultured human ECs, indicating both a role for intra-cellular 11HSD and independence from blood pressure elevation *in vivo* [95]. Care is required in interpreting these results, however, as some 11HSD inhibitors can directly alter contractile function by damaging the endothelium [101].

These pharmacological studies have been extended by the use of arteries from 11HSD knockout mice. Aortic function (and blood pressure) are unaltered in 11HSD1$^{-/-}$ mice suggesting that intravascular regeneration of active glucocorticoids has no effect on vascular contractility [102, 103]. This indicates that despite the ability of glucocorticoids to enhance vascular contraction, impaired corticosterone generation in the arterial wall does not re-

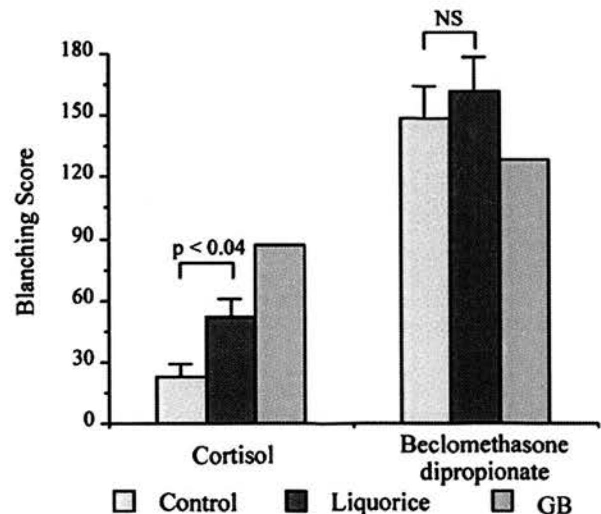

Figure 2. The effect of congenital and acquired 11HSD deficiency on dermal vasoconstrictor sensitivity to cortisol and beclomethasone dipropionate. Inhibition of 11HSD activity with liquorice-enhanced dermal vasoconstriction (measured by skin blanching) in response to cortisol but not to beclomethasone diproprionate. A similar result was obtained in a patient (GB) with the syndrome of apparent mineralocorticoid excess type 1 (11HSD2 deficiency). These data indicate that local regulation of glucocorticoid activity in the vascular wall contributes to contractile tone. Bars are s.e. NS, not significant. Reproduced from [53] with permission. © The Biochemical Society, 1992.

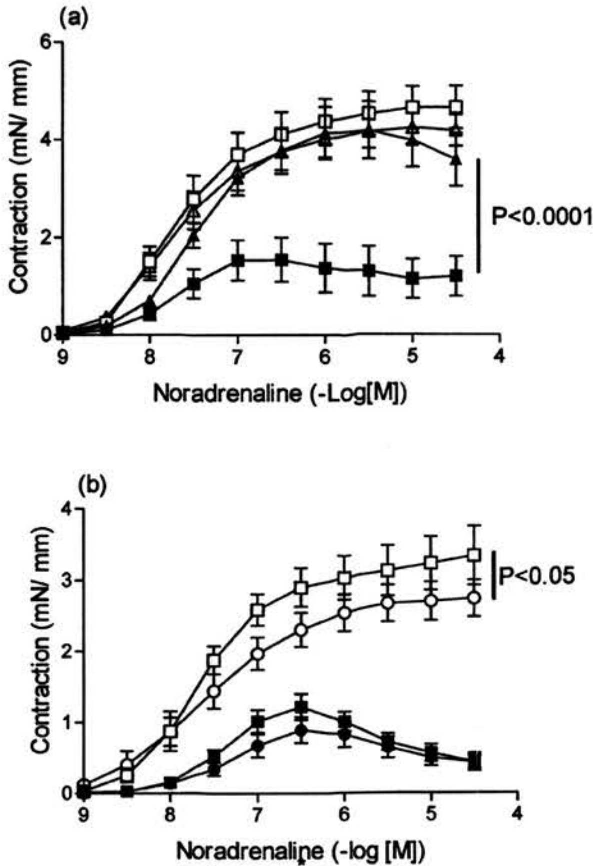

Figure 3. Effect of (*a*) transgenic deletion of 11HSD2 and (*b*) exposure to glucocorticoids on mouse aortic endothelial cell function. In aortic rings isolated from control mice (squares), release of endothelium-derived nitric oxide acts as a physiological antagonist of noradrenaline-mediated contraction; thus, removal of the endothelium (open symbols) results in enhanced contraction. In aortae from $11HSD2^{-/-}$ mice (*a*; triangles) this ability of the endothelium to modulate contraction has been lost, suggesting glucocorticoid-mediated impairment of endothelial cell function. This is contested, however, by the demonstration that (*b*) *in vitro* incubation (24 h, 37 °C; 10^{-7} M corticosterone) of aortic rings from control animals with glucocorticoids (circles) did not produce a similar endothelial cell dysfunction. Values are mean ± s.e. mean; n=8. Adapted with permission from [103] and [18]. © Lippincott, Williams and Wilkins.

duce contractile function. In contrast, enhanced contractility was demonstrated in thoracic aortae from mice lacking 11HSD2 as a result of impaired endothelium-derived NO activity [103], rather than changes in the VSMC (fig. 3a). This suggests that 11HSD2 activity in the endothelium may serve to protect endothelium-dependent relaxation from the detrimental effects of glucocorticoids. This appears to be consistent with *in vivo* studies which suggest that non-selective inhibitors of both isozymes of 11HSDs, such as liquorice derivatives, potentiate rather than impair the vascular actions of glucocorticoids, suggesting inhibition of inactivation rather than reactivation of steroids within the vessel wall. More recent data, however, suggest that the concept of protection of ECs by 11HSD2 may be an oversimplification. In mouse aortic rings, we could not induce EC dysfunction by incubating isolated rings with glucocorticoids (fig. 3b), even in the absence of 11HSD2 [18]; whether dehydrogenase activity of 11HSD1 [38] served to protect the endothelium is not clear, although our data suggest that 11HSD1 does not act as a dehydrogenase in intact arteries. This raises the possibility that *in vivo* differences in vascular function in 11HSD2 knockout mice are dependent on indirect mechanisms, e.g. related to hypertension or sodium retention. Cell-specific manipulation of 11HSDs would be the most attractive means to dissect this biology further, but has yet to be reported for vascular cells.

Glucocorticoids, 11HSDs and vascular inflammation

Whereas studies in transgenic mice have suggested that 11HSD2 activity may influence vascular function, whilst 11HSD1 does not, a much clearer role for 11HSD1 has been identified in regulation of vascular inflammation. The anti-inflammatory and immunosuppressive effects of glucocorticoids, which account for their most common therapeutic applications, are due to GR-mediated interactions with blood vessels, inflammatory cells and mediators of inflammatory responses [104]. For example, glucocorticoids decrease expression of adhesion factors, cytokines and chemokines, and so alter the recruitment of immune cells such as neutrophils and macrophages to sites of inflammation. This also results in a decrease in leukocyte activation and proliferation. Furthermore, the glucocorticoid receptor mediates lymphocyte apoptosis [105] and suppresses the synthesis of inflammatory mediators (e.g. prostanoids), and hydrocortisone stimulates the synthesis of anti-inflammatory mediators (e.g. lipocortins) [106]. Glucocorticoids, but not mineralocorticoids, can also promote the phagocytosis of apoptotic leukocytes [107], and so contribute to the resolution of inflammation. The expression of 11HSD1 in VSMCs [50] and in activated macrophages [108] suggests that generation of glucocorticoid within these cells may contribute to regulation of inflammation.

Influence of 11HSDs on vascular inflammation

The demonstration that pro-inflammatory cytokines selectively upregulate 11HSD1 activity in human VSMCs suggests that glucocorticoid generation within the vascular wall provides a mechanism for local feedback regulation of inflammation [50]. However, this has not been examined *in vivo*. The ability of inflammatory mediators to regulate 11HSD activity in VSMCs may be dependent upon the phenotypic state of the cells (with enzyme ac-

tivity upregulated in actively proliferating, but not in quiescent, cells), the anatomical origin of a particular vessel, the prevailing local glucocorticoid concentrations and the modulation of the inflammatory response by neighbouring tissues [A. R. Dover et al., unpublished data]. Further work is required to clarify the significance of cytokine-mediated regulation of 11HSD1 in arterial cells, particularly given the importance of inflammation in the vascular response to injury [109].

Alternatively, 11HSD1 may regulate inflammation by controlling generation of glucocorticoids within the inflammatory cells. Certainly, the ability of inflammatory cytokines to upregulate 11HSD1 activity in activated human macrophages [108] suggests, as in human VSMC [50], a means of feedback regulation of inflammation within these cells.

Glucocorticoids and vascular remodelling

The term 'vascular remodelling' has been used to cover a range of structural changes in the arterial wall, and its correct definition is the subject of debate (for review see Bund and Lee [110]). In this review, the term 'vascular remodelling' encompasses medial hypertrophy (thickening of the vessel wall caused by increase in cell size) and hyperplasia (thickening of the cell wall caused by an increase in cell number), as well as the intimal remodelling seen in neointimal proliferation and the development of atherosclerotic lesions. It has also been extended to include angiogenic growth of new blood vessels.

The relationship between glucocorticoids and arterial remodelling is well-established; for example, one year following remission, patients with Cushing's syndrome show reduced intimal/medial thickness and increased lumen diameter in the carotid artery [111]. It should be noted, however, that remodelling may be the indirect result of systemic changes (e.g. increased blood pressure) rather than direct interactions of glucocorticoids with the vessel wall. Glucocorticoids may induce vascular remodelling by altering expression of genes for relevant growth factors or by inhibiting processes that modulate growth factor activity. For example, dexamethasone induces a GR-dependent upregulation of endothelin-1 expression [76], and cortisol attenuates the activity of nitric oxide [77] (itself a potent inhibitor of cell growth). Similarly, dexamethasone- and hydrocortisone-mediated increases in ACE activity in VSMCs [112] and ECs [75] may enhance local generation of angiotensin II (a stimulant of VSMC growth both *in vitro* [113] and *in vivo* [114]).

Hypertrophy

Vascular hypertrophy in rats exposed to mineralocorticoids, predominantly deoxycorticosterone acetate, and salt [115] has been attributed to upregulation of the endothelin-1 gene [116]. Similarly, glucocorticoids (dexamethasone, hydrocortisone) have the ability to induce vascular hypertrophy by augmenting the production of [112, 117], and hypertrophic response to [118, 119], angiotensin II. The significance of this hypertrophy is unclear, however, as many investigations that demonstrate enhanced vascular contractility in response to corticosterone involved a duration of exposure that would be insufficient for vascular hypertrophy to occur [120]. Furthermore, glucocorticoid-mediated stimulation of growth in the vascular wall is counterintuitive given that dexamethasone inhibits VSMC growth in culture [121–123] and glucocorticoids prevent neointimal hyperplasia (see below). Thus, the direct influence of glucocorticoids on vascular hypertrophy/hyperplasia is unclear, and any role of local glucocorticoid metabolism by 11HSDs in the process has yet to be investigated.

Neointimal proliferation

The development of neointimal lesions (e.g. in atherosclerosis and in restenosis following revascularisation) is a consequence of an excessive wound healing response in the vessel wall [124, 125]. Vascular injury results in infiltration by inflammatory cells and subsequent migration and proliferation of VSMCs [109]. Consequently, inhibition of either the inflammatory response [126] or VSMC proliferation/migration [127] inhibits lesion development in a variety of models. Since glucocorticoids (dexamethasone) can inhibit inflammation and VSMC proliferation [121–123] and migration [128], it is not surprising that their potential as anti-atherosclerotic [129] and anti-restenotic agents [130] has been investigated [6]. It is also possible, however, that the action of glucocorticoids on the vessel wall is deleterious in patients with vascular disease. For example, given that ACE inhibition limits neointima formation following balloon injury [131, 132], stimulation of ACE activity by dexamethasone [75, 112] could exacerbate lesion development. Also, inhibition of endothelium-derived nitric oxide activity by glucocorticoids could increase both VSMC proliferation and vascular contraction. Further, the systemic effects of glucocorticoids on cardiovascular risk factors (glucose, insulin, lipids and blood pressure) may offset beneficial effects within the vessel wall.

Dexamethasone reduces cholesterol ester accumulation in the aorta [133], and glucocorticoids (dexamethasone, hydrocortisone) inhibit neointimal lesion formation in rats [134, 135], rabbits [136–138] and dogs [139] (with a few contradictory reports [140, 141]). Clinical trials in humans, by contrast, have proved disappointing (with notable exceptions [130]): methylprednisolone did not inhibit restenosis after coronary angioplasty [142] or stent implantation [143], whilst the combination of a glucocor-

ticoid with colchicine increased the risk of coronary aneurysm following stent placement [144]. Discrepancies between clinical studies and animal models could be attributed to species differences or, more probably, to methodological variation (e.g. small sample size; inappropriate patient selection, dose, duration of treatment, route of administration). Exacerbation of lesion development by glucocorticoids could be explained by systemic effects (e.g. weight gain with elevated blood pressure and plasma lipids, which may be more prominent in humans than in other species) or by a net stimulation of vascular cell proliferation. Alternatively, changes in plasma lipids could influence the ability of glucocorticoids to interact with vascular cells. Lipoprotein(a) can downregulate GR gene expression in human VSMCs, thus inhibiting any protective actions of glucocorticoids and, possibly, representing a novel atherogenic mechanism [145].

Angiogenesis

Angiogenesis, in which new blood vessels are formed from an existing vascular network, is a complex process regulated by a balance between counteracting endogenous activators and inhibitors [146]. Physiological angiogenesis is an essential component of reproduction and embryonic development. In postnatal and adult life, it is a discrete process (e.g. in the reproductive tract, wound healing and exercised skeletal muscle) of relatively short duration [147]. In contrast, pathological angiogenesis is usually persistent and unabated and often continues for months or years [147]. Numerous disorders are characterised by excessive angiogenesis, including neoplasia, rheumatoid arthritis and diabetic retinopathy [148]. Consequently, modulation of angiogenesis is regarded as an attractive therapeutic goal in a variety of conditions.

A comprehensive review of the mechanisms of angiogenesis is beyond the scope of this article (for recent reviews see [146, 149]). For the present purposes it is useful to consider angiogenesis to be a stepwise process comprising four distinct phases: (i) basement membrane disintegration, (ii) endothelial cell migration, (iii) channel formation and (iv) maturation. Of the numerous factors that control this process, vascular endothelial cell growth factor (VEGF) is widely considered to be of central importance, since it is crucial for vascular development both in the embryo and in adult tissues and it is EC specific.

Since its first demonstration by Folkman and colleagues, over 20 years ago [150], the ability of glucocorticoids to inhibit angiogenesis has been confirmed *in vitro*, *in vivo* and in tumour-bearing animals [150]. It was suggested that inhibition of angiogenesis in the rabbit cornea was independent of classical GR and MR activity [151]. For example, 17α-hydroxyprogesterone and tetrahydro-S, which have no glucocorticoid or mineralocorticoid activity, retained an anti-angiogenic capability equivalent to, or greater than, that of hydrocortisone. Taken together, these studies demonstrated a class of steroids for which inhibition of angiogenesis appears to be the principal function and hence were named 'angiostatic steroids.' [151].

Despite considerable research, the mechanisms through which glucocorticoids inhibit angiogenesis have not been identified. Indeed, the role of GR is still controversial, as some of the 'angiostatic steroids' may actually have the ability to stimulate these receptors. For example, we have recently shown that inhibition of angiogenesis by tetrahydrocorticosterone, one of the original angiostatic steroids, is dependent upon GR activation in mouse aortic ring explants [G. R. Small et al., unpublished]. Some indication of mechanism was provided by early studies which demonstrated, using nude mice or the non-anticoagulant hexasaccharide fragment of heparin, that the combination of glucocorticoid and heparin was independent of an immune response and anti-coagulant function, respectively [150]. At present, however, there are still several possible pathways through which glucocorticoids may inhibit angiogenesis: (i) Degradation of extracellular matrix, (ii) modification of cytokine production, (iii) inhibition of protease activity, (iv) impairment of vessel maturation and stabilisation, (v) inhibition of growth factor activity, (vi) inhibition of the arachidonic acid cascade, (vii) inhibition of EC-leukocyte interactions and (viii) non-transcriptional effects. The relative significance of these pathways has not been established.

11HSDs and vascular remodelling

Although the initial focus was on 11HSD2 and vascular function, the most recent work in the field of intra-vascular glucocorticoid metabolism has highlighted novel roles for 11HSD1 in influencing vascular structure and remodelling.

Neointimal remodelling and atherogenesis

The potential link between atherosclerosis and tissue-specific generation of glucocorticoids by 11HSDs has been underlined by recent demonstrations that selective upregulation of 11HSD1 in the adipose produces features of the metabolic syndrome, including central obesity, hypertension and hypertriglyceridaemia [10, 152]. This supports the concept that similarities between the metabolic syndrome and Cushing's syndrome are explained by tissue-specific increases in 11HSD1 activity resulting in tissue-specific elevation of glucocorticoid generation [153]. This link between 11HSD1 activity in glucocorticoid-target tissues and atherosclerotic risk factors is not limited to the adipose, as hepatic overexpression of 11HSD1 also results in elevated blood pressure and dyslipidaemia

[154]. It has been proposed, therefore, that 11HSD1 inhibition may reduce atherogenesis. Very recently, systemic administration of a selective 11HSD1 inhibitor was reported to virtually abolish lipid accumulation in the aorta of atherosclerotic (apolipoprotein $E^{-/-}$) mice. However, inhibition of 11HSD1 in $ApoE^{-/-}$ mice produced only a relatively modest reduction in serum triglycerides and cholesterol [155], suggesting that mechanisms over and above amelioration of systemic cardiovascular risk factors may be responsible. It may be that inhibition of 11HSD1 within the vessel wall or within invading macrophages [156] is crucial, but these mechanisms require further clarification.

11HSD2-dependent protection of MR from inappropriate occupation by glucocorticoids may also influence atherogenesis. The role of MR activation in the pathogenesis of atherosclerosis [157] is demonstrated by aldosterone-induced enhancement of lesion development in atherosclerotic (apolipoprotein $E^{-/-}$) mice (probably by increasing oxidative stress in macrophages and cells of the vascular wall [157, 158]). This potentiation of lesion development by aldosterone, which is largely independent of blood pressure, is attenuated by MR antagonists [157], as is constrictive remodelling following angioplasty [159].

The potential importance of 11HSD activity to the development of atherosclerotic lesions was recently extended by the demonstration that, in addition to glucocorticoid metabolism, 11HSDs catalyse the conversion of the atherogenic oxysterol 7-ketocholesterol to 7*β*-hydroxycholesterol [160]. 7-Ketocholesterol is present in micromolar concentrations in human atherosclerotic lesions and in nanomolar concentrations in the plasma [161]. Its

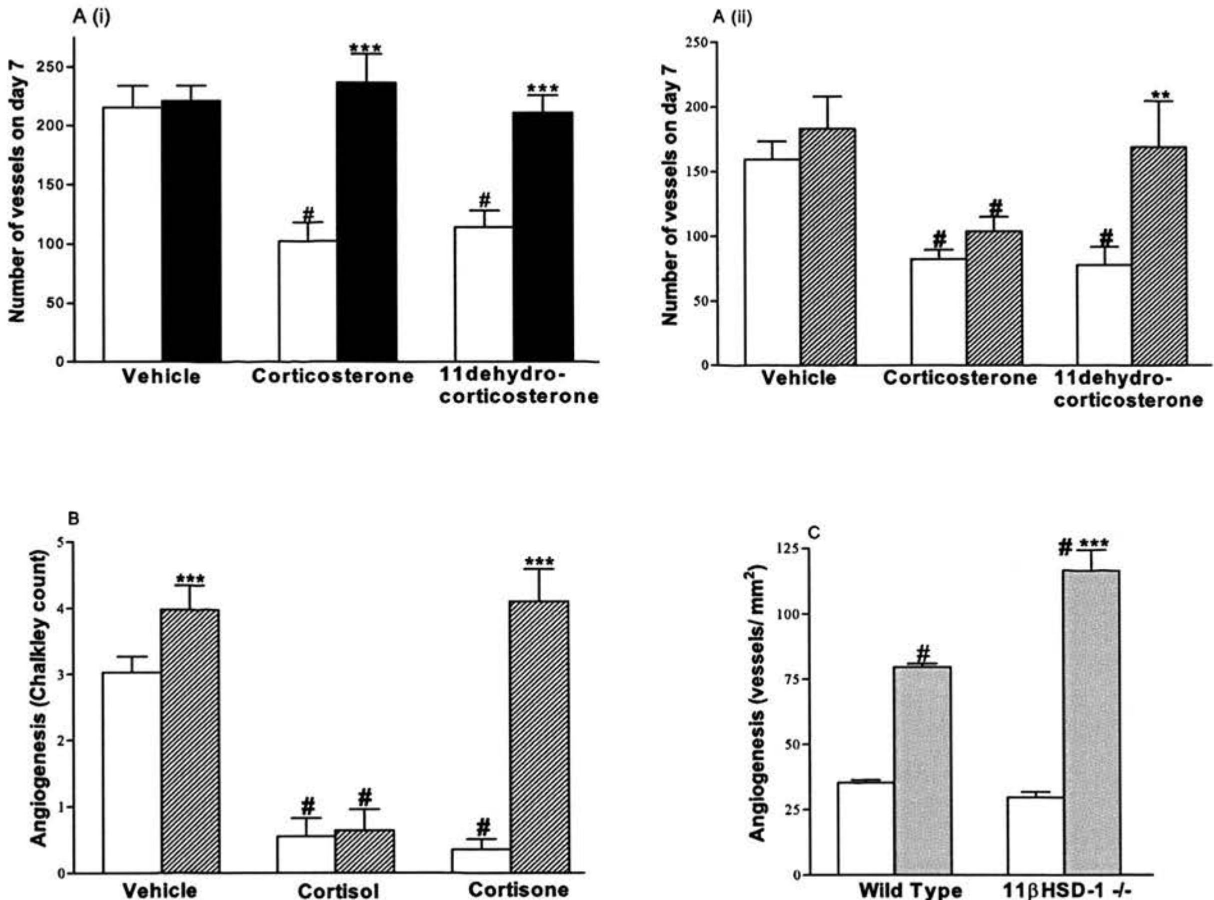

Figure 4. The influence of 11HSD1 on glucocorticoid-mediated angiogenesis. (*A*) In mouse aortic rings cultured in Matrigel, corticosterone and its inactive metabolite 11-dehydrocorticosterone attenuated new vessel growth. Glucocorticoid receptor antagonism (with RU38486, filled columns) abolished the angiostatic response to both compounds (*Ai*), but 11HSD1 deletion (hatched columns) selectively prevented 11-dehydrocorticosterone-mediated angiostasis (*Aii*). Similar results were obtained *in vivo*, using subcutaneous sponge implants (*B*), with 11HSD1 deletion (hatched columns) increasing angiogenesis under basal conditions and abolishing cortisone-, but not cortisol-, mediated inhibition of vessel growth. This mechanism contributed to regulation of myocardial angiogenesis following coronary artery ligation (*C*) with increased vessel growth in $11HSD1^{-/-}$ mice (coronary artery ligation, shaded bars; sham, open bars). #$p < 0.05$ compared with relevant vehicle-treated control; **$p < 0.01$ compared with wild-type mice; ***$p < 0.001$ compared with relevant wild-type control or sham-operated mouse. Reproduced with permission from [168]. © The National Academy of Sciences of the USA, 2005.

association with atherosclerosis is demonstrated in the condition cerebrotendinous xanthomatosis, in which patients who have normal circulating cholesterol levels but increased 7-ketocholesterol develop atherosclerosis prematurely [162]. Conversion of 7-ketocholesterol to 7*β*-hydroxycholesterol by 11*β*-HSD1 may represent the rate-limiting step in a clearance pathway: *in vivo* inhibition of 11HSD1 in rats resulted in an accumulation of 7-ketocholesterol in the liver and increased concentrations in the plasma [163]. In addition to these hepatic effects, reduction of 7-ketocholesterol by 11HSD1 within the vascular wall may also be important. 7-Ketocholesterol and 7*β*-hydroxycholetserol are both toxic to cells of the vascular wall [164] and are potent inhibitors of endothelium-dependent relaxation [165–167]. Consequently, reduction of 7-ketocholesterol, and subsequent clearance of 7*β*-hydrocholesterol, by protecting the vascular wall from damage may have a role in preventing lesion development.

The influence of 11-HSD activity on angiogenesis

Since inflammatory cytokines can promote angiogenesis, we hypothesised that 11*β*-HSD1 in the vessel wall may regulate new vessel formation by controlling the local regeneration of active glucocorticoids. This possibly was addressed using a combination of *in vitro*, *in vivo* and pathological models of angiogenesis [168]. Using a model of tube formation from mouse aortic rings cultured in Matrigel [169], we demonstrated (fig. 4A) that angiogenesis was inhibited by physiological concentrations of active glucocorticoid (corticosterone) but also by its inactive metabolite (11-dehydrocorticosterone). Both these responses were blocked by RU38486, but not by spironolactone, indicating GR dependence. However, whereas 11HSD1-inhibition (with carbenoxolone) or deletion (aortic rings from 11HSD1$^{-/-}$ mice) had no effect on the response to corticosterone, they abolished the ability of 11-dehydrocorticosterone to inhibit angiogenesis (fig. 4B). This indicated that 11HSD1-dependent regeneration of active glucocorticoid within the vascular wall regulates new vessel growth. Application of a model of angiogenesis in sub-cutaneous sponge implants, confirmed this role for 11HSD1 *in vivo*, showing that 11HSD1 deletion produced increased angiogenesis in untreated sponges and blocked the ability of cortisone (but not cortisol) to inhibit new vessel formation. The pathophysiological significance of these observations was emphasised in healing cutaneous wounds and in the myocardial response to coronary artery ligation (fig. 4C). In both cases, 11HSD1 deletion resulted in increased angiogenesis, demonstrating that 11HSD1 regulates the growth of new blood vessels in healing tissues.

Altered angiogenesis in 11HSD1$^{-/-}$ [168] mice could, conceivably, be the result of changes in macrophage activity. Given that 11HSD1 is expressed in macrophages [170], and regeneration of glucocorticoids enhances phagocytosis of apoptotic neutrophils [107], absence of 11HSD1 may confer a prolonged and enhanced acute inflammatory response, thus stimulating angiogenesis. The use of *ex vivo* models such as isolated aortic rings cultured in extracellular matrices [171] has made it possible to differentiate between these two intimately related pathways, angiogenesis and inflammation, and specifically address the effects of glucocorticoids on angiogenesis in the absence of a systemic response. This has produced evidence that glucocorticoids regulate angiogenesis by direct interaction with the vessel wall [168].

Conclusions

It is apparent that glucocorticoids have the ability to regulate both the structure and the function of the artery wall, with significant implications for vascular physiology and pathophysiology. Emerging evidence suggests that pre-receptor metabolism of glucocorticoids within vascular ECs and VSMCs provides a mechanism for regulating these interactions. Relatively few studies have addressed the role of intravascular 11HSD activity, and most of those available have focussed on vascular function. There is, however, a growing body of evidence to suggest that 11HSD isozymes within the arterial wall modulate vascular contractility, the angiogenic growth of new blood vessels, and the atherosclerotic process. Whether these isozymes also influence the inflammatory response to vascular injury and the inter-conversion of atherogenic oxysterols in vascular smooth muscle has still to be determined. Further clarification of the role of 11HSDs in vascular cells is likely to increase our understanding of the link between glucocorticoids and a variety of vascular diseases, and to demonstrate their potential as therapeutic targets for treatment of these conditions.

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Intravascular Glucocorticoid Metabolism during Inflammation and Injury in Mice

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11β-Hydroxysteroid dehydrogenases (11βHSDs) catalyze interconversion of 11-hydroxy-glucocorticoids with inactive 11-keto metabolites. In blood vessel walls, loss of 11βHSD1 is thought to reduce local glucocorticoid concentrations, reducing the progression of atheroma and enhancing angiogenesis. Conversely, on the basis that 11βHSD1 is up-regulated approximately 5-fold by inflammatory cytokines in cultured human vascular smooth muscle cells, it has been proposed that increased 11βHSD1 during vascular inflammation provides negative feedback suppression of inflammation. We aimed to determine whether inflammation and injury selectively up-regulate 11βHSD1 reductase activity *in vitro* and *in vivo* in intact vascular tissue in mice. In isolated mouse aortae and femoral arteries, reductase activity (converting 11-dehydrocorticosterone to corticosterone) was approximately 10-fold higher than dehydrogenase activity and was entirely accounted for by 11βHSD1 because it was abolished in vessels from 11βHSD1$^{-/-}$ mice. Although 11βHSD1 activity was up-regulated by proinflammatory cytokines in cultured murine aortic smooth muscle cells, no such effect was evident in intact aortic rings *in vitro*. Moreover, after systemic inflammation induced by ip lipopolysaccharide injection, there was only a modest (18%) increase in 11β-reductase activity in the aorta and no increase in the perfused hindlimb. Furthermore, in femoral arteries in which neointimal proliferation was induced by intraluminal injury, there was no change in basal 11βHSD1 activity or the sensitivity of 11βHSD1 to cytokine up-regulation. We conclude that increased generation of glucocorticoids by 11βHSD1 in the murine vessel wall is unlikely to contribute to feedback regulation of inflammation. (*Endocrinology* 148: 166–172, 2007)

THE LINK BETWEEN glucocorticoids and cardiovascular disease is well established: systemic glucocorticoid excess (1) and exogenous glucocorticoid therapy (2, 3) are associated with an increase in cardiovascular risk. Epidemiological studies have associated higher cortisol secretion with risk factors for cardiovascular disease, including hypertension, insulin resistance, impaired glucose tolerance, and dyslipidemia (4, 5). Furthermore, the local action of glucocorticoids within the blood vessel wall may be important because glucocorticoids influence the regulation of vascular tone (6, 7) and the vascular response to inflammation and injury (8, 9). This may have therapeutic as well as pathophysiological significance: for example, glucocorticoid therapy has been shown to prevent neointimal proliferation after intraluminal vascular injury (10, 11).

Recent studies demonstrated the importance of tissue-specific regulation of glucocorticoid concentrations (12, 13). Interconversion of glucocorticoids (corticosterone in rodents) and their 11-keto-metabolites (11-dehydrocorticosterone) is catalyzed in target tissues by the 11β-hydroxysteroid dehydrogenases (11βHSDs). 11βHSD type 1 acts predominantly as a reductase *in vivo*, catalyzing the regeneration of active glucocorticoids and thereby promoting activation of glucocorticoid receptors in target tissues. 11βHSD type 2 is an exclusive dehydrogenase (14–16) and prevents illicit activation of mineralocorticoid receptors by glucocorticoids. Both isozymes are expressed in the vessel wall (17–20), as are both mineralocorticoid and glucocorticoid receptors (6, 21). In mouse, 11βHSD1 is predominantly localized to the smooth muscle, whereas 11βHSD2 is found in the endothelium (21). 11βHSD2 prevents glucocorticoids from inhibiting endothelium-dependent vasodilatation (22), but a role for 11βHSD1 in the vessel wall has only recently been elicited and appears to relate to vascular remodeling. By increasing local glucocorticoid concentrations, 11βHSD1 amplifies the angiostatic actions of glucocorticoids (9). Conversely, inhibition of 11βHSD1 protects against atherogenesis in ApoE$^{-/-}$ mice; this effect is disproportionate to changes in serum lipid profile and may reflect alterations in local regeneration of glucocorticoids within the vessel wall (23).

It has been suggested that 11βHSD activity is regulated in diseased blood vessels, contributing to local feedback regulation of inflammation. This hypothesis is based on the observation that proinflammatory cytokines (*e.g.* TNFα) up-regulate 11βHSD1 activity and expression and down-regulate 11βHSD2 expression in cultured human aortic smooth muscle cells (24), favoring increased local glucocorticoid concentrations. However, whereas cytokines alter 11βHSD activity in vascular and other cultured cell types (24–31), the relevance of this observation during inflammation in intact blood vessels has not been established. Here we use models of systemic and local vascular inflammation *in vitro* and *in vivo* to address the hypothesis that exposure to proinflammatory cytokines selectively up-regulates 11βHSD1 activity in intact blood vessels.

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Abbreviations: FCS, Fetal calf serum; 11βHSD, 11β-hydroxysteroid dehydrogenase; LPS, lipopolysaccharide; MA, murine aortic; SMC, smooth muscle cell.

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Materials and Methods

Mice

Male, C57B6J (Charles River, Kent, UK) and 11βHSD $1^{-/-}$ mice on a C57B6J genetic background (Harlan Orlac, UK) (32) were maintained under controlled conditions of light (on 0800–2000 h) and temperature (21 C) with free access to chow (Special Diet Services, Witham, UK) and water. Animal experiments were carried out under Home Office license and conformed to standards defined in The Principals of Animal Care (National Institutes of Health publication 85–23, revised 1985).

Materials

Salts were obtained from BDH (Dorset, UK). 1,2,6,7-$[^3H]_4$-corticosterone was obtained from Amersham Biosciences (Buckingham, UK). 1,2,6,7-$[^3H]_4$-11-dehydrocorticosterone was synthesized in house from 1,2,6,7-$[^3H]_4$-corticosterone using rat placental homogenate (33) and was more than 99% pure when assessed by HPLC. Murine recombinant TNFα, IL-1β, IL-4, and IL-13 (R&D Systems, Abingdon, UK) were stored in PBS containing 0.1% fetal calf serum (FCS) in aliquots at −20 C until required. Etanercept (Wyeth, UK) was dissolved in sterile water. Lipopolysaccharide (LPS), derived from *Escherichia coli* (serotype 0111:B4; Sigma, Poole, UK), was dissolved in sterile 0.9% saline and stored at −20 C. Bioactivity of TNFα was confirmed by means of a neutrophil apoptosis assay (data not shown) (34).

Effects of IL-1β on 11βHSD1 activity in cultured vascular smooth muscle cells

Mice were killed by cervical dislocation. The thoracoabdominal aortae and the iliofemoral vessels were immediately removed into ice-cold DMEM F12 (Invitrogen, Renfrewshire, UK) and cleaned of periadventitial tissue. Primary murine aortic (MA) smooth muscle cells (SMCs) were cultured using a modification of the method of Ray *et al.* (35). The cells were maintained in DMEM containing 20% FCS, 1% penicillin/streptomycin, and 1% L-glutamine 200 mM (Life Technologies, Inc., Paisley, UK) in a humidified oxygenated (95% O_2-5% CO_2) atmosphere.

To demonstrate that 11βHSD1 activity in murine-cultured SMCs is up-regulated after cytokine stimulation as in human SMCs (24), MA-SMCs (passage 2) were seeded onto 6-well plates at 1.75×10^5 cells/well in 2 ml of assay medium. The following day, the medium was replaced with basal medium [containing 0.5% FCS plus IL-1β (20 ng/ml) or vehicle], and cells were incubated for 48 h. $[^3H]_4$-11-dehydrocorticosterone (10 pmol) was then added to the appropriate wells and the cells incubated for a further 24 h. Steroids were extracted from the culture medium supernatant using C_{18} Sep-pak columns (Waters Millipore, Watford, UK). $[^3H]_4$-corticosterone and $[^3H]_4$-11-dehydrocorticosterone were separated by HPLC and quantified by on-line liquid scintillation counting. Enzyme activity was expressed as conversion per 1.75×10^5 cells after subtraction of apparent conversion in negative control wells.

Effects of cytokines on 11βHSD activity in intact vascular and hindlimb tissues in vitro

11βHSD activities were measured in murine aortic rings and iliofemoral vessels by adapting the method of Souness *et al.* (36). Briefly, vessels were incubated (24 h, 37 C) in DMEM-F12 (1 ml) containing $[^3H]_4$-steroid and supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and amphotericin (0.25 μg/ml). 11β-Reductase activity was determined in vessels from wild-type and 11βHSD$1^{-/-}$ mice by adding 10 pmol $[^3H]_4$-11-dehydrocorticosterone. 11β-Dehydrogenase activity was determined in vessels from wild-type mice by adding 10 pmol $[^3H]_4$-corticosterone. 11β-Reductase activity was also determined using the method described for arteries in hindlimb tissues (dissected pieces of quadriceps muscle and skin with subcutaneous fat) from wild-type mice.

To assess the influence of cytokines *in vitro* on 11β-reductase activity, single aortic rings from C57B6J mice were preincubated with murine recombinant TNFα (10–1000 ng/ml); IL-1β (1–100 ng/ml); IL-4 (50 ng/ml); IL-13 (50 ng/ml); Etanercept [a fusion protein that antagonizes human and murine TNFα and that ameliorates the cardiovascular effects of murine TNFα (37), 0.1–10 μg/ml]; or vehicle. After 16 h incubation (24), 10 pmol $[^3H]_4$-11-dehydrocorticosterone was added and incubation continued for a further 24 h. Control wells without tissue contained medium alone, $[^3H]_4$-11-dehydrocorticosterone, or $[^3H]_4$-11-dehydrocorticosterone with cytokines.

To observe the effect of cytokines *in vitro* on 11β-dehydrogenase activity, three to five aortic rings were preincubated for 16 h with murine recombinant TNFα, IL-1β, or vehicle. At 16 h, 10 pmol $[^3H]_4$-corticosterone was added and incubation continued for a further 24 h. Control wells contained $[^3H]_4$-corticosterone alone, medium alone, and $[^3H]_4$-corticosterone with cytokines.

After incubation, steroids were extracted from the culture medium and assayed as described for cultured cells. Aortic ring tissue, which contains only 2–3% of the added radioactivity (36), was not included in the extraction.

Effects of LPS in vivo on vascular 11βHSD1 activity in aorta and perfused murine hindlimb

As described by Brandes *et al.* (38), the aorta at the thoracoabdominal transition was isolated, after cervical dislocation, and a cannula (24-gauge; Neoflon, Ohmeda, Sweden) introduced (distal to the renal arteries) and secured at the iliac bifurcation with a 3–0 prolene suture (Surgical Supplies Ltd., Cumbernauld, UK). The distal inferior vena cava was also cannulated (18-gauge cannula; Venflon, BD, UK) and secured with a 3–0 prolene suture. The hindlimb was constantly perfused, via the aortic cannula, with warmed oxygenated modified Krebs-Henseleit solution containing 2% BSA (Sigma) at flow rates of between 0.8 and 1.2 ml/min to achieve a perfusion pressure of approximately 40 mm Hg. Effluent was collected from the venous cannula. After a 10-min equilibration period, $[^3H]_4$-11-dehydrocorticosterone (for determination of reductase activity) or $[^3H]_4$-corticosterone (for determination of dehydrogenase activity) was added to the perfusion buffer (final concentration 5 nM). Perfusion was maintained for up to 60 min and aliquots of effluent collected at regular intervals. Steroids were extracted from the effluent and analyzed as described for aortic homogenates. To determine enzyme kinetics for 11β-reductase activity, the above procedure was replicated using concentrations of $[^3H]_4$-11-dehydrocorticosterone between 5 and 500 nM.

The effects of inflammation on 11βHSD1 activity in the perfused hindlimb were assessed 6 h after ip administration of LPS (10 mg/kg) or vehicle (physiological saline; 20 ml/kg).

To assess the effects of *in vivo* LPS on aortic 11βHSD activities, aortic rings were obtained from C57B6J mice that had received either LPS (10 mg/kg ip) or vehicle 6 h before the animals were killed. 11β-Reductase and dehydrogenase activities were determined, *ex vivo*, by incubation with 10 pmol $[^3H]_4$-11-dehydrocorticosterone or $[^3H]_4$-corticosterone for 24 h, as described above.

Effects of vascular injury on 11βHSD1 enzyme activity in femoral arteries

Femoral artery injury was performed in anesthetized (halothane) C57B6J mice using the method of Sata *et al.* (39). Briefly, a guidewire (0.014 in. diameter; Cook Inc., UK) was advanced from the descending genual artery to more than 5 mm along the femoral artery, left in place for 1 min, and then removed. The genual artery was ligated, blood flow restored to the femoral artery, and the skin incision closed with 6–0 silk sutures. For histological evaluation, arteries were excised at various time points after injury, fixed in 10% neutral buffered formalin, and embedded in paraffin. Four-micrometer sections were stained with the United States trichrome stain (40).

To assess the effects of vascular injury on basal 11βHSD1 activity, injured and contralateral uninjured femoral arteries (from the descending genual artery to the bifurcation of the iliac artery, therefore containing the entire injured section of vessel) removed 7 d after surgery were incubated *in vitro* with vehicle for 16 h after which 11β-reductase activity was assessed as described above. In addition, to examine the effect of cytokines on 11βHSD1 activity in injured arteries, injured and contralateral uninjured femoral arteries were removed 7 d after injury and incubated *in vitro* with IL-1β (10 ng/ml) for 16 h after which 11β-reductase activity was assessed as described above.

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Data analysis and statistics

In vitro experiments were performed in duplicate or triplicate and the mean in each experiment used in statistical analyses. Enzyme activity was expressed per 1.75×10^5 cells or, in the case of intact tissue, per wet weight of tissue after subtraction of apparent conversion in negative control samples. Data are expressed as mean ± SEM and analyzed by Student's *t* test or ANOVA followed by *post hoc* tests where appropriate.

Results

Effects of IL-1β on 11βHSD activity in cultured mouse aortic smooth muscle cells

As previously reported in human SMCs (24), IL-1β increased 11β-reductase activity by approximately 40% (87 ± 2% conversion in 24 h), compared with controls (62 ± 3%; $P < 0.05$, n = 6).

Effects of cytokines on 11βHSD activity in intact vascular tissue in vitro

11β-Reductase and dehydrogenase activities were detected in aortic rings and femoral arteries from C57B6J mice (Fig. 1). 11β-Reductase activity was higher in aorta than femoral artery ($P < 0.005$), whereas 11β-dehydrogenase activity was similar in aorta and femoral artery (Fig. 1, $P = 0.48$; n = 6). 11β-Reduction was the predominant reaction direction, and was completely abolished in arteries from $11\beta HSD1^{-/-}$ mice (<0.1pmol/mg per 24 h, n = 6).

11β-Reductase activity in aortic rings from wild-type mice (8.30 ± 1.20 pmol product/mg per 24 h, n = 18) was unaffected after incubation with the proinflammatory cytokines IL-1β and TNFα or the antiinflammatory cytokines IL-4 and IL-13 (Fig. 2). To exclude the possibility that 11βHSD1 in aortic rings was already maximally induced by endogenous TNFα, rings were incubated with the TNFα antagonist, Etanercept, which had no effect on 11β-reductase activity (Fig. 2). 11β-Dehydrogenase activity in wild-type aortic rings (0.21 ± 0.08 pmol product/mg per 24 h) was unaffected by preincubation with either IL-1β (10 ng/ml; 0.33 ± 0.04 pmol product/mg per 24 h) or TNFα (100 ng/ml; 0.30 ± 0.01 pmol product/mg per 24 h, $P = 0.25$, n = 4).

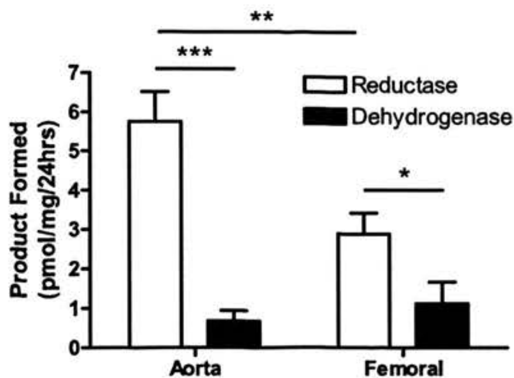

FIG. 1. 11βHSD activities in intact vascular tissues *in vitro*. 11β-Reductase (*open bars*) and dehydrogenase (*closed bars*) activities in intact arteries from C57B6J mice. Reductase and dehydrogenase activities are expressed as the amount of $[^3H]_4$-corticosterone or $[^3H]_4$-11-dehydrocorticosterone formed, respectively. Results are mean ± SE, n = 6. 11β-Reductase activity was higher than dehydrogenase activity (***, $P < 0.0001$ for aorta; *, $P < 0.05$ for femoral artery). 11β-Reductase activity was higher in aorta than femoral artery (**, $P < 0.005$).

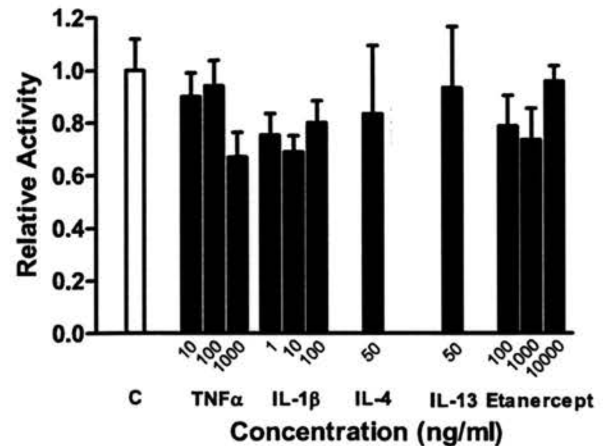

FIG. 2. Effect of cytokines on 11β-reductase activity in the mouse aorta. Aortic rings were incubated for 48 h with TNFα, IL-1β, IL-4, IL-13, Etanercept, or vehicle and then for a further 24 h in the presence of $[^3H]_4$-11-dehydrocorticosterone. 11β-Reductase activity is expressed as $[^3H]_4$-corticosterone formed relative to activity in control incubations without cytokine manipulation. Results are mean ± SE. There were no differences between groups (n = 4–10).

Effect of LPS in vivo on 11βHSD1 activity in aorta and perfused murine hindlimb

In perfused murine hindlimb, both reductase and dehydrogenase activities were detected, with reductase predominating by approximately 10:1 (Fig. 3A). In $11\beta HSD1^{-/-}$ mice, reductase activity was abolished (Fig. 3A). The apparent Michaelis constant was 1.064 μM for 11β-reductase activity in the perfused hindlimb (Fig. 3B). *Ex vivo* assays of hindlimb tissues confirmed reductase activity in the hindlimb vasculature (11.4 ± 31.4 pmol/mg per 24 h), which was substantially greater than in skeletal muscle (0.14 ± 0.02 pmol/mg per 24 h; $P < 0.0001$) or skin with sc fat (0.27 ± 0.05 pmol/mg per 24 h; $P < 0.0001$, n = 4).

Intraperitoneal LPS administration to wild-type mice induced weight loss (1.4 ± 0.2 *vs.* 0.7 ± 0.2 g, $P < 0.05$, n = 6) and splenomegaly (0.12 ± 0.01 *vs.* 0.08 ± 0.01 g, $P < 0.05$, n = 6), compared with vehicle. LPS induced a small increase in 11β-reductase ($P < 0.05$, n = 6) but not dehydrogenase ($P = 0.16$, n = 3) activity in aortic rings analyzed *ex vivo* (Fig. 4A) but did not significantly increase 11β-reductase activity in the perfused hindlimb ($P = 0.12$ by repeated measures ANOVA, n = 6; Fig. 4B).

Influence of vascular injury on 11βHSD activity in femoral artery

Vascular injury produced extensive stretching of the vascular wall and atrophy of medial SMCs, with subsequent development of a proliferative, smooth-muscle rich neointima, evident at 7 d and peaking in size at 21 d (Fig. 5).

11β-Reductase activity was not altered in femoral arteries after vascular injury *in vivo* ($P = 0.33$, n = 6; Fig. 6). Furthermore, vascular injury did not alter the effect of *in vitro*

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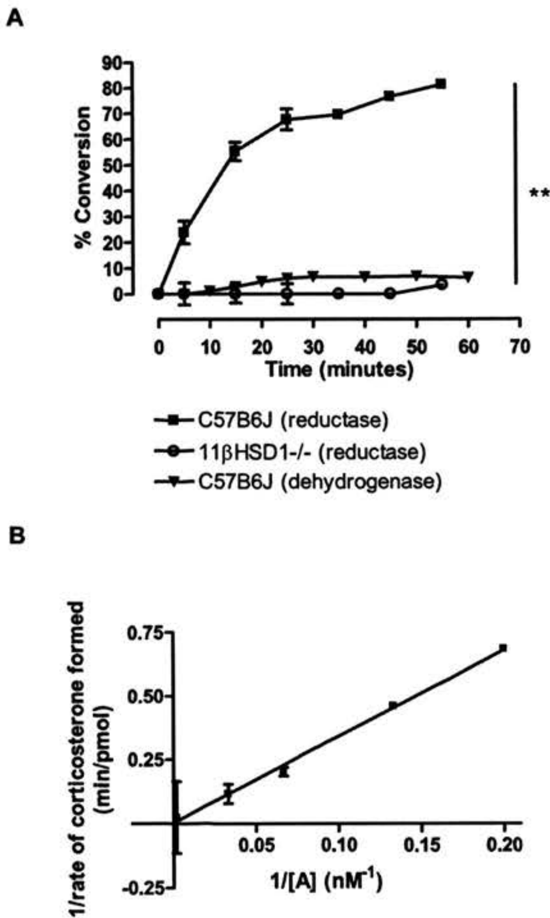

FIG. 3. 11βHSD activities in the perfused murine hindlimb. A, 11β-Reductase (*closed squares*) and dehydrogenase (*closed triangles*) activities in perfused hindlimbs of C57B6J mice and 11β-reductase activity in perfused hindlimbs of $11\beta HSD1^{-/-}$ mice (*open circles*). Enzyme activity is expressed as the percentage of $[^3H]_4$-corticosterone or $[^3H]_4$-11-dehydrocorticosterone formed. 11β-Reductase activity was abolished in $11\beta HSD1^{-/-}$ mice. Results are mean ± SE, n = 3–4; **, $P < 0.005$, comparing reductase activity in $11\beta HSD1^{-/-}$ mice with controls. B, Kinetics of 11β-reductase activity in perfused hindlimbs of wild-type C57B6J mice. Using a time point of 11 min after the commencement of the perfusion, Michaelis constant was calculated from this Lineweaver-Burke plot as 1.064 μM and maximum velocity 313 pmol/min. Results are mean ± SE, n = 3 at each concentration. A, 11-Dehydrocorticosterone.

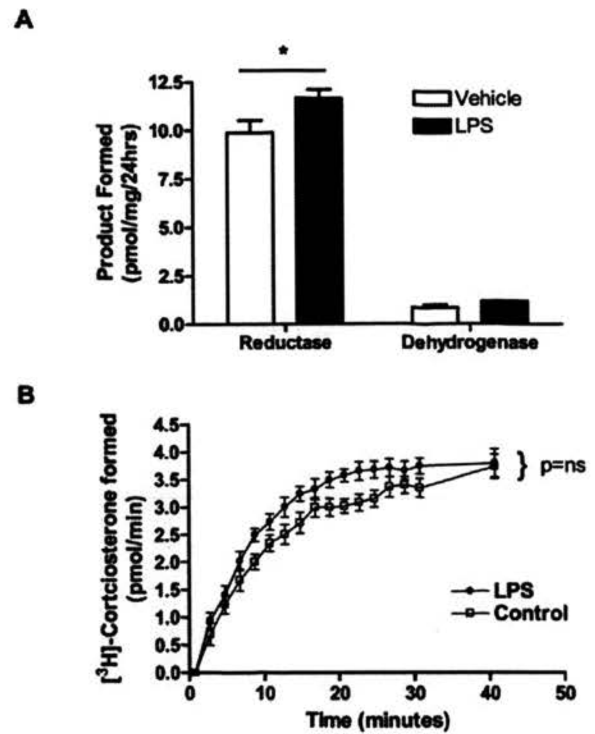

FIG. 4. Effects of *in vivo* LPS on 11βHSD activity in vascular tissue. A, Aortic rings from C57B6J mice were incubated for 24 h in the presence of either $[^3H]_4$-11-dehydrocorticosterone or $[^3H]_4$-corticosterone, 6 h after *in vivo* ip administration of LPS (10 mg/kg) or vehicle. 11β-Reductase and -dehydrogenase activities are expressed as the amount of $[^3H]_4$-corticosterone or $[^3H]_4$-11-dehydrocorticosterone formed, respectively. Results are mean ± SE. 11β-Reductase activity was increased after LPS injection (*, $P < 0.05$, n = 6). 11β-Dehydrogenase activity was similar in both groups ($P = 0.16$, n = 3). B, 11β-Reductase activity in perfused hindlimbs of C57B6J mice 6 h after *in vivo* ip administration of LPS (10 mg/kg, *closed circles*) or vehicle (*open squares*). 11β-Reductase activity is expressed as the amount of $[^3H]_4$-corticosterone formed. Results are mean ± SE. There were no differences between groups ($P = 0.12$ by repeated-measures ANOVA, n = 6).

incubation with IL-1β on 11β-reductase activity ($P = 0.20$, n = 6; Fig. 6).

Discussion

We and others previously established that both isozymes of 11βHSD are present in the vascular wall (17–21), in which they may regulate local glucocorticoid availability and hence activation of glucocorticoid and mineralocorticoid receptors. Here we assessed whether vascular inflammation increases local generation of active glucocorticoid by selectively altering the activity of 11βHSD1. Our results indicate that, in contrast to cultured SMCs (24), 11βHSD1 in intact arteries is not up-regulated by proinflammatory cytokines *in vitro* or *in vivo* or the inflammatory response to injury *in vivo*. This suggests that endogenous glucocorticoid generation in the murine arterial wall does not provide a mechanism for feedback regulation of inflammation.

Although both isozymes of 11βHSD are expressed in murine vessels (21, 22), the net balance between active 11-OH and inactive 11-keto steroids was unpredictable. Whereas 11βHSD2 is an exclusive dehydrogenase and 11βHSD1 a predominant reductase, 11βHSD1 might operate as a dehydrogenase in the absence of cofactor generated by the neighboring hexose-6-phosphate dehydrogenase in the endoplasmic reticulum (41, 42). Here we show that the predominant reaction direction, by approximately 10-fold, in intact vessels is the reductase and that this can be accounted for entirely by 11βHSD1 because it is absent in $11\beta HSD1^{-/-}$ mice. Moreover, the kinetics of reductase activity in the perfused hindlimb are consistent with the 11βHSD1 isozyme (43). Furthermore, whereas dehydrogenase activity was similar in all vessels examined, regional differences in basal 11β-reductase activity (44) were suggested, with activity higher in aortae than femoral arteries. Our studies of regulation during inflammation were therefore focused on the reductase activity attributable to the 11βHSD1 isozyme.

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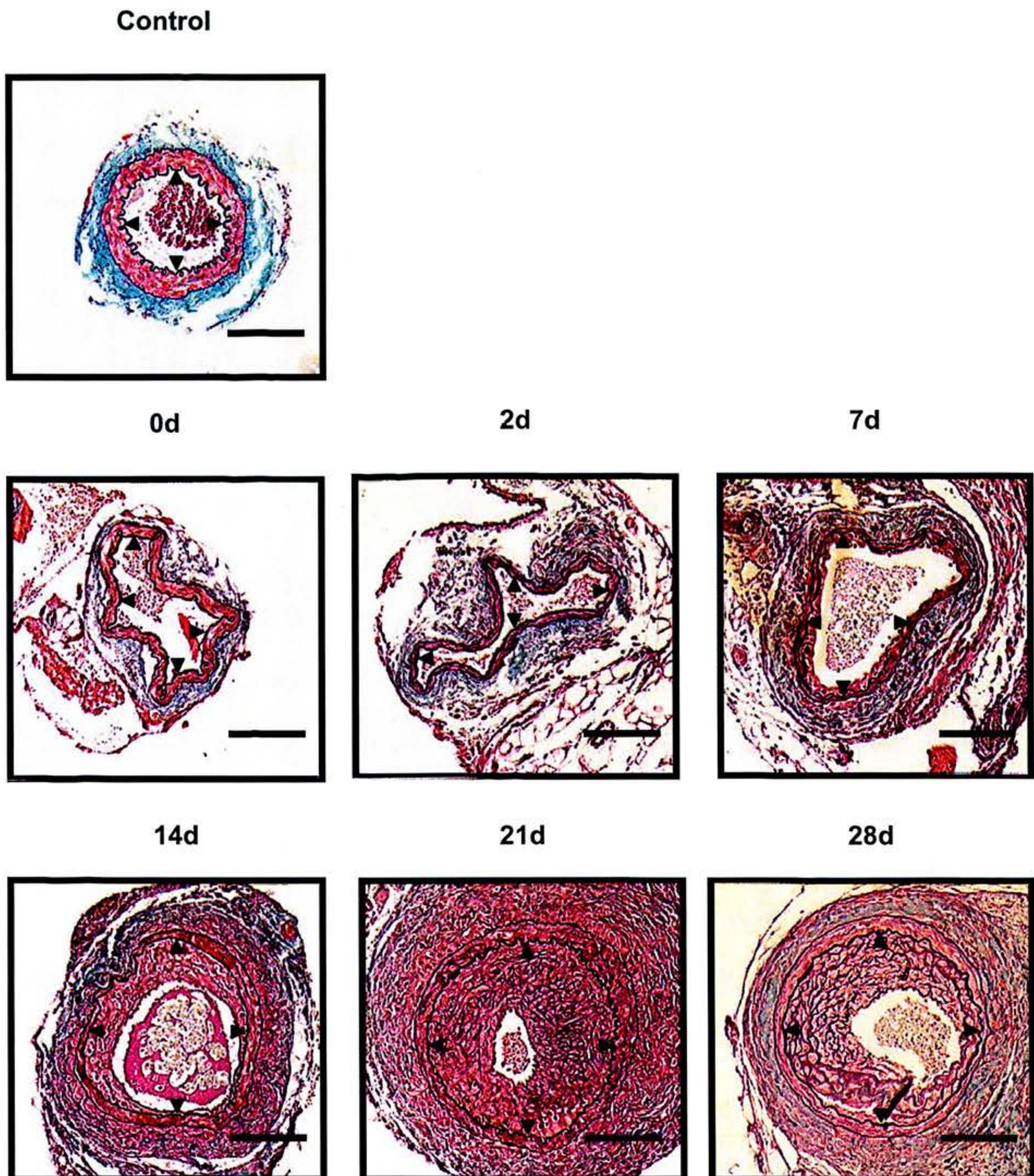

FIG. 5. Development of neointimal lesions over time after intraluminal injury of the femoral artery. After wire injury of the femoral artery, arteries were excised at the time points indicated and stained with the United States trichrome stain. This indicated initial stretching of the vessel wall, followed by the time-dependent formation of neointimal lesions, which are first observed at 7 d and peak in size after 21 d. A control uninjured vessel is also shown. *Scale bar*, 100 μm. *Arrowheads* indicate internal elastic lamina.

Consistent with a previous report in human cultured SMCs (24), 11β-reductase activity in cultured murine vascular SMCs was up-regulated after exposure to the proinflammatory cytokine, IL-1β. The extent of up-regulation (40% increase in activity), however, was less dramatic than the 5-fold enhancement reported with human SMCs (24) and was difficult to replicate at later passages (data not shown). This may be indicative of a species difference in the regulation of 11βHSD1 activity by cytokines in cultured SMCs.

In contrast to cultured MA-SMCs, vascular 11βHSD1 reductase activity was not up-regulated by several different cytokines (IL-1β, IL-4, IL13, TNFα) in intact arteries *in vitro*. This lack of effect was not due to preexisting up-regulation of 11HSD1 activity because the TNFα antagonist, Etanercept,

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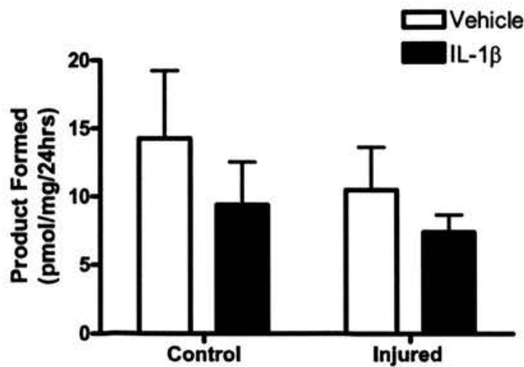

FIG. 6. Influence of injury and inflammation on 11β-reductase activity in femoral arteries. Whole injured and uninjured femoral arteries obtained 7 d after surgery were incubated for 16 h with 10 ng/ml IL-1β or vehicle and then for a further 24 h in the presence of [^{3}H]11-dehydrocorticosterone. 11β-Reductase activity is expressed as the amount of [^{3}H]corticosterone formed. Results are mean ± SE. There were no differences between groups ($P = 0.33$ for effect of injury, $P = 0.20$ for effect of cytokine, n = 6).

also had no effect on glucocorticoid generation. In addition, IL-1β and TNFα failed to produce the down-regulation of 11β-dehydrogenase activity reported in human SMCs (24).

In contrast to our *in vitro* findings, there was a small (18%), selective increase in 11β-reductase activity in aortic rings from mice that had received LPS *in vivo*. Thus, although individual cytokines were ineffective at enhancing 11βHSD1 activity in intact tissue, the result of *in vivo* LPS may be to produce an altered inflammatory milieu that favors a modest increase in 11β-reductase activity. Although this effect of LPS was not evident in perfused hindlimbs, there was a similar trend toward an increase in 11β-reductase activity, raising the possibility that there is a small effect of LPS on hindlimb vascular 11βHSD1 activity, which may be masked by the contributions from other tissue types within the regional perfused territory. Additionally, there may be regional differences in the inflammatory regulation of vascular 11βHSD1 activity. It is uncertain whether the resultant change in glucocorticoid availability after these modest effects would have physiologically relevant consequences, but importantly the magnitude of effect is substantially smaller than that observed in cultured cells (24).

The majority of reports detailing regulation of 11βHSD1 activity by inflammatory cytokines (in a variety of different cell types) demonstrate a selective increase in 11β-reductase activity and/or expression (24–31). This effect is not universal, however, because TNFα has no effect on 11β-reductase activity in cultured human hepatocytes (28). Furthermore, in circulating monocytes, 11βHSD1 expression is not up-regulated by TNFα or IL-1β but is induced during differentiation into macrophages and after exposure to IL-4 and IL-13 (25). All studies reported to date used cell culture systems, which undoubtedly alter the natural cell phenotype. The differences that we observed in the ability of cytokines to up-regulate 11βHSD1 activity in cell culture but not intact tissue preparations suggests that the regulation of 11βHSD1 by inflammatory stimuli may not only be tissue specific but may also depend on the degree of cell proliferation and/or differentiation. In vascular lesions, contractile SMCs dedifferentiate and take on a proliferative phenotype (45). Thus, the absence of inflammatory up-regulation of 11βHSD1 in healthy intact arteries cannot be extrapolated to arteries undergoing proliferative remodeling. This issue was addressed using the model of injury/proliferation in the mouse femoral artery.

Insertion of a wire in the femoral artery produces extensive stretching of the vascular wall followed by acute inflammation and the temporal development of a proliferative, smooth muscle-rich neointima (39). In our hands small lesions are first evident 7 d after injury and peak in size after 21 d. This is consistent with the pattern of remodeling reported by Sata *et al.* (39), in which significant medial and neointimal SMC proliferation was demonstrated at 7 d. Even under these circumstances, however, 11βHSD1 activity was not increased in the femoral artery wall. Furthermore, the remodeling process did not increase the sensitivity of 11βHSD1 to up-regulation by IL-1β. This supports the concept that inflammatory regulation of 11βHSDs in whole-tissue preparations, even during extensive inflammatory and proliferative remodeling, does not mirror that found in cell culture.

We conclude, therefore, that increased local generation of endogenous glucocorticoids by 11βHSD1 in vascular SMCs does not contribute to regulation of vascular lesion development by feedback inhibition of inflammation in mice.

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