Role of intra-cellular glucocorticoid regulation in vascular lesion development

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DECLARATION

I declare that all the work presented in this thesis was carried out by me with the following exceptions:

Femoral wire-angioplasty was initially performed, taught and subsequently supervised by Dr. Patrick Hadoke. Coronary sinus sampling in humans was performed by Professor David Newby, PCR analysis of human atrial and arterial samples by Ms Eileen Miller and assays of renin, angiotensinogen and aldosterone by Dr. Chris Kenyon.

I can confirm that this thesis has not been previously submitted for any other degree at any institute.

Dr. Javaid Iqbal

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

11β-HSD	11beta-hydroxysteroid dehydrogenase
ACE	Angiotensin converting enzyme
ApoE	Apolipoprotein E
BCT	Brachiocephalic Trunk
BHF	British Heart Foundation
BP	Blood pressure
CHD	Coronary heart disease
CVD	Cardiovascular disease
DES	Drug eluting stents
D-DES	Dexamethasone drug eluting stent
DKO	Double knockout
DNA	Deoxyribonucleic acid
EEL	External elastic lamina
FGF	Fibroblast growth factor
GR	Glucocorticoid receptors
HDL	High density lipoprotein
HMGR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPA	Hypothalamic pituitary adrenal
HPLC	High Performance Liquid Chromatography
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule 1
IEL	Internal elastic lamina
IL	Interleukin
IMT	Intima-media thickness
KO	Knockout
LDL	Low density lipoprotein
MCP	Monocyte chemo-attractant protein
MI	Myocardial infarction
MR	Mineralocorticoid receptors

NO	Nitric oxide
NOS	Nitric oxide synthase
NHS	National Health Service
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAME	Syndrome of apparent mineralocorticoid excess
SLE	Systemic lupus erythematosus
TGF	Transforming growth factor
TNF	Tissue necrosis factor
UST	United States trichrome
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand factor

POSTERS & PRESENTATIONS

BMUS 2010 **Assessment of arterial lesions using high frequency ultrasound**
Javaid Iqbal, A Thomson & PWF Hadoke
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Javaid Iqbal, PWF Hadoke & BR Walker
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Javaid Iqbal, PWF Hadoke, C Yau, BR Walker
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Javaid Iqbal, N Kirkby, R Andrew, PWF Hadoke & BR Walker
Poster presentation, European Society of Cardiology, Barcelona

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Javaid Iqbal, DE Livingstone, R Andrew, BR Walker & PWF Hadoke
Moderated poster presentation, British Cardiovascular Society, London

SSEM 2008 **Effect of intravascular glucocorticoid metabolism on vascular remodelling in a rodent model of angioplasty**
Javaid Iqbal, LJ Macdonald, BR Walker & PWF Hadoke
Poster presentation, Scottish Society of Experimental Medicine, Edinburgh

BAS 2008 **Role of intravascular glucocorticoid metabolism by 11β-HSD isozymes in neointimal lesion formation**
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PEER REVIEWED PAPERS

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ABSTRACT

Atherosclerosis and post-angioplasty neointimal proliferation, which are leading causes of cardiovascular morbidity and mortality, develop as a result of chronic or acute vascular injury producing inflammatory and proliferative responses in the vessel wall.

Glucocorticoids, the stress hormones produced by the adrenal cortex, have anti-inflammatory and anti-proliferative characteristics and can also influence systemic cardiovascular risk factors. The systemic levels of these hormones are controlled by the hypothalamic pituitary adrenal axis. However, there is also a tissue-specific pre-receptor regulation of these hormones by the two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD); type 1 regenerates active glucocorticoids within the cells and type 2 inactivates glucocorticoids. Whilst it has been shown that the inhibition of 11β-HSD1 has favourable effect on cardiovascular risk factors and the inhibition of 11β-HSD2 results in hypertension; the effect of these enzymes on vascular lesion development is not known. The work described in this thesis tested the hypothesis that 11β-HSD1 inhibition reduces vascular lesion development due to improvement in cardiovascular risk factors, whereas 11β-HSD2 inhibition leads to adverse vascular remodelling.

Apolipoprotein-E deficient (*ApoE*$^{-/-}$) mice fed on western diet were used to study atherosclerosis, whereas neointimal proliferation was investigated using a well-established mouse model of wire-angioplasty. Vascular lesions were assessed using novel imaging and standard histological techniques.

11β-HSD1 inhibition reduced the size of atherosclerotic lesions and improved markers of plaque stability with a reduction in lipid content and increase in collagen content of the plaques. This was associated with a reduction in weight gain and blood pressure but without any effect on lipid profile. 11β-HSD1 inhibition did not produce any significant effect on neointimal proliferation in C57Bl/6J mice. However in *ApoE*$^{-/-}$ mice, 11β-HSD1 inhibition reduced neointimal proliferation with corresponding increase in size of patent lumen and with an associated reduction in macrophage content of neointimal lesions. 11β-HSD2 deletion produced an outward remodelling in un-injured vessels but there was no effect on neointimal proliferation after wire-angioplasty. Administration of a selective mineralocorticoid antagonist, eplerenone, reduced neointimal lesions

significantly but to a similar degree in both C57Bl/6J and 11β-HSD2$^{-/-}$ mice, associated with a significant reduction in macrophage content of lesions but without any effect on blood pressure.

Data in this thesis highlight the potential therapeutic application of 11β-HSD1 inhibition in reducing the size and vulnerability of atherosclerotic plaques and also reduction in neointimal proliferation (and hence post-angioplasty restenosis) in high risk patients with 'metabolic syndrome' phenotype. The results also indicate that 11β-HSD2 has a limited, if any, role to play in the development of neointimal lesions.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality across the world. In the UK, more than one in three people (36%) die from CVD, accounting for over 200,000 deaths each year (British Heart Foundation, BHF, www.heartstats.org). Despite a decline in atherosclerosis-related deaths in the western world in recent years [Ford et al., 2007], the burden of CVD, both in terms of cost and human suffering, remains immense [Lim et al., 2007]. The overall financial cost for the National Health Service (NHS) in healthcare provision for these patients exceeds £25 billion a year (BHF, www.heartstats.org). The WHO estimates that CVD mortality will remain the number one killer worldwide in the next decades (12.2% deaths attributable to CVD in 2004 vs. 14.3% projected in 2030) [Iqbal and Fox, 2010].

The major underlying cause of CVD is the formation of atherosclerotic lesions in the coronary circulation leading to limitation of blood flow. These lesions, if clinically significant, are usually treated with percutaneous angioplasty and stenting. In-stent restenosis, however, remains a significant problem, affecting up to 20% of patients [Welt and Rogers, 2002c]. This is perhaps not surprising, since the iatrogenic arterial injury during angioplasty creates an intense inflammatory response [Welt and Rogers, 2002] and also induces migration and proliferation of vascular smooth muscle cells (VSMCs) [Edelman and Rogers, 1996] leading to an increase in neointimal lesion formation.

Glucocorticoids, steroid hormones formed in the adrenal gland, can influence the development of atherosclerotic and neointimal lesions due to their interaction with systemic risk factors, inflammatory cells and VSMCs. The intracellular activity of glucocorticoids is regulated by the isozymes of 11beta-hydroxysteroid dehydrogenase (11β-HSD). The work described in this thesis was designed to determine whether activity of the isozymes of 11β-HSD influences the development of atherosclerotic and neointimal lesions. This chapter will summarise the current understanding of the pathogenesis of atherosclerosis and neointimal proliferation and describe how regulation of glucocorticoids by 11β-HSD may influence these processes.

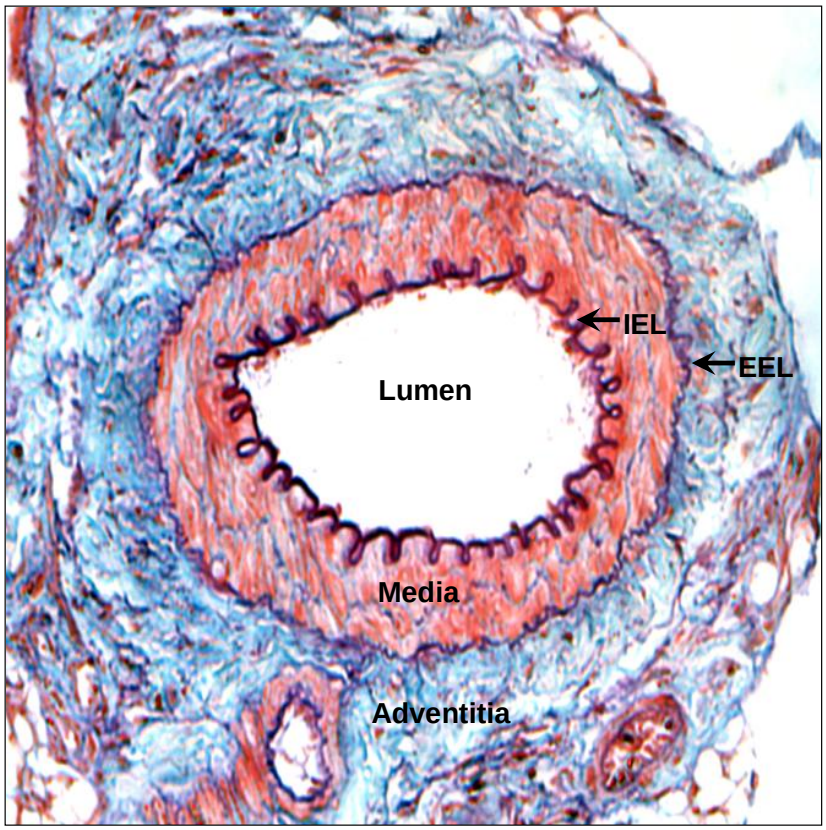
1.1 Atherosclerosis

Atherosclerosis (from the Greek words athero (gruel or paste) and sclerosis (hardness)) is a chronic inflammatory disorder characterized by the formation of lesions in the large and medium sized arteries at the sites of injury-induced endothelial cell dysfunction [Benditt, 1977]. It begins with damage to the innermost layer of the artery (endothelium) which leads to accumulation of lipids, platelets, smooth muscle cells, inflammatory cells, connective tissue, calcium and cellular waste products in the arterial wall. The resultant lesion (plaque or atheroma) can grow large enough to significantly reduce the blood flow through an artery. However, most of the clinically-significant consequences of atherosclerosis occur when plaques become fragile and rupture leading to thrombosis and arterial occlusion. Occlusion of a coronary artery will result in a myocardial infarction (MI) [Shah and Forrester, 1991].

1.1.1 Structure of a healthy artery

The healthy arterial wall (Figure 1.1) consists of three layers: the intima, media, and adventitia [Florey, 1969]. The intima is the innermost layer of the arterial wall and is defined as the region of the arterial wall from and including the endothelial surface at the lumen to the luminal margin of the media [Stary et al., 1992]. The internal elastic lamina (IEL), a fenestrated sheet of elastin fibres, is generally considered part of the media. The middle layer or media contains several layers of SMCs intermixed with elastic fibres and embedded in connective tissue rich in proteoglycans. The media is bound on the outside by the external elastic lamina (EEL) that separates it from the adventitia. The outermost layer or adventitia is formed of irregularly arranged collagen bundles, scattered fibroblasts, a few elastic fibres and small vessels that supply blood to the vessel wall itself and hence termed vasa-vasorum [Heistad et al., 1981].

Figure 1.1: Structure of a healthy artery



The cross-section of a healthy murine artery (stained with United States trichrome, UST) is shown here to illustrate the three layers of arterial wall. UST stains internal and external elastic lamina (IEL, EEL) in dark purple (indicated by arrow-head) and therefore defines intima (between lumen and IEL), media (between IEL and EEL) and adventitia (outside EEL).

1.1.2 Risk factors for atherosclerosis

Large scale epidemiological studies, such as the Framingham Heart study [Wilson et al., 1998] and the INTER-HEART study [Yusuf et al., 2004], have provided valuable data for identification of the major risk factors for CVD. Some of these risk factors, such as age, sex and ethnic origin, are non-modifiable but help in clinical diagnosis and risk stratification. On the other hand, the modifiable risk factors including hypertension, diabetes mellitus, obesity and dyslipidaemia serve as targets for prevention and treatment of atherosclerotic diseases. A substantial body of evidence confirms that reducing risk factors reduces the morbidity and mortality associated with atherosclerotic diseases [Critchley and Capewell, 2003b;Ford et al., 2007].

However, it is notable that conventional risk factors predict less than 50% of cardiovascular events [Heller et al., 1984]. Consequently, a number of novel risk factors (Table 1.1) have emerged as potential predictors of atherosclerosis and its complications. Identification of these novel risk factors allows better insight into pathophysiology of the disease, and facilitates development of novel preventive and therapeutic interventions.

1.1.3 Pathogenesis of atherosclerosis

Whilst speculations about the pathogenesis of atherosclerosis date back to the 18th Century, the first scientific evidence was obtained from studies conducted in the second half of the 19th century. This initial work was based mostly on the gross and microscopic appearance of lesions from human autopsy specimens. Temporal studies of atherogenesis could only be carried out from the early 20th Century, following the introduction of suitable animal models.

Table 1.1: Risk factors for atherosclerosis

Conventional risk factors	Non-modifiable	Age Gender Ethnic origin Family history
	Modifiable	Smoking Obesity Hypertension Hypercholesterolemia Diabetes mellitus Insulin resistance
Emerging risk factors/markers	Inflammatory markers	C-reactive protein (CRP) Interleukins (e.g. IL-1, IL-6) Inter-cellular adhesion molecule-1 (ICAM-1) Serum amyloid A Soluble CD40 ligand
	Haemostasis factors	Platelet activation Platelet aggregation Fibrinogen von Willebrand factor Plasminogen activator inhibitor-1 (PAI-1) Tissue plasminogen activator
	Lipid related factors	Low-density lipoprotein (LDL) Oxidized LDL Remnant lipoproteins Apolipoproteins A1 and B Lipoprotein a
	Other factors	Homocysteine Angiotensin-converting enzyme Micro-albuminuria Viral infections Psychosocial stress Air pollution

There were various theories proposed in 19th Century to explain pathogenesis of atherosclerosis: for example, the encrustation theory (Rokitansky, 1851) suggesting that atherosclerosis begins in the intima with deposition of thrombus and its subsequent organisation by the infiltration of fibroblasts and secondary lipid deposition; the insudation theory (Virchow, 1856) proposing that lipids insudate into the arterial wall, where they interact with cells of the vessel wall and extracellular matrix, leading to initiation and development of atherosclerosis; haemodynamic theories of atherogenesis; and the response to injury hypothesis (reviewed by [Tegos et al., 2001]). The currently accepted "response-to-injury hypothesis" was proposed by Russell Ross in the 1970s, suggesting that injury to the endothelium could lead to platelet aggregation and intimal thickening, resulting eventually in atherosclerosis [Ross and Glomset, 1976]. In the intervening years, observational and experimental data from a number of studies have further elaborated this response to injury theory, which now presents the generally accepted view on atherogenesis [Fuster et al., 1992]. A variety of local and systemic factors (such as disturbances of blood flow at branch points, hyperglycaemia, oxidised lipids, cigarette smoking and viruses) can result in vascular insult leading to initiation and progression of atherosclerosis [Fishman et al., 1998].

1.1.4 The stages of atherogenesis

The American Heart Association's committee on vascular lesions has defined six different stages of development of atherosclerotic plaques [Stary et al., 1995]:

- Type I (initial) lesion comprises isolated macrophages and foam cells in the intima
- Type II (fatty streak) lesion is an accumulation of intra-cellular lipids and foam cells in the intima
- Type III (intermediate) lesion includes type II changes and a small extra-cellular lipid pool
- Type IV (atheroma) lesion has type II changes and a well defined core of extra-cellular lipids

- Type V (fibro-atheroma) lesion is defined as a lesion in which prominent new fibrous connective tissue has formed. This type of lesion may be subdivided into the Va lesion (in which the fibrous tissue is part of a lesion with a lipid core), the Vb lesion (in which part of the lesion is calcified) and the Vc lesion (in which the lipid core is absent).
- Type VI (complicated) lesion has features of plaque erosion/rupture, haemorrhage or thrombosis. It is subdivided by the superimposed features. Thus, disruption of the surface may be labelled as type VIa; haematoma or haemorrhage as type VIb; and thrombosis as type VIc. Type VIabc indicates the presence of all three features.

1.1.5 Atherosclerosis - an inflammatory disorder

Atherosclerosis was originally considered to be a lipid storage disease; but over the recent years, there has been a shift in thinking and it is now believed to represent a chronic inflammatory disorder [Ross, 1999].

Inflammation is considered to play a key role in plaque initiation, progression and disruption [Corrado et al., 2010]. Indeed, level of C-reactive protein (CRP), an acute phase protein released from liver in response to infection or inflammation, is a predictor of cardiovascular events [Danesh et al., 2000;Rutter et al., 2004]. The various forms of vascular injury, including elevated levels of modified low-density lipoproteins (LDL), incite pro-inflammatory effects leading to the recruitment of monocytes and T-lymphocytes which is the earliest feature of lesion formation [Munro and Cotran, 1988]. This infiltration by inflammatory cells is mediated by various chemokines including monocyte chemo-attractant protein type 1 (MCP-1) and interleukins [Gerszten et al., 1999]. These monocytes then differentiate into macrophages and up-regulate their scavenger receptor expression, allowing them to accumulate lipid and, thus, leading to the formation of foam cells and fatty streaks. These activated macrophages release numerous cytokines and growth factors including interleukins (IL, especially IL-1β, IL-6 and IL-8), tumour necrosis factor-α (TNF-α) and MCP-1. These factors, which can induce adhesion molecule expression, vascular permeability, leukocyte migration, macrophage activation and VSMC proliferation, are normally expressed in the vessel

wall but their concentrations are significantly higher in atherosclerotic plaques [Dewberry et al., 2000]. Inhibiting these inflammatory cytokines, for example IL-1, has been shown to reduce atherosclerosis in experimental models [Kirii et al., 2003] and their clinical potential is being investigated [Crossman et al., 2008].

1.1.6 Plaque stability

There are robust data to suggest that the major cause of mortality in atherosclerotic diseases is the disruption of atherosclerotic plaques leading to an athero-thrombotic occlusion of vessels, and not merely the size of plaques or degree of luminal obstruction [Zaman et al., 2000]. This growing evidence over the last decade has shifted the focus from just reduction in lesion size to reduction in both the size and vulnerability of lesions [Weissberg, 2000]. The significance of plaque stability has been emphasised, for example, by the demonstration that the clinical benefit provided by 3-hydroxy-3-methyl-glutaryl-CoA reductase **(**HMGR) inhibitors (statins) is greater than is predicted from the modest improvements in lumen diameter [Brown et al., 1993;Libby and Aikawa, 2003]. A number of factors can influence plaque stability as discussed here.

1.1.6.1 Systemic risk factors

Although the conventional cardiovascular risk factors (Section 1.1.2) robustly correlate with the extent of plaque formation, they are not strong predictors of plaque disruption and development of acute cardiac events [Body et al., 2008]. There is a suggestion of gender-related changes in plaque composition and a difference in type of plaque disruption [Farb et al., 1996]. Whilst deep plaque rupture reaching into the lipid core is more common in men, women tend to have plaques rich in smooth muscle cells and matrix proteins rather than lipids and macrophages and plaque rupture usually results in superficial erosions only [Farb et al., 1996]. Smoking tends to increase the oxidised LDL content and thrombogenic potential of plaques [Wissler, 1994]. The clinical data also highlight the effect of dyslipidaemia and statin treatment on stability of plaques [Takano et al., 2003]. However, the therapeutic benefit of statins may not only be via reduction in systemic cholesterol [Libby and Aikawa, 2003]. Diabetic patients are also more prone to plaque erosions and rupture, independent of severity or extent of lesions [Schwartz and Bornfeldt, 2003].

1.1.6.2 Haemodynamic factors

Haemodynamic factors such as local disturbances in flow velocity and alterations in shear stress can also affect plaque stability. Circumferential tensile stress on the fibrous cap is recognised as an important mechanical factor involved in plaque rupture [Cheng et al., 1993]. However, these mechanical effects can also modulate plaque composition by affecting endothelial cell function and consequently recruitment of inflammatory cells, expression of adhesion molecules and production of growth factors [Walpola et al., 1995]. It has been shown that upstream parts of plaques have more macrophages whereas the downstream margin (distal shoulders) has more smooth muscle cells [Dirksen et al., 1998].

1.1.6.3 Plaque composition

A typical atheromatous plaque has a fibrous cap and a central core which contains extracellular lipids, inflammatory cells, necrotic tissue and fibrous components. Plaques rich in fibrous tissue are stable whereas vulnerable plaques are characterized by large lipid pools [Davies, 1996]. Post-mortem studies have revealed that lipid-rich plaques are frequently found beneath coronary thrombosis [Falk, 1989]. Furthermore, atherectomy samples obtained from patients with unstable coronary artery disease frequently contain more extracellular debris than those of patients with stable angina [Rosenschein et al., 1994]. Lipid-rich plaques are, therefore, considered “rupture prone”.

The thickness or strength of the cap is another important predictor of plaque stability [Li et al., 2006]. Similarly the composition of cap is important: collagen provides the biomechanical strength to the cap [Libby, 2000], whereas infiltration by inflammatory cells makes it prone to rupture [Lendon et al., 1991].

Plaques contain variable amounts of inflammatory cells which have an inverse correlation with plaque stability. Macrophages are a major cellular source of matrix metalloproteinases (MMPs) within the atherosclerotic lesion. MMPs can contribute to degradation of the stabilizing matrix and digestion of the fibrous cap [Galis et al., 1994]. Macrophages within a vulnerable plaque also express tissue factor on their surface. Tissue factor is an essential component for activation of the coagulation cascade when

plaque components are exposed to the blood following rupture and, therefore, increases athero-thrombotic potential leading to vascular occlusion [Hatakeyama et al., 1997].

Smooth muscle cells also form an important component of atherosclerotic plaque. They produce extracellular matrix and fibrous components and thereby serve to encapsulate the soft atheroma and, thus, stabilise plaque. Various inflammatory cytokines, growth factors (such as transforming growth factor beta, TGF-β) and thrombin (generated during episodes of local thrombosis) act as stimulants for smooth muscle cell proliferation/migration and connective tissue production [Newby, 1997].

1.1.7 Treatment and prevention of atherosclerosis

1.1.7.1 Primary prevention

Atherosclerosis is a chronic progressive disease that starts in childhood and adolescence, but usually remains asymptomatic until middle age. This has led to a growing emphasis on primary prevention. The American Heart Association has issued guidelines for primary prevention of atherosclerotic disease in childhood [Kavey et al., 2003]. Primary prevention is generally targeted towards risk factor modification by adopting a healthy life style (healthy eating, exercise, smoking cessation etc), controlling glycaemic and lipid profile by pharmacological and non-pharmacological measures and blood pressure control [Pearson et al., 2002].

1.1.7.2 Secondary prevention

Although there is no definite role for anti-platelet drugs (such as aspirin or clopidogrel) in primary prevention, there are robust data to suggest that these drugs reduce recurrent ischemic events in patients with established atherosclerotic disease [Budaj et al., 2002;Baigent et al., 2009]. Based on the guidelines from the expert panel of the National Cholesterol Education Programme, patients with established atherosclerosis should also be treated with lipid-lowering drugs [ATP III, 2001]. Although niacin therapy may be used for patients with low levels of high-density lipoproteins (HDL) and fibrates for those with high triglycerides, the mainstay of lipid lowering is treatment with statins[Maron et al., 2000]. The patients with established atherosclerotic disease also need an aggressive management of other risk factors which may require use of anti-

diabetic and anti-hypertensive drugs. Most of these patients with cardiovascular disease require a combination of drugs, including treatment with beta-blockers and inhibitors of angiotensin converting enzyme (ACE), for symptomatic and prognostic reasons [Hippisley-Cox and Coupland, 2005]. Finally, advanced or symptomatic lesions can be treated with percutaneous (angioplasty and stenting) or surgical (coronary artery bypass grafting, CABG) revascularisation procedures [Schofield, 2003].

1.1.7.3 Need for novel therapeutic targets

Recent epidemiological data have shown a decline in mortality from atherosclerotic diseases in the developed countries as a consequence of better prevention and treatment [Fox et al., 2007]. However, despite recent advances in pharmacological and non-pharmacological interventions, there are warning signs that the decline in cardiovascular mortality may be attenuated or even reversed, as a late consequence of the rising prevalence of obesity and diabetes. Evidence that the decline in mortality has attenuated is already apparent in the younger (35-44 years) age group [Critchley and Capewell, 2003]. Hence, there is a growing need to identify and develop novel therapeutic targets for treatment of metabolic syndromes and atherosclerosis.

1.1.8 The *ApoE*$^{-/-}$ mouse as an experimental model of atherosclerosis

Animal models are necessary for pre-clinical research to study the mechanism of atherogenesis and the effects of anti-atherosclerotic interventions. Ideally, a model should exhibit similar plaque morphology and biology to humans, and a similar response to various therapies. Since the first use of fat-fed rabbits in the early 20th Century, numerous animal species have been used to study atherosclerosis [Bocan, 1998]. Although rabbits do not develop spontaneous atherosclerosis, they are sensitive to fat feeding and promptly develop fatty streaks. However, there is a striking (~ 40x) difference in cholesterol levels between humans and fat-fed rabbits [PRIOR et al., 1961]. Rats and dogs are not good models because they do not develop spontaneous lesions and require profound dietary modifications to produce vascular lesions [THOMAS et al., 1960]. The cholesterol fed pig is a reasonably good model and develops lesions similar to those seen in humans. However, diet induced atherosclerosis (without surgical/genetic interventions) can take months to develop and, therefore, there are cost, maintenance and

handling issues. Non-human primates may develop lesions similar to humans with modest dietary interventions. These lesions can be assessed with techniques used in clinical practice and, therefore, they are better suited to model human atherosclerotic lesions [Bush and Verlangieri, 1989;Takai et al., 2005]. However, they are not widely used due to cost and ethical concerns.

The use of mice is desirable in research because they are economical to maintain, well-characterized and ideal for genetic modifications. However, rodents are generally resistant to atherogenesis, which usually has to be induced by genetic manipulation and/or mechanical injury; often combined with feeding a high fat diet [Kashyap et al., 1995;Russell and Proctor, 2006]. In 1992, apolipoprotein E deficient (*ApoE*$^{-/-}$) mice were generated by inactivating the *ApoE* gene in mouse embryonic stem cells by homologous recombination [Piedrahita et al., 1992]. Apolipoprotein E is present on the surface of several lipoproteins, including chylomicrons, very-low-density lipoprotein (VLDL) and HDL particles. It is an important modulator of lipoprotein interactions with several receptors, including LDL receptors and LDL-related receptors [Mahley, 1988]. *ApoE*$^{-/-}$ mice have, therefore, defective hepatic clearance of cholesterol resulting in a five-fold increase in total plasma cholesterol levels, 68% higher triglyceride levels but only 45% higher HDL levels [Plump et al., 1992]. This model has been studied extensively for the effects of genetic and pharmacological interventions on the development of lesions. However, this model has its own limitations; the response to different pharmacological interventions may not always mirror the response seen in humans (reviewed by [Zadelaar et al., 2007]) and the small size of mice may pose some technical challenges, such as the imaging of atherosclerotic plaques and acquisition of sufficient blood to characterise biochemical events [Daugherty, 2002].

The lesion formation in *ApoE*$^{-/-}$ mice has been characterized during feeding of both standard chow and high-fat diet and resembles atherogenesis in humans [Zhang et al., 1992;Nakashima et al., 1994]. Monocyte adhesion to intact endothelium, one of the earliest cellular events in atherogenesis, can be detected in 2-month old *ApoE*$^{-/-}$ mice fed on chow. Lesions predominantly containing foam cells can be seen from 3-months onwards and fibro-fatty lesions became apparent from 4-months onwards [van Ree et al., 1994]. *ApoE*$^{-/-}$ mice fed on high-fat diet show accelerated progression of atherogenesis

and can also develop complex lesions in many vascular areas, including the aortic sinus, aortic arch, brachiocephalic trunk (BCT), renal arteries and coronary arteries [van Ree et al., 1994;Rosenfeld et al., 2000]. Although intra-plaque haemorrhage has been demonstrated in advanced lesions in the BCT, there is no convincing evidence that these mice develop spontaneous plaque rupture [Rosenfeld et al., 2000].

The advanced, complicated or symptomatic atherosclerotic lesions are often treated with percutaneous revascularisation (angioplasty and stenting) procedures [Keeley et al., 2003;Smith, Jr. et al., 2001]. However, the long term outcome of these revascularisation procedures is hampered by development of restenosis due to neointimal proliferation [Bennett, 2003].

1.2 Neointimal proliferation

Although neointimal proliferation shares many pathophysiological components with atherogenesis, it is a distinct pathological and clinical entity [Wang and Paigen, 2002]. Whilst atherosclerosis represents a chronic response to a systemic vascular insult, neointimal proliferation in response to acute and focal (usually iatrogenic) vascular injury comprises an acute inflammatory response followed by a substantial fibro-proliferative response. This results in lesions rich in fibro-cellular elements rather than the lipid rich plaques seen in atherosclerosis. Atherosclerosis and neointimal proliferation may also have different genetic determinants [Kuhel et al., 2002].

1.2.1 Pathogenesis of neointimal proliferation

Angioplasty (± stenting) results in an iatrogenic injury to intimal and medial layers with activation of haemostatic, inflammatory and fibro-proliferative processes leading to the formation of neointimal lesions [Bennett, 2003].

1.2.1.1 Platelet activation

Angioplasty-induced endothelial cells denudation and medial tearing exposes circulating blood cells to the sub-endothelial matrix containing numerous platelet-activating factors including thrombin, thromboxane, platelet-activating factor and collagen. This leads to platelet activation, aggregation and release of their contents into the injured arterial wall

[Chandrasekar and Tanguay, 2000]. Anti-platelet therapy has been shown to not only reduce acute thrombosis following angioplasty [Barnathan et al., 1987;Mishkel et al., 1999] but also to reduce neointimal proliferation [Akbulut et al., 2004] and, therefore, strongly recommended in all patients undergoing angioplasty [King, III et al., 2008].

1.2.1.2 Inflammatory cell infiltration

Inflammatory cells are involved in the acute response to arterial injury. After balloon injury, monocyte-derived macrophages are seen within the neointimal lesions [Ferns et al., 1991c]. In the cholesterol-fed rabbit these macrophages are transformed into macrophage-derived foam cells, and constitute a substantial part of the neointima [Ferns et al., 1992]. The importance of inflammatory cytokines in the vascular response to injury has been further highlighted in experiments where blocking various cytokines, such as CD11b/CD18 [Rogers et al., 1998], IL-1 [Morton et al., 2005], or IL-10 [Feldman et al., 2000], produced a reduction in neointimal proliferation. IL-1, an extensively studied pleotropic cytokine, has been shown to stimulate VSMC proliferation [Raines et al., 1989] and expression of cell adhesion molecules on endothelial and smooth muscle cells [Wang et al., 1995]. It has been shown that a genetic polymorphism in the IL-1 receptor antagonist (IL-1ra) gene correlates with post-angioplasty remodelling in humans [Francis et al., 2001]. SMC-derived TNF-α serves as a marker for an altered VSMC phenotype and may contribute to local cellular activation and proliferation of VSMCs at sites of arterial injury [Tanaka et al., 1996]. Increased MCP-1 expression following angioplasty has also been linked to restenosis and it has been reported that patients with raised plasma levels of MCP-1 have higher incidence of restenosis [Oshima et al., 2001]. Glucocorticoids can interact with inflammatory process (section 1.3.5) and therefore modulate neointimal lesion formation.

1.2.1.3 VSMC migration and proliferation

Neointimal lesions that develop after angioplasty are characterised by smooth muscle cell accumulation within the neointima [Waller et al., 1991]. Balloon injury is initially associated with medial smooth muscle cell death [Clowes et al., 1983]. This is followed by a phase of coordinated proliferation of medial smooth muscle cells and subsequent migration of medial smooth muscle cells into the intima in response to the release of

chemo-attractants such as platelet-derived growth factor (PDGF) [Ferns et al., 1991]. About 80% of the migrating cells are reported to be in the G1 and S phases of the cell cycle resulting in further proliferation of these intimal smooth muscle cells [Yoshida et al., 1997]. Data are also accumulating to suggest that the circulating bone marrow-derived progenitor cells serve as a complementary source of intimal smooth muscle (and smooth-muscle like) cells during vascular remodelling [Han et al., 2001;Kumar et al., 2010].

Among the various growth factors involved in post-angioplasty vascular remodelling, PDGF, TGFβ1 and fibroblast growth factor (FGF) are considered to play the main role. The importance of PDGF as a VSMC chemo-attractant has been demonstrated in several species including rats, rabbits and non-human primates [Ferns et al., 1991a;Levitzki, 2005]. TGFβ1 plays a central role in vascular lesion development and tissue repair owing to its effects on cell growth and extracellular matrix synthesis [McCaffrey, 2009]. It also stimulates adventitial fibroblast differentiation into myofibroblasts after injury, a process that may affect arterial remodelling [Shi et al., 1996]. The binding of cytokines and growth factors to their respective receptors stimulates mitogen-activated protein (MAP) kinases. MAP kinases are involved in nuclear signalling and their expression increases after angioplasty [Hu et al., 1997] with resultant increase in cellular proliferation.

Inhibition of VSMC proliferation is, therefore, an attractive mechanism for preventing neointimal lesion formation and, indeed, the pharmacological agents (sirolimus or paclitaxel) in currently used drug eluting stents (DES) have anti-proliferative characteristics.

1.2.1.4 Re-endothelialisation

Endothelial injury during angioplasty initiates a vascular response to injury and re-endothelialisation helps to curtail this response. Whilst, small/superficial injuries may heal without neointimal proliferation, deep/wide injuries are usually associated with substantial neointimal thickening [Reidy and Silver, 1985]. Arteries that have sustained extensive endothelial damage may not become fully re-endothelialised but instead become lined by phenotypically-modified smooth muscle cells [Reidy and Silver, 1985].

It has also been reported that endothelium dependent vascular wall reactivity may be affected after balloon angioplasty, and that the duration of impaired responses are related to the extent of injury [Weidinger et al., 1990].

Delayed re-endothelialisation after angioplasty increases the risk of late thrombosis [Ertas et al., 2009]. Currently used DES (containing sirolimus and paclitaxel) may have unfavourable effects on re-endothelialisation due to the non-specific action of these drugs on cell proliferation [Bavry et al., 2006] and, therefore, can increase the risk of late thrombosis [McFadden et al., 2004]. Endothelial progenitor cells, stem cells derived from various sources, and other promoters of endothelial regeneration are being evaluated to improve re-endothelialisation [Wu et al., 2008;Barsotti et al., 2009].

1.2.2 Prevention and treatment of neointimal proliferation (restenosis)

1.2.2.1 Stents

Stents were first introduced in 1986 and their use has increased exponentially over the last decade, as they are the most effective mechanical means for reducing post angioplasty restenosis [Erbel et al., 1998]. Stents are thin cylindrical wire meshes (diameter 3-5mm; length 1.5-3.0cm) and are commonly made of stainless steel (bare metal stent, BMS). Coating stainless-steel stents with gold, carbide, phosphorylcholine or heparin does not appear to confer any additional benefit compared with BMS [Birkenhauer et al., 2004]. In contrast, randomised clinical trials have shown that coating stents with various anti-proliferative drugs reduces restenosis rates compared with BMS [Weisz et al., 2009;Ellis et al., 2009].

1.2.2.2 Anti-proliferative drugs

In the era of stents, restenosis is mainly due to neointimal proliferation, rather than elastic recoil and constrictive remodelling [Bennett, 2003]. The recent research has, therefore, mainly focused on reducing neointimal proliferation.

Sirolimus and paclitaxel are the two most commonly used drugs for stent coating in clinical practice. Sirolimus acts by receptor inhibition of the mammalian target of rapamycin (mTOR), resulting in the cessation of cell-cycle progression in the late G1 to

S phases and, consequently, inhibiting VSMC proliferation [Poon et al., 1996]. Paclitaxel inhibits cell proliferation and migration by disrupting cellular microtubule organisation [Axel et al., 1997]. There are robust data from multi-centre trials to suggest that stents coated with these drugs prevent restenosis [Stone et al., 2004;Ellis et al., 2009;Moses et al., 2003;Weisz et al., 2009]. Various other agents, for example inhibitors of growth factors or transcriptional factors, are being evaluated [Bilder et al., 2003].

1.2.2.3 Anti-inflammatory agents

Inflammation is a key mediator of the vascular response to injury and, therefore, anti-inflammatory agents have been tested to limit neointimal proliferation [Welt and Rogers, 2002]. There has been some success with IL-1 inhibition in a porcine coronary angioplasty model [Morton et al., 2005] and cyclo-oxygenase 2 inhibition in humans [Koo et al., 2007]. It has also been suggested that part of the benefit seen from sirolimus or paclitaxel eluting stents is also derived through an anti-inflammatory mechanism [Suzuki et al., 2001]. However, the use of anti-inflammatory steroids (discussed in section 1.3) has produced conflicting results.

1.2.2.4 Anti-thrombotic agents

As the steel matrix used in BMSs offers a highly thrombogenic surface, anti-platelet drug therapy is necessary to prevent thrombotic events. Dual anti-platelet treatment (aspirin and clopidogrel) is currently recommended for 6-12 months after stent deployment, followed by indefinite use of low dose aspirin [King, III et al., 2008].

Anti-platelet treatment with abciximab (a glycoprotein IIb/IIIa inhibitor) has been shown to be effective in a porcine model of neointimal proliferation [Hong et al., 2007] and anti-thrombotic recombinant pegylated-hirudin and the prostacyclin analogue iloprost have also been shown to reduce neointimal proliferation in pigs and sheep (Alt et al 2000). Heparin, although it inhibits VSMC proliferation *in-vitro* (Au et al 1993; Chajara & Capron 1993; Yang et al 1999), did not prevent restenosis when administered systemically (Brack et al., 1995) or from DESs (Semiz et al 2003).

1.2.2.5 Hormonal agents

Oestrogen reduces VSMC proliferation and stimulates endothelial regeneration after angioplasty [Geraldes et al., 2002]. In a porcine model, oestrogen eluting stents were associated with reduced neointimal proliferation without affecting endothelial regeneration [New et al., 2002]. Although the first study in humans (EASTER trial) suggested that oestrogen-eluting stents may reduce restenosis [Abizaid et al., 2004], subsequent multicentre trials (ETHOS-1 and ISAR-PEACE) failed to show any benefit [Ryu et al., 2009]. The effect of glucocorticoids on neointimal proliferation is complex. Experimental studies in general have suggested that glucocorticoids (administered systemically or locally) can reduce neointimal proliferation [Villa et al., 1994c;Guzman et al., 1996;Poon et al., 2001;Ribichini et al., 2007;Strecker et al., 1998;Van Put et al., 1995]. However, clinical trials have shown variable and often contradictory results. Oral prednisone reduced restenosis after stenting (the IMPRESS trial) [Versaci et al., 2002;Ferrero et al., 2007] and low dose dexamethasone was associated with low restenosis rate in the STRIDE trial [Liu et al., 2003]. On the other hand, methylprednisolone did not inhibit restenosis after coronary angioplasty [Pepine et al., 1990] or stenting [Reimers et al., 1998] and dexamethasone-drug eluting stents (D-DES) did not reduce the incidence of restenosis [Hoffmann et al., 2004;Ribichini et al., 2007]. The role of glucocorticoids in neointimal lesion formation is discussed further in section 1.3.9.

1.2.2.6 Need for novel therapeutic targets

The currently used DESs containing sirolimus and paclitaxel have greatly reduced the prevalence of post angioplasty restenosis [Ellis et al., 2009;Weisz et al., 2009]. However, these anti-proliferative drugs lack selectivity with respect to the targeted cell types. Therefore, they not only inhibit VSMC proliferation underlying neointima formation, but also compromise endothelial repair and, hence, increase the risk for late thrombosis [Bavry et al., 2006]. Accordingly, there is a need for novel agents capable of inhibiting restenosis without clinically-relevant, adverse effects. In the recent years, a number of drug types have been tested and reported as therapeutic targets to prevent restenosis. Such agents target inflammation [Morton et al., 2005], VSMC proliferation

[Selvendiran et al., 2009] and ion channels [Kohler et al., 2010]. However, randomised clinical trials and long-term outcome data are yet to support use of such agents.

1.2.3 Experimental models of neointimal proliferation

Experimental models are needed to develop and test novel therapeutic agents for neointimal proliferation. Carotid artery injury in rabbits/rats was one of the earliest models used to study the mechanisms of restenosis [Groves et al., 1979;Clowes et al., 1983]. A variety of animal models are currently available; however, there are anatomical/procedural and pathophysiological/biochemical differences between these models and humans [Lafont and Faxon, 1998]. Whilst coronary angioplasty in humans is used to dilate arteries with advanced atherosclerotic disease, animal models usually employ angioplasty of arteries with no pre-existing stenosis. The force applied to the vessel wall also differs in magnitude and nature. The compliance of human arteries containing advanced atherosclerotic and calcified lesions is usually poor, and angioplasty can cause considerable tissue damage, including intimal tears and splitting of the tunica media (Waller 1989; Brady & Warren 1991). Finally, experimental animals usually lack the variety of risk factors seen in patients undergoing angioplasty (e.g. diabetes, hypertension, obesity). Attempts have been made to improve the study of the pathogenesis and treatment of restenosis by combining models of atherosclerosis and acute injury [Leidenfrost et al., 2003;Weingartner et al., 2005;Abela et al., 1995;Skinner et al., 1995;Aikawa et al., 1998], in which angioplasty is performed in arteries with existing lesions. However, these models are complex, require a longer time period and are technically challenging [Lardenoye et al., 2000;Ivan et al., 2002]. The animal models, despite their limitations, have been useful for understanding restenosis in humans, and for testing novel therapies. However, care must be exercised when interpreting these results and extrapolating them to humans.

The models of neointimal lesion formation can be categorised into large animal (such as pigs, dogs and non-human primates) and small animal (including rabbits , rats and mice) models [Mehta et al., 1996;Narayanaswamy et al., 2000].

1.2.3.1 Large animal models

The obvious advantage of large animals is their anatomical and physiological resemblance to humans. The response to vascular injury in pigs [Schwartz et al., 1990], dogs [Lafont and Faxon, 1998] and primates [Geary et al., 1996] resembles that in humans. The clinical relevance of large animal models is demonstrated by their ability to predict the effect of therapeutic interventions in humans (not without exceptions [Lafont and Faxon, 1998]). Moreover, it is possible to use the same catheters and devices that are used for angioplasty in humans. However, the use of large animals imposes issues such as higher purchase and maintenance costs, difficulties associated with husbandry and handling, and slower development of lesions.

1.2.3.2 Small animal models

Small animal models are useful in mechanistic experiments, for example, to study inflammation or VSMC proliferation in response to vascular injury [Johnson et al., 1999]. These animals are readily available, cost less to purchase and house, are easy to handle and their small size reduces the amount of drug needed for interventional 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 reliable statistical analysis. However, the small size of arteries studied, the need to assess lesion formation in arteries that are not relevant to the clinical situation (e.g. femoral, iliac), and poor replication of the condition in humans are obvious disadvantages [Lafont and Faxon, 1998;Johnson et al., 1999].

1.2.3.3 Mouse models of neointimal proliferation

Mouse is a useful animal to investigate neointimal proliferation due to the availability of genetically modified strains [Allayee et al., 2003], making it possible to assess lesion development in animals deficient in, or over-expressing, key factors involved in the vascular response to injury. Although various interventions (such as application of a peri-vascular cuff, arterial ligation, and chemical or electrical injury) have been used to induce neointimal lesion formation, these models are poor representative of post-

angioplasty neointimal proliferation. Intra-luminal injury models have, therefore, been developed over recent years [Lindner et al., 1993;Sata et al., 2000;Dover et al., 2007].

Intra-luminal mechanical injury in mice using a flexible guide-wire introduced into the common carotid artery was first described by Linder *et al.* [Lindner et al., 1993]. However, the lesions produced in mouse carotid arteries are small, have a high inter-animal variability and may produce intra-cerebral complications, including stroke [Lindner et al., 1993]. Therefore, the same technique has been applied to the femoral arteries [Roque et al., 2000;Sata et al., 2000] producing large, often occlusive, lesions and fewer procedural complications. Intra-luminal injury causes endothelial denudation, and stretches the arterial 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 endothelial 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, rich in macrophages and VSMCs, are first evident one week after the injury and continue to grow in size until 3-4 weeks [Lindner et al., 1993;Sata et al., 2000]. More recently, a method of balloon injury to the mouse aorta [Petrov et al., 2005] and a stent model [Chamberlain et al., 2010] have also been developed.

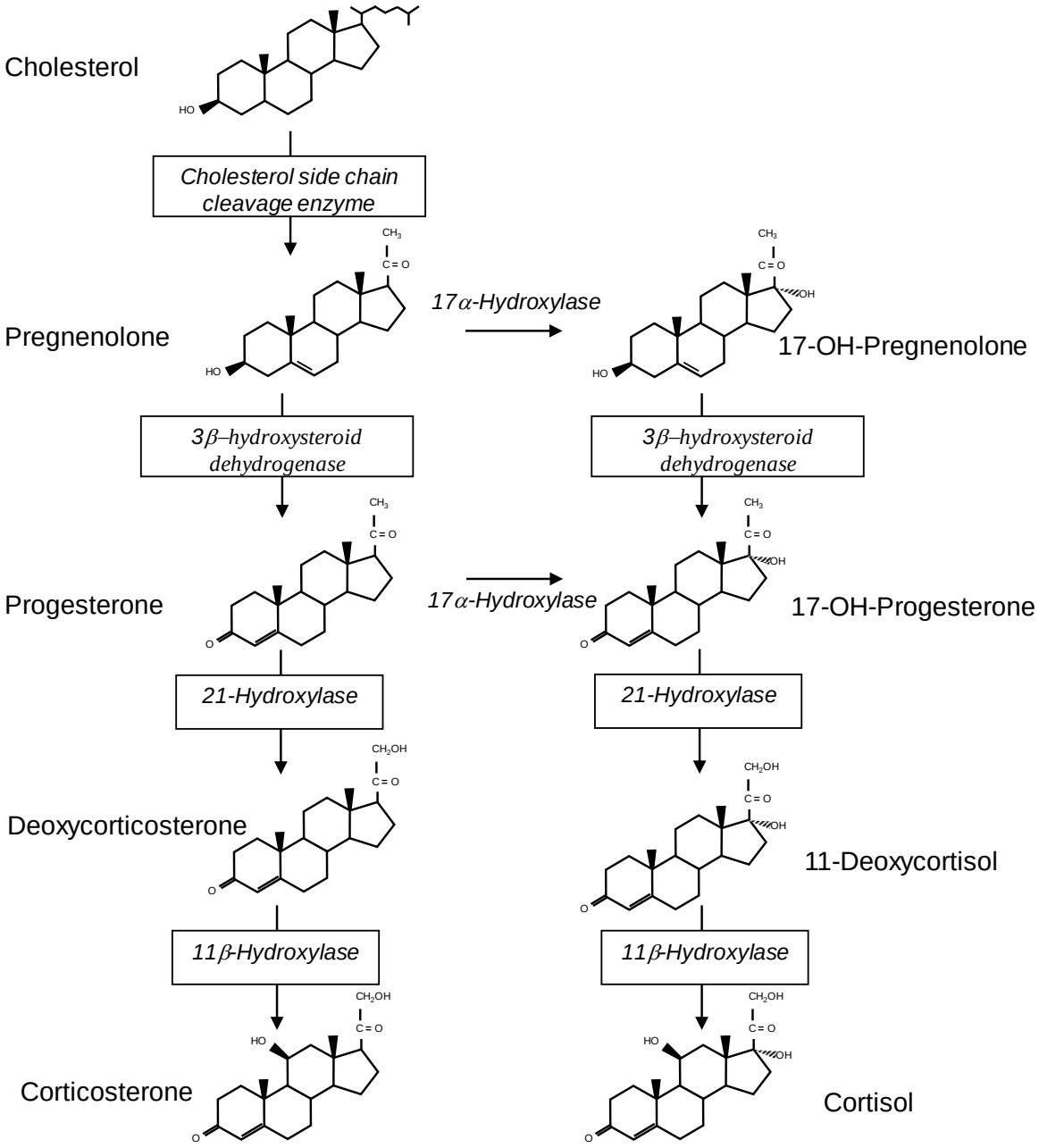
1.3 Glucocorticoids and the cardiovascular system

Glucocorticoids are stress hormones with a key role in the regulation of metabolic and immune responses. The name glucocorticoid (glucose + cortex + steroid) derives from their role in the regulation of the metabolism of glucose, their synthesis in the adrenal cortex, and their steroidal structure.

1.3.1 Synthesis and metabolism

Glucocorticoid synthesis from cholesterol (Figure 1.2) occurs in the zona fasciculata of the adrenal cortex and is tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Cortisol is the major glucocorticoid in man, whereas corticosterone predominates in rodents due to lack of the enzyme 17α-hydroxylase.

Figure 1.2: Synthesis of glucocorticoids



Glucocorticoids are synthesised *de novo* from cholesterol in the zona fasciculata of the adrenal cortex. In humans, cortisol is the principal glucocorticoid hormone; however, rodents lack the enzyme 17α-hydroxylase (required to convert pregnenolone or progesterone to their hydroxy metabolites), and, therefore, corticosterone is their predominant glucocorticoid.

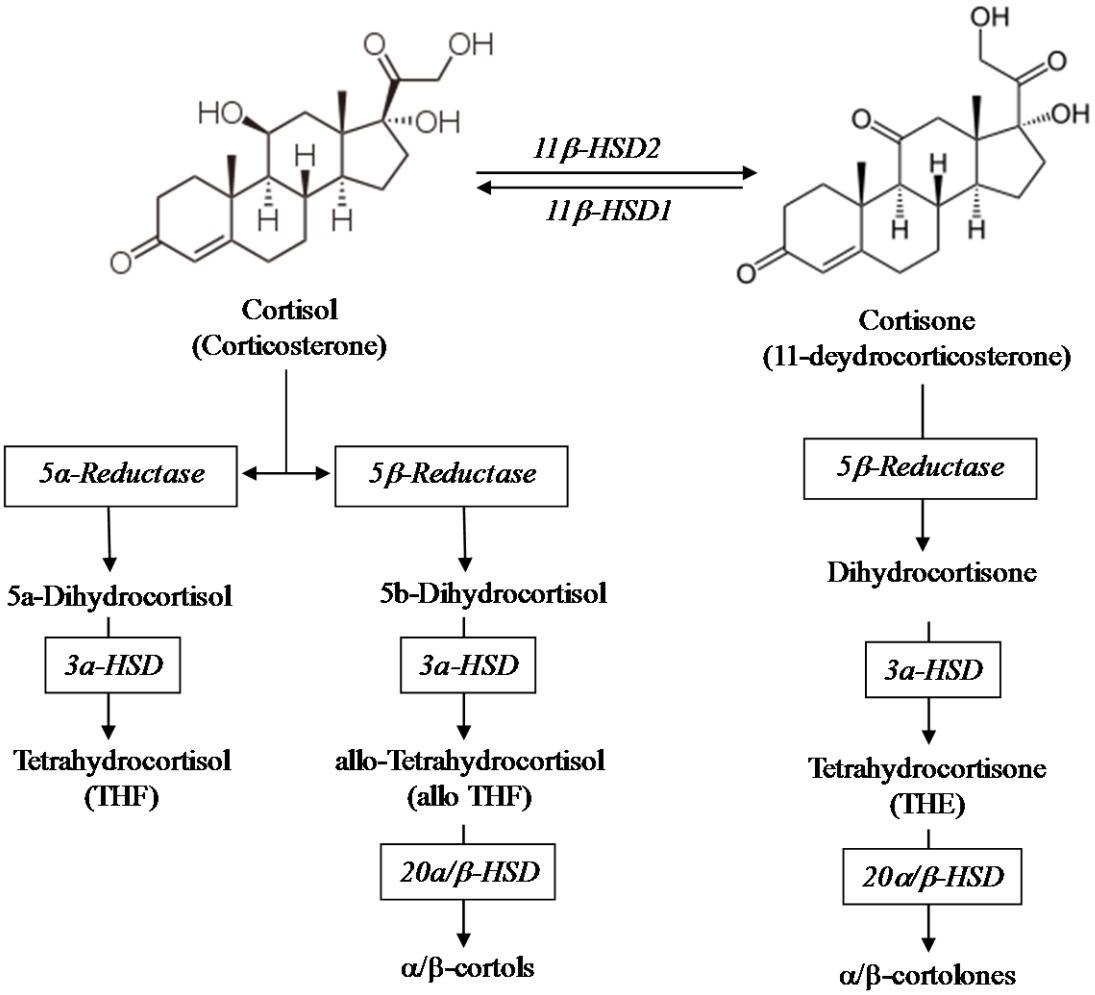
Glucocorticoids are produced *de novo* and released into the blood as required, exhibiting a diurnal variation with concentrations reaching peak levels in the early morning and diminishing in the evening [Dallman et al., 1993]. On secretion into the blood, approximately 90% of glucocorticoids are bound to corticosteroid-binding globulin and albumin, and only the unbound fraction is available to interact with receptors [Hammond et al., 1990]. Metabolic inactivation of glucocorticoids (Figure 1.3) occurs predominantly in the liver with inactive metabolites excreted in the urine.

1.3.2 Molecular actions of glucocorticoids

The classic glucocorticoid actions are mediated by the ubiquitously expressed glucocorticoid receptors (GR). GR are members of the nuclear receptor super family of ligand activated transcription factors and form multi-protein complexes with numerous proteins (such as heat-shock proteins). Glucocorticoids enter a cell probably via passive diffusion and bind to their receptors, prompting dissociation of heat shock proteins, receptor dimerisation and translocation to the nucleus. The ligand-receptor complex then binds to glucocorticoid response elements in the promoter region of target genes, and activates transcription (trans-activation) or inhibits the function of transcription factors (trans-repression) [Gower, Jr., 1993;Schaaf and Cidlowski, 2002]. In addition to these transcriptional or 'genomic' effects, glucocorticoids can also induce rapid, so-called non-genomic effects, by affecting the physicochemical properties of cell membranes, or through membrane-bound GR or by initiation of signal transduction within the cytosol [Hafezi-Moghadam et al., 2002].

The main actions of glucocorticoids mediated by GR stimulation are: regulation of carbohydrate and protein metabolism, negative feedback on the HPA axis, regulation of cell proliferation and differentiation, and anti-inflammatory and immunosuppressive effects [Munck et al., 1984]. Glucocorticoids are also required for blood pressure maintenance [Ullian, 1999] although the mechanisms involved are complex and incompletely understood [Raff, 1987;Shields et al., 1988;Montrella-Waybill et al., 1991;Ullian, 1999].

Figure 1.3: Metabolism of glucocorticoids



Metabolic inactivation of glucocorticoids occurs predominantly in the liver, and also in the kidney, with inactive metabolites excreted in the urine. This involves a complex modification process in which glucocorticoids and their 11-keto-metabolites (cortisone, 11-dehydrocorticosterone)] are reduced in a pathway involving 5α- and 5β-reductases, 3α-hydroxysteroid dehydrogenase (HSD), 20α- and 20β-hydroxysteroid dehydrogenases followed by conjugation with glucuronic acid or sulphates. Metabolism by 11β-HSD enzymes is discussed in detail in next section.

1.3.3 Systemic glucocorticoid regulation by HPA axis

Glucocorticoid concentration in systemic circulation is regulated by hypothalamic-pituitary-adrenal (HPA) axis which provides a feedback loop. The main hormones that activate the HPA axis are corticotropin-releasing hormone (CRH) and adrenocorticotropin hormone (ACTH). The loop is completed by the negative feedback of cortisol on the hypothalamus and pituitary [Dallman et al., 1994]. The HPA axis exhibits a well-characterized circadian rhythm that is under the control of the suprachiasmatic nuclei of the hypothalamus, whose activation, in turn, is regulated by light [Moore and Eichler, 1972]. Cortisol is released by the adrenal cortex in a pulsatile manner, driven by the pulsatile release of CRH by the hypothalamus, which then leads to the release of ACTH by the pituitary. Increased glucocorticoid secretion at the circadian peak depends on increased hypothalamic-pituitary activity and on increased sensitivity of the adrenal cortex to ACTH [Jacobson, 2005].

Activation of the HPA axis, with increased cortisol secretion rate and elevated morning plasma cortisol levels, has been associated with central obesity, insulin resistance, hyperglycaemia, dyslipidaemia and hypertension [Walker, 2007]. Relationships of plasma cortisol with obesity, however, are more complex, since obesity is associated with increased metabolic clearance rate of cortisol, which tends to lower plasma cortisol levels despite enhanced cortisol production rate [Walker, 2007]. Given the similarities between Cushing's syndrome and the metabolic syndrome, subtle abnormalities of HPA have been sought and found in subjects with cardiovascular risk factors [Rosmond and Bjorntorp, 2000].

Although systemic circulatory levels of glucocorticoids are regulated by the HPA axis, there is another level of regulating glucocorticoid activity which is regulated at intra-cellular level and is the main theme of this thesis.

1.3.4 Intra-cellular glucocorticoid regulation by 11β-HSD enzymes

The intra-cellular, pre-receptor regulation is catalysed by the 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes, originally described more than 50 years ago [Amelung et al., 1953;Hubener et al., 1956]. Two isozymes of 11β-HSD, type 1 and type

2, have been identified, both of which are microsomal enzymes of the short-chain alcohol dehydrogenase super family [Stewart and Krozowski, 1999] and catalyse the inter-conversion of active glucocorticoids and their inert 11-keto forms [Amelung et al., 1953]. These isozymes can also catalyse the inter-conversion of the oxysterols, 7-ketocholesterol and 7β-hydroxycholesterol [Hult et al., 2004].

1.3.4.1 11β-HSD1

11β-HSD1 is a low affinity (Km in the μM range for both cortisol and corticosterone [Lakshmi and Monder, 1988]) nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme. 11β-HSD1 is widely expressed in many glucocorticoid-target tissues (including, liver, lung, adipose tissue, brain, vascular smooth muscle, skeletal muscle, anterior pituitary, gonads and adrenal cortex) [Stewart and Krozowski, 1999], where it amplifies local glucocorticoid concentrations [Seckl and Walker, 2001]. 11β-HSD1 synthesis and activity are regulated by a complex interaction of many factors, including: glucocorticoids [Hammami and Siiteri, 1991;Takeda et al., 1994;Low et al., 1994], stress [Jamieson et al., 1997;Walker et al., 1994], sex steroids [Low et al., 1994], growth hormone [Painson et al., 1992], cytokines [Cai et al., 2001] and peroxisome proliferator activated receptor agonists [Tomlinson et al., 2001].

11β-HSD1, although originally described as bi-directional enzyme, acts predominantly as an oxo-reductase *in vivo*. Intact cells or organs, including liver [Ricketts et al., 1998;Jamieson et al., 1995;Jamieson et al., 2000], adipose tissue [Bujalska et al., 1997], neurones [Rajan et al., 1996] and vascular smooth muscle [Hatakeyama et al., 1999], do not generally exhibit dehydrogenase activity of this isozyme. NADPH is the co-factor required for oxo-reductase activity and is generated by an enzyme hexose-6-phospate dehydrogenase (H6PDH). 11β-HSD1 and H6PDH are co-expressed in the inner microsomal compartment of cells and H6PDH confers oxo-reductase activity upon 11β-HSD1 [Hewitt et al., 2005]. It has been shown that a direct relationship exists between 11β-HSD1 reducatse activity and H6PDH expression [Bujalska et al., 2005].

1.3.4.2 11β-HSD2

11β-HSD2, in contrast to 11β-HSD type 1, is a high affinity (Km for cortisol and corticosterone in the nM range) nicotinamide adenine dinucleotide (NAD)-dependent, exclusive dehydrogenase and converts active glucocorticoids into inactive 11-ketosteroids. It is expressed mainly in mineralocorticoid target tissues (for example, kidney, sweat glands, salivary glands and colon [Stewart and Krozowski, 1999]), where it protects MR from illicit occupation by glucocorticoids [Edwards et al., 1988;Funder et al., 1988]. 11β-HSD2 is also expressed in the heart and blood vessels, although the specific cellular localization and *in-vivo* activity in vascular tissues remains debatable. There are reports suggesting the presence of 11β-HSD2 in VSMC (Hatakeyama et al., 1999; Cai et al., 2001) and also in ECs (Brem et al., 1998) but a comprehensive analysis showed that in rodents 11β-HSD2 is predominantly localized to ECs [Walker et al., 1991;Christy et al., 2003] where it can influence vascular function by modulating endothelium-derived nitric oxide activity [Hadoke et al., 2001]. In these sites 11β-HSD2 is constitutive; its synthesis and activity are not highly regulated. It has also been noted that 11β-HSD2 is expressed in tissues (such as lung, lymph nodes, heart, blood vessel wall and placenta) which are not classic MR targets [Stewart et al., 1995;Waddell et al., 1998;Slight et al., 1996]. In the placenta 11β-HSD2 protects the foetus from exposure to maternal glucocorticoids [Murphy et al., 1974;Brown et al., 1996].

Inactivation of cortisol by 11β-HSD2 explains 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 circulating concentrations [Sheppard and Funder, 1987]. Thus, a key physiological role of 11β-HSD2 is to protect MR from inappropriate activation by glucocorticoids. Therefore, 11β-HSD2 inhibition (using liquorice or its derivatives), transgenic disruption in mice [Kotelevtsev et al., 1999], or congenital deficiency in man [Walker et al., 1992], produces the glucocorticoid-mediated 'syndrome of apparent mineralocorticoid excess' (SAME) with its characteristic hypertension [Walker and Edwards, 1994]. Excess MR activation is associated with hypertension and other cardiovascular risk factors (reviewed by [Connell et al., 2008]).

1.3.5 GR expression and activity in the cardiovascular system

GR (and also MR) have been demonstrated in cultured VSMC [Meyer and Nicholls, 1981;Scott et al., 1987], in endothelial cells (EC) [Inoue et al., 1999;Jun et al., 1999;Golestaneh et al., 2001;Newton et al., 2002] and in intact arteries [Kornel et al., 1982;Christy et al., 2003]. Vascular GR have been shown to be active as demonstrated by dexamethasone (a potent synthetic glucocorticoid) -mediated induction of ACE activity in rat aortic ECs [Sugiyama et al., 2005], cortisol-mediated inhibition of prostacyclin synthesis in rat aorta [Jeremy and Dandona, 1986] and both dexamethasone and cortisol -mediated increase in protein kinase C in porcine coronary arteries. All these responses are sensitive to GR antagonism [Maddali et al., 2005]. Whether membrane binding sites for corticosteroids are present, or have a role, in the vascular wall has not been established [Fernandez-Perez et al., 2008]. The nature of the interaction between glucocorticoids and vascular cells is complex and can also be affected by the duration of exposure; prolonged exposure can inhibit proliferation of cultured VSMCs whereas short exposures (2min-6 hours) can stimulate proliferation [Kawai et al., 1998].

Both GR and MR are also expressed in the murine and human myocardium [Lombes et al., 1995;Pujols et al., 2002]. Their relationship to cardiac function has been demonstrated by conditional GR over-expression in the murine heart which induces atrio-ventricular block [Sainte-Marie et al., 2007]. Similarly, MR knockdown in mice induces severe heart failure and fibrosis [Beggah et al., 2002], whilst mice over-expressing cardiac MR develop ventricular arrhythmias [Ouvrard-Pascaud et al., 2005].

1.3.6 Glucocorticoids and cardiovascular risk factors

It was suggested by Adlersberg and colleagues (more than half a century ago) that cortisone administration can cause dyslipidaemia [Adlersberg et al., 1950a;Adlersberg et al., 1950]. Glucocorticoids have the potential to regulate the cardiovascular system by modulation of systemic risk factors [Walker et al., 1998]. Glucocorticoid excess can affect virtually all classical cardiovascular risk factors, including obesity [Dallman et al., 2004], insulin resistance [Andrews and Walker, 1999], hyperglycaemia [Clore and Thurby-Hay, 2009], hypertension [Whitworth et al., 1997] and dyslipidaemia [Walker,

2007] and, therefore, it is not surprising to see higher cardiovascular mortality in patients with Cushing's syndrome [Etxabe and Vazquez, 1994].

The association between excess glucocorticoids and hypertension has been known for many years but the underlying mechanisms remain poorly understood [Krakoff and Elijovich, 1981]. Glucocorticoid induced hypertension may involve multiple complex pathways influencing renal homeostasis, cardiac, vascular and central nervous systems [Simmons et al., 1996;Whitworth et al., 1997;Brem, 2001]. Glucocorticoid exposure during intra-uterine life has also been linked to the development of hypertension (and other cardiovascular risk factors) in adult life [Benediktsson et al., 1993]. The cardiovascular effects of glucocorticoids have been reviewed by Whitworth *et al* [Whitworth et al., 2005].

1.3.7 Glucocorticoids and vascular inflammation

Since inflammation is a key mediator of cardiovascular pathologies, inhibition of inflammation would be expected to reduce the development of fibro-fatty lesions in atherosclerosis, remove the stimulus for smooth muscle cell migration/proliferation in restenosis and also attenuate myocardial scarring and fibrosis [Wainwright et al., 2001;Miller et al., 2001].

Although glucocorticoids are generally considered to be anti-inflammatory hormones this is a rather simplistic notion. There are considerable data to suggest that glucocorticoids can also have pro-inflammatory / immuno-stimulatory actions [Dhabhar and McEwen, 1997;Yeager et al., 2004]. Indeed, it appears that glucocorticoids have dynamic interactions with inflammatory cells which may result in diametrically opposite effects on several components of the inflammatory and immune response [Meagher et al., 1996;Dhabhar and McEwen, 1999]. These opposing effects of glucocorticoids probably stem from their marked pleiotropy, pharmacokinetic properties, physiological versus pharmacological effects, systemic vs. tissue type-specific actions, and the timing and duration of the exposure. Glucocorticoids can influence both the innate and the adaptive immune responses as outlined below.

1.3.7.1 Effects of glucocorticoids on innate immunity

Glucocorticoids play a major role in leukocyte trafficking during local or systemic inflammation, by promoting the survival / proliferation of neutrophils and inducing apoptosis of eosinophils and basophils [Meagher et al., 1996]. Glucocorticoid-induced neutrophilia is enhanced by the increased release of bone-marrow polymorphonuclear cells and inhibition of neutrophil transmigration to inflammatory sites. This occurs through down-regulation of leukocyte adhesion molecules, such as leukocyte factor adhesion-1 (LFA-1) and L-selectin, and endothelial adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), as well as through inhibition of chemokine release (for example IL-8) at inflamed sites [Cronstein et al., 1992;Jilma et al., 2000;Nakagawa et al., 1999]. However, the influence of glucocorticoids on adhesion molecules is complex; for example, glucocorticoids inhibit NF-kappaB induced vascular cell adhesion molecule-1 (VCAM-1) expression, but they also stabilise VCAM-1 mRNA [Simoncini et al., 2000].

Glucocorticoids suppress inflammation by enhancing the clearance of foreign antigens, toxins, micro-organisms and dead cells by improving opsonisation and the activity of scavenger systems and by stimulating macrophage phagocytotic ability and antigen uptake [Liu et al., 1999]. They also suppress the synthesis of many inflammatory mediators, such as several cytokines and chemokines, prostaglandins, leukotrienes, proteolytic enzymes, nitric oxide (NO) and free oxygen radicals [Franchimont et al., 2002].

1.3.7.2 Effect of glucocorticoids on adaptive immunity

Glucocorticoids can impair maturation of dendritic cells resulting in a decreased ability to present antigens to T-lymphocytes and to elicit an immune response [Piemonti et al., 1999]. However, once an immune response has developed, glucocorticoids may modulate the type of immune response by regulating the differentiation of $CD4^+$ T-cells into Th1 lymphocytes, which drive cellular immunity, or into Th2 lymphocytes, which drive humoral immunity [Cousins et al., 2002]. Glucocorticoids have been shown to block IL-12 secretion from monocyte/macrophages and dendritic cells, which is an early crucial step in the development of the Th1-directed cellular immune response and,

hence, is a key link between innate and cellular immunity [Blotta et al., 1997]. Glucocorticoids may promote Th2 development by enhancing IL-10 secretion by macrophages or Th2 cells [Ramirez et al., 1996]. On the other hand, glucocorticoids may also promote the development of T-regulatory cells to suppress immune responses, prevent self-reactivity and encourage immune tolerance [Barrat et al., 2002]. Thus, by acting directly either on leukocytes or on dendritic cells, endogenous glucocorticoid hyper-secretion or chronic, excessive administration causes a progressive shift from a Th1-directed cellular immune response to a Th2-driven humoral immune response.

1.3.7.3 Effect of glucocorticoids on T-cell apoptosis and survival

Glucocorticoids can influence inflammation in either direction by inducing T-lymphocyte apoptosis or survival, depending on the cell sub-type and differentiation stage [Zacharchuk et al., 1990]. Also, both the degree of activation and the timing of glucocorticoid exposure (before, during or after activation) can make T-cells either sensitive or resistant to glucocorticoid-induced apoptosis [Ashwell et al., 2000]. Several studies have shown that concomitant T-cell receptor (TCR) and glucocorticoid signalling promotes T-cell survival, whereas either TCR signalling alone or GR signalling alone induces T-cell apoptosis.[Ashwell et al., 2000] Glucocorticoids might also attenuate TCR signalling through non-genomic actions [Van et al., 2001]. There is growing evidence that endogenous glucocorticoids play a role in T-cell development and selection in the thymus [Ashwell et al., 2000]. Glucocorticoids can also influence peripheral T-cell development and selection in the extra-thymic immune system, where glucocorticoids might be essential in shaping the immune response and maintaining peripheral T-cell pool homeostasis by preventing TCR-induced T-cell deletion and enhancing T-cell survival simultaneously [Gonzalo et al., 1993].

1.3.8 Glucocorticoids and haemostasis

Glucocorticoids can directly affect haemostasis. Dexamethasone has been shown to increase production of von Willebrand Factor (vWF), endothelin and PAI-1 in human umbilical vein endothelial cells (HUVEC) [Huang et al., 1995] and of PAI-1 in cultured human adipose tissue [Halleux et al., 1999]. Patients with Cushing's syndrome (excess of endogenous glucocorticoids) have elevated levels of vWF, PAI-1, thrombin-

antithrombin and plasmin-antiplasmin complexes and factor VIII. These haemostatic problems may resolve after curative surgical treatment [Patrassi et al., 1985;Fatti et al., 2000]. Patients with Cushing's syndrome also have increased platelet count, fibrinogen, PAI-1, and decreased tissue factor pathway inhibitor levels which represents a potential hypercoagulable and hypofibrinolytic state and may increase the risk for atherosclerotic and athero-thrombotic complications [Erem et al., 2009].

1.3.9 Direct effects of glucocorticoids on the cardiovascular system

Whilst much of the impact of glucocorticoids on the cardiovascular system is due to exacerbation of the cardiovascular risk factors and interaction with renal homeostasis, [Bjorntorp, 1991], there is evidence for additional, direct effects on the cells of the cardiovascular system.

1.3.9.1 Effects of glucocorticoids on the vessel wall

Glucocorticoids can increase peripheral vascular resistance in animals devoid of renal mass [Langford and Snavely, 1959] suggesting a direct interaction with the cells of the heart and vascular wall. In man, topical administration of glucocorticoids induces dermal vasoconstriction [Walker et al., 1991] possibly due to glucocorticoid-mediated enhancement of contractile responses to noradrenaline and angiotensin-II [Ullian, 1999;Hadoke et al., 2006]. In VSMCs, glucocorticoids have been shown to up-regulate contractile receptors, alter intracellular second messenger activation and modulate the activity and synthesis of vasoactive substances leading to an augmentation of contraction. Increased contractility may be due to increased release of endothelium-derived vasoconstrictors (such as angiotensin II, endothelin-1) [Mendelsohn et al., 1982;Morin et al., 1998] and/or impaired endothelium-dependent relaxation [Mangos et al., 2000] due to impaired vasodilators (e.g. prostaglandins, nitric oxide) activity [Gerritsen and Rosenbaum, 1985;Wallerath et al., 1999;Simmons et al., 1996]. Glucocorticoids can also inhibit migration and proliferation of VSMCs [Longenecker et al., 1982;Longenecker et al., 1984] and tube formation by endothelial cells [Nicosia and Ottinetti, 1990]. Glucocorticoids have also been shown to inhibit angiogenesis [Folkman et al., 1983;Crum et al., 1985;Folkman and Ingber, 1987] which can be abolished by GR receptor antagonism [Small et al., 2005].

1.3.9.2 Effect of glucocorticoids on the heart

Glucocorticoids may help in maintaining normal cardiac contractile function. Dexamethasone treatment enhances the development of contractile tension and increases contraction and relaxation velocities in cardiac muscles [Penefsky and Kahn, 1971]. The decrease in contractile force of rat papillary muscles following adrenalectomy can be prevented by dexamethasone administration [Lefer, 1968] which can modulates Ca^{2+} transport in cardiac myocytes [Whitehurst, Jr. et al., 1999;Narayanan et al., 2004] and activity of K^{+} channels [Lefer, 1968;Wang et al., 1999;Penefsky and Kahn, 1971]. Therefore, glucocorticoids may also influence cardiac electrical activity. Over-expression of cardiac GR in mice leads to reduced heart rate and depressed cardiac conduction with atrio-ventricular blocks despite no major changes in cardiac structure [Sainte-Marie et al., 2007]. Short-term dexamethasone treatment has also been shown to decrease resting heart rate in healthy human volunteers [Brotman et al., 2006].

Several experimental and human studies have also revealed harmful effects (e.g. cardiomyopathy) of glucocorticoids on the myocardium [Zecca et al., 2001;Mitsuya et al., 2004]. Glucocorticoid-induced cardiomyopathy is characterized by accumulation of lipid droplets in myocardium, cardiomyocyte hypertrophy, and dissolution of myofibrils [Clark et al., 1982;de Vries et al., 2002]. Dexamethasone treatment in adulthood leads to hypertrophy and precocious degeneration of cardiomyocytes [de Vries et al., 2002]. Glucocorticoid treatment in the neonatal period can also induce hypertrophy of the myocardium with changes in the protein-to-DNA ratio and in the actin content of the heart [Werner et al., 1992;La Mear et al., 1997]. The role of glucocorticoid signalling in the development of cardiomyopathy is not entirely understood. Some studies, however, suggest that cardiomyocyte hypertrophy is probably related to cross-talk between glucocorticoid signalling and hypertrophic signalling pathways [Lister et al., 2006]. In this model the enhanced glucocorticoid signal up-regulates kinase-1, which may augment α-adrenoceptors-mediated hypertrophy [Lister et al., 2006]. Dexamethasone treatment also significantly increases ACE activity [Barreto-Chaves et al., 2000] which has direct and indirect effects on the myocardium, resulting in myocardial fibrosis and heart failure.

1.3.10 Glucocorticoids and atherosclerosis

Chronic glucocorticoid excess is associated with increased cardiovascular mortality in humans. Retrospective analysis of the association between plasma cortisol concentrations and atherosclerosis shows a correlation of elevated morning plasma cortisol levels with moderate to severe coronary atherosclerosis [Troxler et al., 1977;Alevizaki et al., 2007]. These results are consistent with epidemiological studies as patients with untreated or non-remitting Cushing's disease exhibit a four-fold increase in mortality, compared with the general population, mainly as a result of vascular disease [Etxabe and Vazquez, 1994]. This is further supported by the demonstration that normalization of glucocorticoid levels in patients with Cushing's disease improves the distensibility-coefficient and intima-media thickness (IMT) [Faggiano et al., 2003]. However, whilst these patients show improvement in atherosclerosis and cardiovascular outcome, the incidence of these conditions remains higher than in the general population [Colao et al., 1999]. In addition, glucocorticoids have been shown to promote foam cell formation by increasing the accumulation of cholesterol esters, by enhancing acyl coenzyme A cholesterol acyltransferase 1 (ACAT-1) expression [Yang et al., 2004]. It has also been proposed that the beneficial effects of lipid lowering drugs on atherosclerosis may partially be due to their effect on synthesis of steroid hormones from cholesterol [Kanat et al., 2007].

Chronic therapeutic administration of glucocorticoids is also linked to increased cardiovascular events, including fatal and non-fatal myocardial infarctions [Wei et al., 2004;Davis et al., 2007]. This increased risk is cumulative and dose-dependent, and is reduced if treatment is discontinued [Souverein et al., 2004;Varas-Lorenzo et al., 2007]. Daily administration of prednisolone in dose equivalents exceeding 10mg, for example, is associated with a two-fold increased risk of acute myocardial infarction [Varas-Lorenzo et al., 2007]. The relationship between chronic glucocorticoid therapy and cardiovascular disease has been addressed most consistently in patients with chronic inflammatory conditions, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In RA, the risk of mortality (predominantly from cardiovascular disease) increases by 14% after 1 year, rising to 69% after 10 years, in patients treated with low-dose, oral glucocorticoids [Sihvonen et al., 2006]. However, when interpreting

these studies, it must be kept in mind that it is difficult to differentiate the effect of glucocorticoid treatment from that of the underlying inflammatory condition [Hadoke et al., 2009].

Experimental studies of the link between glucocorticoids and atherosclerosis, using various animal models, have produced conflicting results. Glucocorticoids prevent or arrest atherosclerosis in fat-fed rabbits, despite increasing hyperlipidaemia [Bailey and Butler, 1985;Asai et al., 1993;Naito et al., 1992;Jain et al., 1965]. This effect was first demonstrated over 50 years ago [Oppenheim and Bruger, 1952;Gordon et al., 1954;Stumpf and Wilens, 1954;Constantinides et al., 1962] and has subsequently proved to be reproducible in this model [Asai et al., 1993;Naito et al., 1992;Cavallero et al., 1976;Bailey and Butler, 1985] and in rabbits with inherited hypercholesterolaemia (Watanabe heritable hyperlipidaemia, WHHL) [Makheja et al., 1989]. The mechanism(s) underlying the apparent anti-atherosclerotic effect of glucocorticoids is (are) incompletely understood. The possible mechanisms include inhibition of DNA synthesis in the cellular component of lesions [Cavallero et al., 1976], inhibition of inflammatory cell proliferation [Asai et al., 1993;Sakai et al., 1999], inhibition of intimal VSMC proliferation [Voisard et al., 1994] and migration [Van Put et al., 1995], and reduced chemotaxis of circulating monocytes / macrophages into the sub-endothelial space [Yamada et al., 1993;Prescott et al., 1989;Dhawan et al., 2007]. However, there are several studies suggesting an atherogenic effect of glucocorticoids. In cockerels, cortisone feeding has been shown to increase atherogenesis [Stamler et al., 1954]. There is also an indication that social stress, which increases cortisol and corticosterone secretion [Szeto et al., 2004], increases atherogenesis in WHHL rabbits [Mccabe et al., 2002]. Furthermore, GR antagonism with RU38486 has been shown to reduce lesion formation by 45% in atherosclerotic mice [Santanam et al., 2004] suggesting a pro-atherogenic effect of GR stimulation.

1.3.11 Glucocorticoids and neointimal proliferation

Several properties of glucocorticoids suggest that they may prevent the intense fibro-proliferative vascular remodelling that occurs following percutaneous revascularisation (angioplasty ± stenting), prompting a number of experimental and clinical studies.

Studies in animals have largely supported the hypothesis that glucocorticoid administration reduces neointimal proliferation. Systemic dexamethasone administration inhibited neointimal lesion formation in rats [Villa et al., 1994;Guzman et al., 1996], rabbits [Van Put et al., 1995;Poon et al., 2001] and dogs [Strecker et al., 1998] with inhibition of macrophage accumulation proposed as a possible mechanism of action [Poon et al., 2001]. Similar results were obtained with either oral or local prednisone in rabbit iliac artery [Ribichini et al., 2007]. Not all studies in animals have yielded positive results, however, as dexamethasone treatment did not reduce neointimal hyperplasia after angioplasty in the rabbit [Karim et al., 1997] or in the pig [Lincoff et al., 1997]. Moreover, early clinical trials were disappointing: methylprednisolone did not inhibit restenosis after coronary angioplasty [Pepine et al., 1990] or stenting [Reimers et al., 1998] whilst dexamethasone-eluting stents (D-DES) have not reduced the incidence of restenosis [Hoffmann et al., 2004;Ribichini et al., 2007]. More recently, interest in the potential of glucocorticoids in prevention of restenosis has been re-awakened [Radke et al., 2004;Liu et al., 2004;Ferrero et al., 2007] with several trials suggesting a beneficial effect of glucocorticoids: oral prednisone produced a dose-dependent reduction in clinical events and angiographic restenosis rate after stenting (the IMPRESS trial) [Versaci et al., 2002;Ferrero et al., 2007]; low-dose dexamethasone was associated with low restenosis rate (the STRIDE trial) [Liu et al., 2003]), D-DES produced a low rate of clinical events (death, myocardial infarction and ischemia driven target vessel revascularization) at 6 months (despite no inhibition of restenosis; the DESIRE trial) [Ribichini et al., 2007]. There are on-going multi-centre trials to further evaluate the effects of local and systemic administration of glucocorticoids [Ribichini et al., 2009].

1.4 11β-HSD isozymes and the cardiovascular system

The ability to regulate glucocorticoid activity within the target tissues has highlighted the possibility of tissue-specific glucocorticoid excess (or deficiency) in the presence of normal circulating concentrations of the enzyme [Wamil and Seckl, 2007]. Altered activity of 11β-HSD isozymes in the adipose tissue, liver, skeletal muscle and brain have been linked to diabetes mellitus, metabolic syndrome and cognitive dysfunction. The association of some of these conditions with hypertension and atherosclerosis has

suggested a role for 11β-HSD activity in the development of cardiovascular disease [Walker, 2007]. The physiology of both isozymes has been explored in greater detail in the recent years, particularly since the generation of mice with targeted disruption of the genes encoding each isozyme [Kotelevtsev et al., 1997;Kotelevtsev et al., 1999].

1.4.1 Non-selective 11β-HSD inhibitors

Inhibition of 11β-HSD activity in experimental and clinical studies depended, until relatively recently, upon the use of several naturally-occurring compounds. Principal among these were compounds derived from glycyrrhizic acid, the principal active component of the liquorice plant, Glycyrrhiza glabra. In addition to these plant-derived compounds, endogenous bile acids, such as lithocholic acid and chenodeoxycholic acid are also non-selective inhibitors of 11β-HSD [Perschel et al., 1991;Latif et al., 1994].

Excessive liquorice ingestion causes a characteristic syndrome, with clinical and biochemical features of pseudo-hyperaldosteronism, which is the result of 11β-HSD2 inhibition [Conn et al., 1968]. Glycyrrhetinic acid [Adamson and Tillman, 1955], the hydrolytic product of glycyrrhizic acid, and its hemi-succinate derivative, carbenoxolone [Csonka and Murray, 1971], have anti-inflammatory properties in the skin and have also been used extensively for pharmacological inhibition of 11β-HSD activity. The usefulness of these compounds is limited, however, as they inhibit both isozymes of 11β-HSD [Schleimer, 1991;Li et al., 1997] although it has been suggested that carbenoxolone is more active against the dehydrogenase activity of 11β-HSD [Morris et al., 2003;Brem et al., 1997].

1.4.2 11β-HSD1 inhibition and the metabolic syndrome

It has been shown that over-expression of 11β-HSD1 in the adipose tissue [Masuzaki et al., 2001;Masuzaki et al., 2003] or liver [Paterson et al., 2004] results in central obesity, hypertension and dyslipidaemia. Conversely, 11β-HSD1 knockout mice have an atheroprotective phenotype, including lower cholesterol and triglyceride levels and improved glucose tolerance [Morton et al., 2005]. Transgenic deletion of 11β-HSD1 has no effect on blood pressure [Kotelevtsev et al., 1997] or vascular contractility [Hadoke et al., 2001d], and this enzyme does not seem to influence vascular reactivity *in-vivo*.

The identification of 11β-HSD1 as a potential target for the treatment of diabetes mellitus and metabolic syndrome has prompted considerable interest in the pharmaceutical industry; leading to the discovery and development of a multitude of novel selective 11β-HSD1 inhibitors. There have been nearly 100 patents filed by ~29 different pharmaceutical companies and research organisations since 2002. These embrace a wide variety of compounds, based predominantly around: triazoles, aryl sulphonamide thiazoles, sulphonamides and adamantyl carboxamides [Hughes et al., 2008].

Initial results from *in-vivo* studies in mouse and rat models have produced promising evidence that selective 11β-HSD1 inhibitors provide an effective treatment for systemic cardiovascular risk factors, including: obesity, type II diabetes and dyslipidaemia. 11β-HSD1 inhibition with BVT 2733 (a thiazole type inhibitor, 200mg/kg twice daily) improved insulin sensitivity and dyslipidaemia [Alberts et al., 2003] and decreased blood glucose (7 days administration by mini-pump) [Alberts et al., 2002] in hyperglycaemic mice. Compound 544 (a triazole type inhibitor, 30mg/kg twice daily for 11 days) reduced body weight, fasting glucose and serum lipids in mice with diet-induced obesity and streptozotocin/high-fat-diet -induced diabetes mellitus [Hermanowski-Vosatka et al., 2005]. Improvements in cardiovascular risk factors are not restricted to these compounds or to mouse models as low dose (3mg/kg/day) of another triazole type 11β-HSD1 inhibitor also improved fasting triglyceride levels and prevented lipid accumulation in tissues of rats with diet-induced obesity [Berthiaume et al., 2007a;Berthiaume et al., 2007].

Selective 11β-HSD1 inhibitors potentially have a variety of clinical applications (for example, in the treatment of wound healing and age-related cognitive impairment (Alzheimer's disease)). Their application in the cardiovascular arena could include treatment of the metabolic syndrome, obesity, type II diabetes and prevention and treatment of atherosclerosis [Hadoke et al., 2009]. The demonstration that these compounds also inhibit 11β-HSD1 in the brain raised a concern of possible side-effects caused by disruption of the HPA axis but no such complications have been identified in animal studies. Several companies have now produced orally-active compounds that achieve inhibition in these tissues in rodents [Hughes et al., 2008], with tissue inhibition

in human adipose also recently demonstrated in a Phase IIa clinical trial where compound INCB13739 reduces fasting plasma glucose, cholesterol and LDL levels in patients with type II diabetes [Rosenstock et al., 2010]. Other selective 11β-HSD1 inhibitors produced by Amgen and Biovitrum (AMG221) are also currently being evaluated in clinical trials [Hughes et al., 2008].

1.4.3 11β-HSD1 inhibition and inflammation

There is evidence to suggest that glucocorticoid metabolism through the 11β-HSD enzymes provides an additional level of regulation of inflammatory response [Chapman et al., 2006]. 11β-HSD1 is expressed in monocytes, macrophages and lymphocytes and can regulate innate and adaptive immune responses.

Circulating monocytes express low levels of 11β-HSD1; however, on differentiation to macrophages or dendritic cells, there is substantial increase in expression [Thieringer et al., 2001]. Expression in macrophages is stimulated further by IL-4 and IL-13 [Thieringer et al., 2001]. Expression of 11β-HSD1 in resident macrophages is detectable *in-vivo* in the mouse, and the activity increases very rapidly during acute inflammation (e.g. peritonitis [Gilmour et al., 2006]) The expression of 11β-HSD1 in macrophages has been shown to be important in the early induction of the capacity of these cells to phagocytose apoptotic neutrophils [Gilmour et al., 2006]. This suggests that glucocorticoid production via 11β-HSD1 can have a very active role in enhancing anti-inflammatory responses and is not solely due to effects on expression of pro-inflammatory cytokines.

11β-HSD1 expression has also been demonstrated in various lymphocyte populations [Zhang et al., 2005]. Resting $CD4^+$ or $CD8^+$ T-cells and B-cells isolated from mice express 11β-HSD1. This activity increases further when naive $CD4^+$ cells are activated by T-cell receptor stimulation or when the cells are differentiated *in-vitro* into Th1 or Th2 subtypes [Zhang et al., 2005;Hu et al., 2009]. Intracellular generation of active glucocorticoids from inactive precursors was associated with a reduction in cytokine production, an effect that did not occur in cells obtained from 11β-HSD1 knockout mice. The expression of 11β-HSD1 is also seen in the thymus. Thymocytes express low levels of 11β-HSD1, but this increases substantially in an experimental burn injury model in

mice, an effect that is associated with an increased rate of thymocyte apoptosis [D'Elia et al., 2009].

Inflammation can also up-regulate expression of 11β-HSD1 in a variety of cells and tissues including VSMCs, adipocytes, osteoblasts, myoblasts, fibroblasts, synovial and colonic tissue [Tomlinson et al., 2001;Cai et al., 2001;Hardy et al., 2006;Bryndova et al., 2004]. This is largely mediated by IL-1 and TNF-α and to a lesser extent by the Th2 cytokines (IL-13 and IL-4). The induction of 11β-HSD1 activity has been shown to have functional effects on the cells (an intracrine effect) but is likely to also increase glucocorticoid levels within the tissue (a paracrine effect). This has been shown in the synovium in which the increased expression in response to inflammation was proposed to reflect an attempt to reduce inflammation within the joint [Hardy et al., 2008]. In such tissues it is likely that the high local glucocorticoid levels could impair the function of immune cells that migrate into the joint, even if they themselves do not express 11β-HSD1. These findings suggest that 11β-HSD1 may play a dynamic role in the regulation of the inflammatory responses in both innate and adaptive immunity.

1.4.4 11β-HSD1 inhibition and atherosclerosis

A striking example of the benefit of selective 11β-HSD1 inhibition in mouse models of disease is provided by a study with an inhibitor (Compound 544, 10mg/kg/day, 8 weeks), that produced a dramatic (~85%) reduction in aortic cholesterol content in *ApoE*$^{-/-}$ mice on a high fat diet [Hermanowski-Vosatka et al., 2005]. Non-selective 11β-HSD1 inhibition with carbenoxolone (4 weeks) significantly reduced atherosclerosis in obese LDLR$^{-/-}$ mice [Nuotio-Antar et al., 2007]. In addition, it is possible that some anti-atherosclerotic interventions owe part of their effectiveness to regulation of 11β-HSD1 as PPAR-α agonists, such as fibrates, which are important agents for treating dyslipidaemia, reduce 11β-HSD1 expression and activity in murine liver [Hermanowski-Vosatka et al., 2005]. However, another selective 11β-HSD1 (compound 2922, 100mg/kg/day for 12 weeks) did not reduce atherosclerosis, despite improvement in glycaemic profile [Lloyd et al., 2009]. Finally, one slight concern is raised, however, by the ability of 11β-HSD1 to metabolise 7-oxysterols, as this may be pro-atherogenic [Schroepfer, 2000]. 7-Ketocholesterol which is present in very small quantities in the

plasma is highly concentrated in human atherosclerotic lesions [Brown et al., 2000] and is linked with an increased risk of developing atherosclerotic plaques at an early age, as demonstrated in patients with cerebrotendinous xanthomatosis [Fujiyama et al., 1991]. 11β-HSD1 inhibition *in-vivo* results in increased 7-ketocholesterol levels in the liver and plasma in rats [Schweizer et al., 2004b;Schweizer et al., 2004].

The mechanisms through which selective 11β-HSD1 inhibitors reduce atherosclerosis have yet to be demonstrated unequivocally. It was notable that the dramatic reduction in lesion formation produced by Compound 544 in atherosclerotic mice was greater than predicted by the more modest improvements in systemic metabolic risk factors [Hermanowski-Vosatka et al., 2005]. It was proposed, therefore, that 11β-HSD1 inhibition may have a direct protective effect on the vascular wall [Hermanowski-Vosatka et al., 2005]. If this is the case, the mechanism has not been established although data were provided to suggest that 11β-HSD1 inhibition reduces concentrations of MCP-1 in the circulation and aortic wall (MCP-1 is not normally found in the arterial media or intima but has been found in human and rodent atherosclerotic plaques [Ylaherttuala et al., 1991]). This is not consistent with the reported GR-dependent inhibition of MCP-1 from arterial SMC by glucocorticoids [Dhawan et al., 2007].

1.4.5 11β-HSD1 inhibition and neointimal proliferation

Glucocorticoids can decrease neointimal proliferation due to anti-inflammatory and anti-proliferative actions or increase neointimal proliferation via a detrimental effect on systemic cardiovascular risk factors. Therefore, the effect of 11β-HSD1 inhibition on neointimal proliferation will depend on the balance between these two factors. However, the role of 11β-HSD1 in regulating neointimal proliferation has not been described in the literature.

1.4.6 11β-HSD1 inhibition and heart function post myocardial infarction

There are emerging data to suggest that 11β-HSD1 inhibition may reduce the size of infarct after acute myocardial infarction and may, therefore, improve left ventricular function and ejection fraction [McSweeney et al., 2010]. The possible mechanisms for this beneficial effect on myocardium are enhanced inflammatory cell recruitment and

angiogenesis [Small et al., 2005;McSweeney et al., 2010]. The impact of 11β-HSD1 inhibition in the context of chronic heart failure, however, has not been studied.

1.4.7 11β-HSD2 inhibition and the cardiovascular system

Mice with transgenic deletion of 11β-HSD2 are hypertensive [Kotelevtsev et al., 1999], and have enhanced vasoconstrictor responses to noradrenaline with impaired NO-mediated, endothelium-dependent vasodilatation [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 are due to a loss in 11β-HSD2-mediated protection of GR within endothelial cells and, hence, to 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*.

Another possible, but controversial, explanation of the effect of 11β-HSD2 on the cardiovascular system may be modulation of the immune response. There are some data suggesting expression of 11β-HSD2 in circulating and tissue monocytes/macrophages in patients with rheumatoid arthritis [Olsen et al., 2004]. Expression of 11β-HSD2 has also been described in synovial tissue from patients with RA [Hardy et al., 2008]. Although, this issue needs further in-depth investigation, it highlights how 11β-HSD2 (and 11β-HSD1) expression can change in pathological conditions and modify immune responses.

11β-HSD2 may also help prevent myocardial fibrosis by protecting MR from glucocorticoids [Konishi et al., 2003]. Mice with transgenic deletion of 11β-HSD2 on *ApoE*$^{-/-}$ background tend to develop extensive atherosclerotic changes without the need for dietary interventions (Deuchar et al., unpublished data). However, the role of 11β-HSD2 in regulating the inflammatory and proliferative responses in a model of intravascular injury has not been studied yet.

1.5 Hypotheses and Aims

1.5.1 Hypotheses

Vascular lesion development (atherosclerosis and neointimal proliferation) represents an inflammatory and proliferative response to chronic or acute vascular injury. Glucocorticoids can modulate inflammation and cellular proliferation within the vessel wall and may contribute to the development of systemic risk factors associated with cardiovascular disease. 11β-HSD1 inhibition improves cardiovascular risk factors and loss of 11β-HSD2 activity results in hypertension. Therefore, the work described in this thesis addressed the hypotheses that 11β-HSD1 inhibition reduces the size and alter the composition of vascular lesions (atherosclerotic and neointimal) by modulating systemic risk factors and vascular inflammation, whereas, 11β-HSD2 deletion results in glucocorticoid-mediated activation of vascular MR leading to an increase in neointimal proliferation which can be blocked with MR antagonism.

1.5.2 Aims

The primary objectives of this project were:

- To establish whether 11β-HSD1 inhibition reduces lesion size and alters plaque composition in a murine model of atherosclerosis
- To determine whether 11β-HSD1 inhibition/deletion reduces neointimal proliferation in a mouse model of wire-angioplasty
- To determine whether 11β-HSD2 deletion results in enhanced neointimal proliferation in a mouse model of wire-angioplasty

2. Materials and Methods

2.1 Animals

All animal work was carried out under UK Home Office licence in accordance with the Animal (Scientific Procedures) Act (UK), 1986 and conforms with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

All non-genetically modified mice used were the C57Bl/6J strain and were either bred in-house or purchased from Harlan Laboratories (Bicester, UK). Apolipoprotein E knockout (*ApoE*$^{-/-}$) mice were either bred in-house or purchased from Charles River Laboratories (Kent, UK). 11β-HSD1$^{-/-}$ mice, 11β-HSD2$^{-/-}$ mice and 11β-HSD1$^{-/-}$, *ApoE*$^{-/-}$ double knockout mice, all congenic on a C57Bl/6J background, were bred in-house.

All animals were housed at the Biomedical Research Facility, University of Edinburgh. Mice were maintained under standard conditions of light (lights on 8am-8pm), humidity (50%) and temperature (21-22ºC).

2.2 Materials

2.2.1 Chemicals and reagents

Unless stated otherwise, all chemicals and solutions were from Sigma-Aldrich (Gillingham, UK) and all solvents were from Fisher Scientific (Loughborough, UK). Harris' haematoxylin was obtained from Thermo Shandon (Runcorn, UK), Weigert's iron haematoxylin A and B solutions from Bios Europe (Skelmersdale, UK), DPX mounting medium from VWR International (Lutterworth, UK), monoclonal rat anti-mouse primary antibody against Mac-2 from Cedarlane (Burlington, Canada) and [^{3}H]$_{4}$ corticosterone from GE Healthcare (Amersham, UK).

2.2.2 Diets and drugs

Animals were fed standard chow (RM1; Special Diet Services, UK) unless specified otherwise. Western diet (OpenSource^{TM} D12079B), containing 21% fat, 20% protein and 50% carbohydrate, was obtained from Research Diets (Brunswick, USA). Animals were allowed to feed *ad libitum* in all studies.

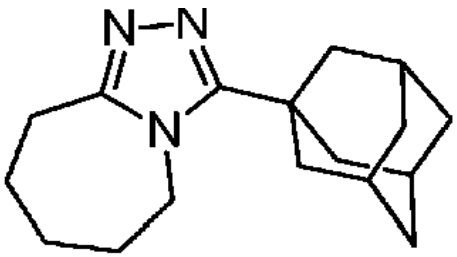
Compound 544 (3-adamantan-1-yl-6,7,8,9-tetrahydro-5H-[1,2,4]triazolo[4,3-α]azepine), a selective 11β-HSD1 inhibitor [Hermanowski-Vosatka et al., 2005], was obtained (as compound T5293658) from Enamine Ltd. (Kiev, Ukraine). This compound is a competitive inhibitor for cortisone, but not NADPH, suggesting that it interacts with the steroid binding site (unpublished data). It has poor water solubility and does cross blood-brain barrier [Hermanowski-Vosatka et al., 2005]. The advantages of this particular compound include published data and good bioavailability with oral administration [Hermanowski-Vosatka et al., 2005]. The molecular weight of compound 544 is 271.4nM; IC_{50} for human 11β-HSD1 is 7.5±0.5nM and IC_{50} for mouse 11β-HSD1: 97±5.1nM. The biochemical structure of compound 544 is shown in Figure 2.1.

Eplerenone, a selective mineralocorticoid receptor antagonist [Rabasseda et al., 1999], was obtained from Pfizer Inc. (New York, USA). The molecular weight of eplerenone is 414.5 nM and IC_{50} for minerelocortcioid receptors is 360nM. The biochemical structure of eplerenone is shown in Figure 2.1.

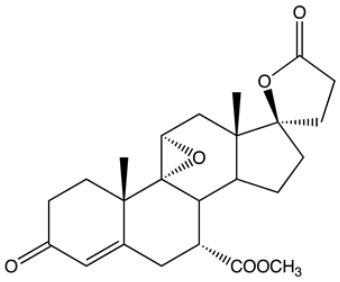
2.2.3 Surgical material

Surgical instruments were purchased from Fine Science Tools (Interfocus Ltd, UK). Angioplasty guide-wires (0.015") were obtained from Cook Inc. (Bloomington, USA), Mersilk 6-0 sutures were from Ethicon Ltd. (Livingston, UK), isoflurane was from Merial Animal Health Ltd. (Harlow, UK), buprenorphine 0.3mg/ml as vetergesic from Alstoe Animal Health (York, UK) and 1% lignocaine from Hameln Pharmaceuticals Ltd. (Gloucester, UK).

Figure 2.1: Biochemical structure of compound 544 and Eplerenone



Compound 544



Eplerenone

Compound 544 "3-adamantan-1-yl-6,7,8,9-tetrahydro-5H-[1,2,4]triazolo[4,3-α]azepine" was used as a selective inhibitor of 11β-HSD1.
Eplerenone "Pregn-4-ene-7,21-dicarboxylic acid, 9,11-epoxy-17-hydroxy-3-oxo-,γ-lactone, methyl ester, (7α,11α,17α)-" was used as a selective inhibitor of mineralocorticoid receptors.

2.3 Preparation of solutions and drugs

2.3.1 Solutions for histology

Alkaline tap water: 2 drops of ammonia were added to 300ml tap water.

Gomori's aldehyde fuchsin: 5g pararosaniline base was dissolved in 500ml of 60% ethanol and then 1ml hydrochloric acid (HCl) and 2ml paraldehyde were added and mixture was allowed to blue at room temperature for 2 days.

Gomori's trichrome: 3g phosphotungstic acid was dissolved in 500ml distilled water and then 3g chromotrope 2R and 1.5g fast green FCF were added and mixed well. Finally 5ml acetic acid was added and the mixture was filtered.

Phosphate buffer saline (PBS): 1 tablet was dissolved in 200ml of distilled water.

PBS/1%BSA: Bovine serum albumin (BSA) was dissolved in PBS to make 1% *w/v* solution.

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

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

0.3% potassium permanganate in 0.3% sulphuric acid: Prepared from stock solutions of 1% potassium permanganate (5g in 500ml distilled water) and 3% sulphuric acid (15ml concentrated sulphuric acid + 485ml distilled water). 1% potassium permanganate (90ml) was mixed with 3% sulphuric acid (30ml) and 180ml distilled water to make 300ml solution.

2% oxalic acid: 100% oxalic acid (10ml) was mixed with distilled water (490ml).

3% hydrogen peroxide (H_2O_2): Commercially-available 30% H_2O_2 solution was diluted 10 fold in PBS.

5% phosphotungstic acid: Commercially-available 10% phosphotungstic acid was diluted with an equal volume of distilled water.

20% goat serum: Commercially available normal goat serum (Vector Labs, UK) was diluted 1:4 with PBS/1%BSA solution.

74 OP: A solution of 99.5% ethanol and 0.5% methanol

2.3.2 Solutions for 11β-HSD1 activity assay

Sucrose buffer: Sucrose (42.79g) was dissolved in distilled water (500ml) to produce a 0.25M solution and the pH adjusted to 7.5 with concentrated NaOH.

Krebs-Henseleit buffer: NaCl (118mM), KCl (3.8mM), KH_2PO_4 (1.19mM), $CaCl_2$ (2.54mM), $MgSO_4$ (1.19mM), $NaHCO_3$ (25mM) were dissolved in water and the pH was adjusted to 7.4 with concentrated NaOH.

HPLC mobile phase: High performance liquid chromatography (HPLC)-grade water (60%, Fisher Scientific, UK) was mixed with methanol (25%) and acetonitrile (15%).

2.3.3 Drugs

11β-HSD1 inhibitor: Compound 544, a triazole compound selectively inhibiting 11β-HSD1 activity, was administered by oral gavage (30mg/kg dissolved in 5% solution of 2-Hydroxypropyl-β-cyclodextrin) for assessing its effect on hepatic 11β-HSD1 reductase activity. The inhibitor was administered by admixture with food to study its effects on atherosclerosis (10mg/kg/day for 8 weeks) or neointimal proliferation (30mg/kg/day for 4 weeks).

Eplerenone: To assess the effects of MR antagonism on neointimal proliferation, a selective MR antagonist, eplerenone, was administered by admixture with food (200mg/kg/day, for 4 weeks).

2.4 Blood pressure measurement

Systolic blood pressure (BP) was measured in conscious, warmed, restrained mice by tail-cuff photo-plethysmography as described [Evans et al., 1994].

2.4.1 Principle

In photo-plethysmography, a progressively inflating/deflating pressurised cuff is applied to the proximal part of the mouse's tail and blood flow through the tail is monitored by light transmittance (inversely proportional to the tail volume) at a point distal to the cuff. The cyclical changes in tail volume (indicating blood flow) cease when cuff pressure exceeds systolic blood pressure.

2.4.2 Protocol

Mice were habituated to the restraining device and heated chamber for one week before starting the studies. In rodents, increasing tail blood flow is one of the methods to dissipate excess body heat. To ensure adequate tail blood flow for photo-plethysmography, mice were put in a heated chamber (37ºC) for 3-5min immediately prior to the experiment. Mice were then transferred to a Perspex tubular restraint device mounted above a heated pad. A tail-cuff (Harvard Apparatus, Kent, UK) with integrated pressure cuff and photo-sensor sleeve was placed around the proximal part of the tail and connected to a computer for data acquisition. Animal restraint force and the position of the tail cuff were adjusted to ensure animal comfort and a good baseline pulse trace of cyclical tail volume changes. The tail cuff was inflated to beyond the point at which the distal pulse stopped, deflated, and the pressure at which the pulse trace reappeared (i.e. systolic blood pressure) was noted. For each mouse, 8-12 cycles of inflation/deflation were performed and the mode value (or the mean value if no mode value) was recorded as systolic blood pressure.

2.5 Biochemical tests

2.5.1 Glucose tolerance test

For glucose tolerance tests (GTT), mice were housed individually and fasted for 6hr. After this period, baseline (time 0min) blood samples were collected by tail tipping, and then glucose (2mg/g) was administered by intra-peritoneal injection. Further blood samples were taken 15, 30, 60 and 90 min after glucose administration. Blood samples

were initially kept in wet ice and then spun at 10,000rpm for 5min to separate plasma. Plasma was collected into Eppendorf tubes and stored below -20°C until assayed.

2.5.2 Glucose assay

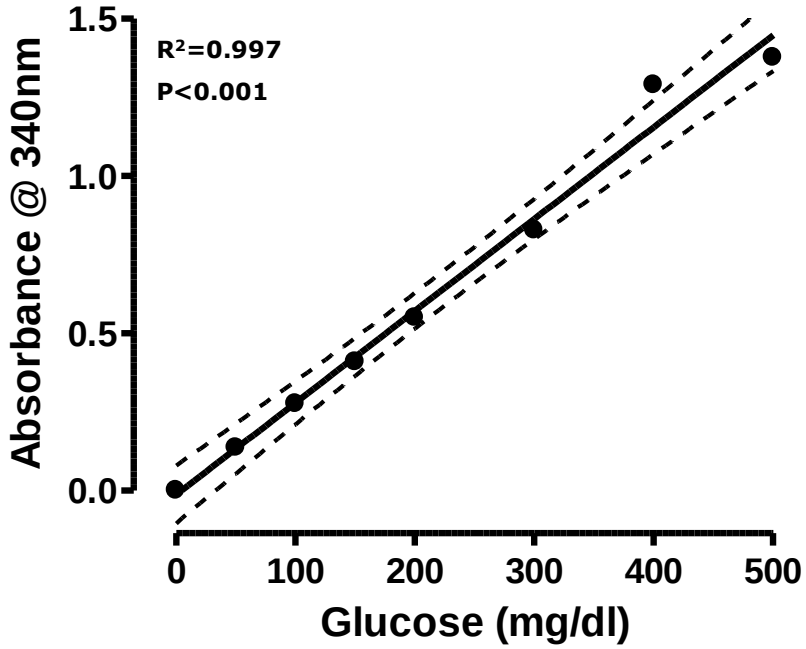
Serum glucose levels were determined spectrophotometrically using the glucose hexokinase reagent (Infinity^{TM}, Thermo Electron Corporation, Middletown, USA). Standard solutions were prepared following manufacturer's instructions. Standards (2µl) or samples (2µl), each in duplicates, were added to a clean 96 well plate and then 200µl of reagent added to each well. Plates were incubated at room temperature for 3-5min and then absorbance was read on a plate reader (OPTImax turnable microplate reader, Molecular Devices, USA) at 340nm and the sample values were then interpolated from the standard curve (Figure 2.1).

2.5.3 Cholesterol assay

Serum cholesterol levels were measured spectrophotometrically using cholesterol liquid stable reagent (Infinity ^{TM}, Thermo Electron Corporation, Middletown, USA).

Standard solutions were prepared following the manufacturer's instructions. Standards (2µl) or samples (2µl), each in duplicates, were added to a clean 96 well plate and then 200µl of reagent added to each well. Plates were incubated at 37ºC for 5mins and absorbance was read on a plate reader (primary wavelength 500nm, secondary wavelength 660nm). A standard curve was generated using standard solutions and was used to calculate unknown sample values.

Figure 2.2: A standard curve from spectrophotometric analysis to calculate unknown sample values



Known concentrations of glucose (x-axis) were used as standards for spectrophotometric analysis using Infinity™ regents. Absorbance was read at 340nm and a standard curve was generated. Absorbance values were also obtained for samples and glucose concentration in samples were interpolated from the standard curve.

2.5.4 Triglyceride assay

Serum triglyceride levels were measured spectrophotometrically using Infinity^{TM} triglyceride reagent (Thermo Electron Corporation, Middletown, USA).

Standard solutions were prepared following the manufacturer's instructions. Standards (2µl) or samples (2µl), each in duplicates, were added to a clean 96 well plate and then 200µl of reagent added to each well. Plates were incubated at 37ºC for 5mins and absorbance was read on a plate reader (primary wavelength 500nm, secondary wavelength 660nm). A standard curve was generated using standard solutions and the sample values were then interpolated from the standard curve.

2.5.5 Serum insulin

Serum insulin levels were measured by ultra-sensitive mouse insulin ELISA kit (Crystal Chem., USA). All reagents were prepared from the kit provided, as per manufacturer's instructions. The assay kit includes a microplate with columns of clip-on wells coated with guinea pig anti-insulin antibodies. Diluent (95µl) and sample (5µl) or standard (5µl) were added to the wells and the plate was incubated at 4˚C for 2hr. Plates were then rinsed and washed with a wash-buffer (5 x 300µl). Anti-insulin enzyme conjugant (100µl) was added to each well and the plate was incubated (room temperature, 30min) and then washed with the wash-buffer (7 x 300µl). Enzyme-Substrate solution (100µl) was added to each well and the plate covered with tin foil and incubated at room temperature for 40min. The reaction was stopped by adding Enzyme Stop solution (100µl) to each well. The optical density of each well was calculated using a microplate reader set to 450nm and the sample values then interpolated from the standard curve.

The limits of detection from these biochemical tests, correlation coefficients and p-values from standard curves are given in Table 2.1.

Table 2.1: Sensitivity and correlation coefficients of biochemical assays

Assay	Sensitivity range	Correlation coefficient	p
Glucose	40-240 mg/dl	0.99	<0.001
Cholesterol	0.1-12 mmol/L	0.96	<0.001
Triglyceride	1.1-4.1 mmol/L	0.98	<0.001
Insulin	0.1-6.4 ng/ml	0.99	<0.001

2.6 Measurement of 11β-HSD1 activity

11β-HSD1 activity was quantified by measuring dehydrogenase activity in liver homogenates (by centrifugation, 1000g, 4°C, 15min). The protein concentration of samples was determined by Bradford method using a calorimetric protein assay kit (Bio-Rad, UK). Hepatic homogenates were then diluted in Krebs-Ringer buffer to obtain a protein concentration of 20µg/ml. Aliquots of collected supernatant (180µl) were then mixed with 50µl cofactor mix (0.2% glucose, 2 mM NADP, dissolved in Krebs-Ringer buffer) and 20µl substrate mix (5nM $[^3H]_4$-corticosterone, 15nM unlabeled corticosterone, dissolved in 25% ethanol: 75% Krebs-Ringer buffer). Reactions were prepared in duplicate with a final volume of 250µl. Reaction mixtures were incubated (1hr, 37^{o}C) and then stopped by adding 10 volumes of ethyl acetate. The tubes were then vortexed and the upper organic phase containing steroids was collected in fresh tubes and dried down under nitrogen (60^{o}C). Steroids were then reconstituted in 800µl mobile phase and analysed by using reverse phase High Performance Liquid Chromatography (HPLC).

The HPLC system consisted of an auto-sampler and a mobile phase pump (Waters, UK) equipped with C_{18} Sunfire Symmetry column (length 15 cm, internal diameter 4.6 mm, pore size 5 µm; Waters, UK), and a radioflow detector (LB509, Berthold, UK).

Steroid samples suspended in mobile phase (60-100µl) were injected into the HPLC system. The flow rate of mobile phase was maintained at 1.0ml/min and of the scintillant (ProFlow, Meridian, UK) at 2.0ml/min. The column temperature was kept at 35^{o}C to stabilise retention times and peak shapes. The elution times of $[^3H]_4$ 11-dehydrocorticosterone and $[^3H]_4$ corticosterone were typically 12.4 and 17.5 min, respectively. Peaks were more than 2 minutes in width and more than 50 times higher than background.

The area under each peak was integrated using Chromeloen v6.7 (Dionex, UK) software and used to calculate the percentage conversion of corticosterone to 11-dehydrocorticosterone. Enzyme activity was calculated from the amount of product

($[^3H]_4$ 11-dehydrocorticosterone) formed from unit hepatic proteins in a unit time and was expressed as reaction velocity (pmol/mg/min).

2.7 Wire angioplasty

Neointimal proliferation was induced by insertion of an angioplasty guide-wire into the femoral artery, as described by Sata *et al.* (2000) and subsequently used by Dover *et al.* (2007) and others.

2.7.1 Surgical preparation

All surgical instruments were sterilized by autoclaving prior to each session, and a bead sterilizer was used to re-sterilise between consecutive operations.

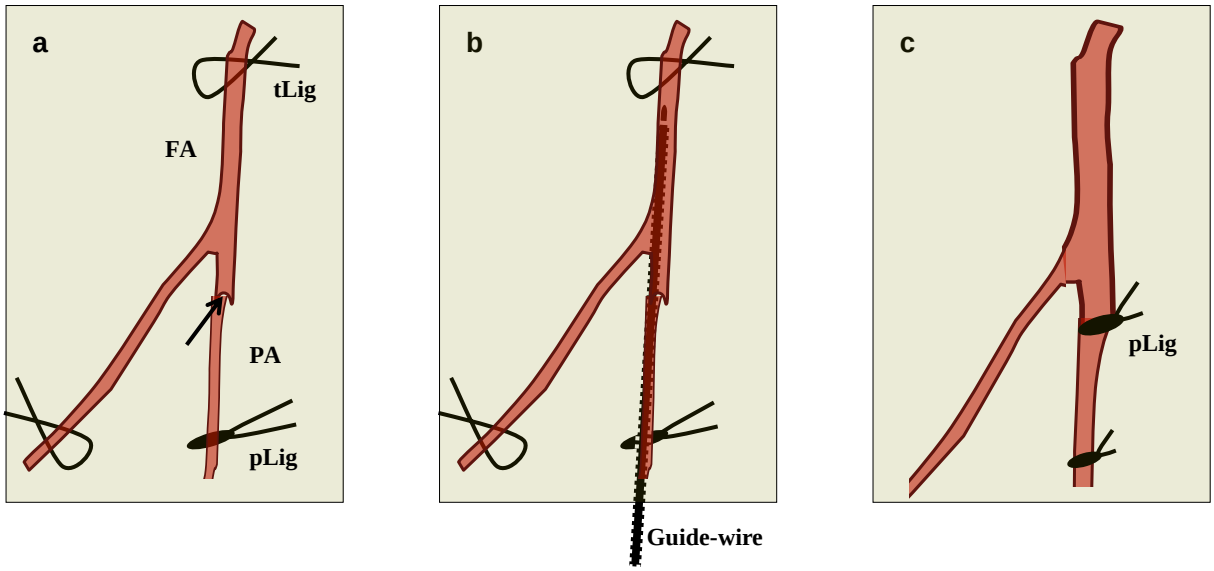
Mice were weighed and anaesthetised by inhaled isoflurane. Animals were first placed in an induction chamber containing 4-5% isoflurane. Upon loss of consciousness, mice were transferred to a heated pad where anaesthesia was maintained with ~2% isoflurane, administered via a nasal cone. Adequate anaesthesia was indicated by loss of pedal withdrawal reflex. To provide post-operative analgesia, 0.05mg/kg buprenorphine was injected subcutaneously. Mice were then placed in a supine position, both hind limbs were secured with adhesive tape and hairs were removed from left groin and upper thigh with an electric shaver.

2.7.2 Operative procedure

A 5mm longitudinal skin incision was made at the proximal thigh. The subcutaneous space was opened by blunt dissection revealing the femoral bundle (containing femoral artery, vein and nerve). The vessels were carefully 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) by blunt dissection. 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 (Figure 2.2). The surgical field was irrigated with 1% lignocaine (lidocaine hydrochloride) to prevent arterial vasospasm. A fixed core straight 0.015" diameter guide-wire was introduced into the popliteal artery via an arteriotomy.

After loosening the proximal temporary ligature, the guide-wire 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 a 6-0 suture proximal to the arteriotomy site, and blood flow restored in the common femoral artery by removing the two temporary ligatures. This procedure 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 2.3: Surgical steps of wire angioplasty of mouse femoral arteries



Temporary ligatures (tLig) were placed on the isolated femoral artery (FA) for vascular control during the procedure, and the popliteal artery (PA) was permanently ligated (pLig) distally (a). An arteriotomy (arrow-head) was performed proximal to pLig. A fixed core straight guide-wire was inserted into the PA via this arteriotomy and advanced into the FA, causing endothelial denudation and stretching of the vessel wall (b). After removal of the wire, the PA was ligated proximal to the arteriotomy site (c).

2.8 *Ex-vivo* 3-dimensional assessment of lesion volume using OPT

Optical Projection Tomography (OPT) is a relatively new technique that allows 3-dimensional imaging and reconstruction of whole tissue samples [Sharpe et al., 2002]. It has superior resolution than other imaging techniques and is non-destructive, allowing subsequent histological analysis.

2.8.1 Principles of OPT imaging

OPT is based on using a light source and an image focusing lens system (optical) to capture raw data and then mathematical transformation to create a 3-dimensional image of original object (projection tomography). OPT is equipped with an apparatus that rotates specimen through 360º around a single axis whilst holding it in position for imaging [Alanentalo et al., 2007].

The optical projection tomograph (Bioptonics 3001, Bioptonics, UK) consists of a diffuse light source, an apparatus for holding and rotating biological samples, adjustable optical lenses to focus light and a charged couple device (CCD) to capture intensity data. The Bioptonics 3001 is equipped with three different light sources, to provide a visible white light, an infrared light and an ultraviolet (UV) light. This device can work in two different modes, transmission mode (tOPT) using visible or infrared light to capture shadows of transmitted light (as in CT scanning) or emission mode (eOPT) using UV light to illuminate the specimen following which photons emitted by excited flourophores within the specimen are focused on to the CCD (as in SPECT scanning).

2.8.2 Trans-cardiac perfusion fixation

To obtain good tissue fixation and to maintain vascular dimensions and lumen size for OPT analysis, trans-cardiac perfusion fixation of whole animals was performed.

Animals were terminally-anaesthetised by intra-peritoneal injection of sodium pentobarbital (80mg/kg, Ceva Animal Health Ltd., UK). Following the onset of deep anaesthesia, indicated by loss of pedal withdrawal reflex, a midline sternotomy and left thoracotomy were performed. The heart was exposed and a 23G needle (Microlane^{TM}, BD Ltd., UK) was introduced into the cavity of the left ventricle. A further incision was

made in the wall of the right atrium to allow the perfusate to escape. PBS containing heparin (10 units/ml) was infused into the left ventricle at a constant rate (7.5 ml/min) using a peristaltic pump (Gilson Inc, USA). Adequate perfusion was indicated by blanching of the liver and other organs and was usually achieved in 3-4 minutes. Afterwards, animals were perfused with 10% neutral formalin to provide internal fixation and tissue preservation. Adequate fixation was indicated by characteristic muscular contractions and subsequent rigidity. The vessels of interest (aortic arch, BCT, femoral arteries etc) were then excised from mice, post-fixed in 10% formalin for 24 hours and then stored in 70% ethanol.

2.8.3 Sample preparation

Samples were embedded in 1.35% *w/v* low melting point agarose (Invitrogen, UK). Agarose was mixed in distilled water, heated to 90ºC and filtered through Whatman 113V paper to eliminate any particulate matter. The agarose was poured into petri dishes and placed on a cold plate (-5ºC) in order to accelerate solidification whilst keeping the vessels in the correct alignment. The gelled agarose blocks were then glued with cyanoacrylate adhesive (Henkel, UK) onto specialized OPT mounts, with the longitudinal axis of the sample in an upright position, and the agarose was then trimmed to a conical shape to reduce back-reflection of light in the scanning plane. The mounted blocks were dehydrated in methanol for 24 hours and then cleared in Murray's clear or BABB (34% *v/v* benzyl alcohol and 66% *v/v* benzyl benzoate).

2.8.4 Scanning and image reconstruction protocol

At the beginning of each session, the tomograph was calibrated using a metal pin to calculate and correct any axial or rotational misalignment.

Tissue samples (attached to OPT mounts and immersed in BABB) were placed in the scanning chamber of the tomograph. Sample positioning, focus and appropriate magnification were set under white light (bright-field condition) and then samples were illuminated by a UV light (dark-field condition). The excitation filter was set at 425/40nm band-pass, the emission filter at 475nm low-pass and the CCD exposure time

as appropriate (typically 200-800ms). Raw data were acquired by capturing images of rotating objects at increments of 0.9º so that a 360º rotation provided 400 images.

Raw images were digitally evaluated and corrected for focus and rotational misalignment and then image reconstruction was performed by filtered back-projection of raw data using NRecon (Skyscan, Belgium) software.

2.8.5 3-dimensional quantification of lesion volume

Volumetric measurements were achieved using CT data analysis (CTan; Skyscan, Belgium) software. A vertical region of interest was defined for each sample and then the position of internal elastic lamina was located (identified by difference in fluorescent signals emitted by media and neointimal) for every 25th scan line by manual tracing and by automated interpolation for the interleaved scan lines. This 3-D volume was further segregated into lumen and neointimal volumes by manually defining intensity threshold for both vascular compartments and automated calculation of volumes (Kirkby et al, unpublished data).

2.9 Cross-sectional analysis of lesion size by histology

2.9.1 Preparation of vessels

Samples fixed in 10% neutral buffered formalin and stored in 70% ethanol 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.).

Paraffin blocks were then cut into 4µm thick serial sections using a Leitz microtome (Leica Microsystem, Germany) and MB35 premier low profile disposable blades (Thermo-Shandon, UK). Ribbons of sections were floated on the surface of a water bath (45ºC) to remove creases and then mounted onto electrostatically coated microscope slides (Superfrost plus; VWR International, UK). Slides were baked (37ºC) overnight to allow good adherence of sections.

2.9.2 Unites States trichrome staining

United States trichrome (UST) staining [Hadoke et al., 1995] was carried out either manually or using an automated staining system (Varistain Gemini; Thermo Shandon, U.K.). The stepwise protocol used for this staining is described in Table 2.1.

Briefly; sections were dewaxed in xylene and rehydrated through graded alcohols to water. Freshly-prepared potassium permanganate was applied and, after rinsing in water, sections were decolourised in aqueous oxalic acid. Sections were washed in tap water, rinsed in 70% alcohol and then immersed in filtered Gomori's 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's trichrome stain (which stains platelets mauve, collagen fibres green and muscle red). Finally, the slides were rinsed in aqueous acetic acid, dehydrated, cleared in xylene and cover-slipped using Di-N-Butyle Phthalate in Xylene (DPX). The result is staining of elastic fibres in deep purple, collagen in green, SMC in red, Nuclei in dark blue, RBCs in green and platelets in mauve (Figure 2.3).

2.9.3 Morphometric analysis of lesion size

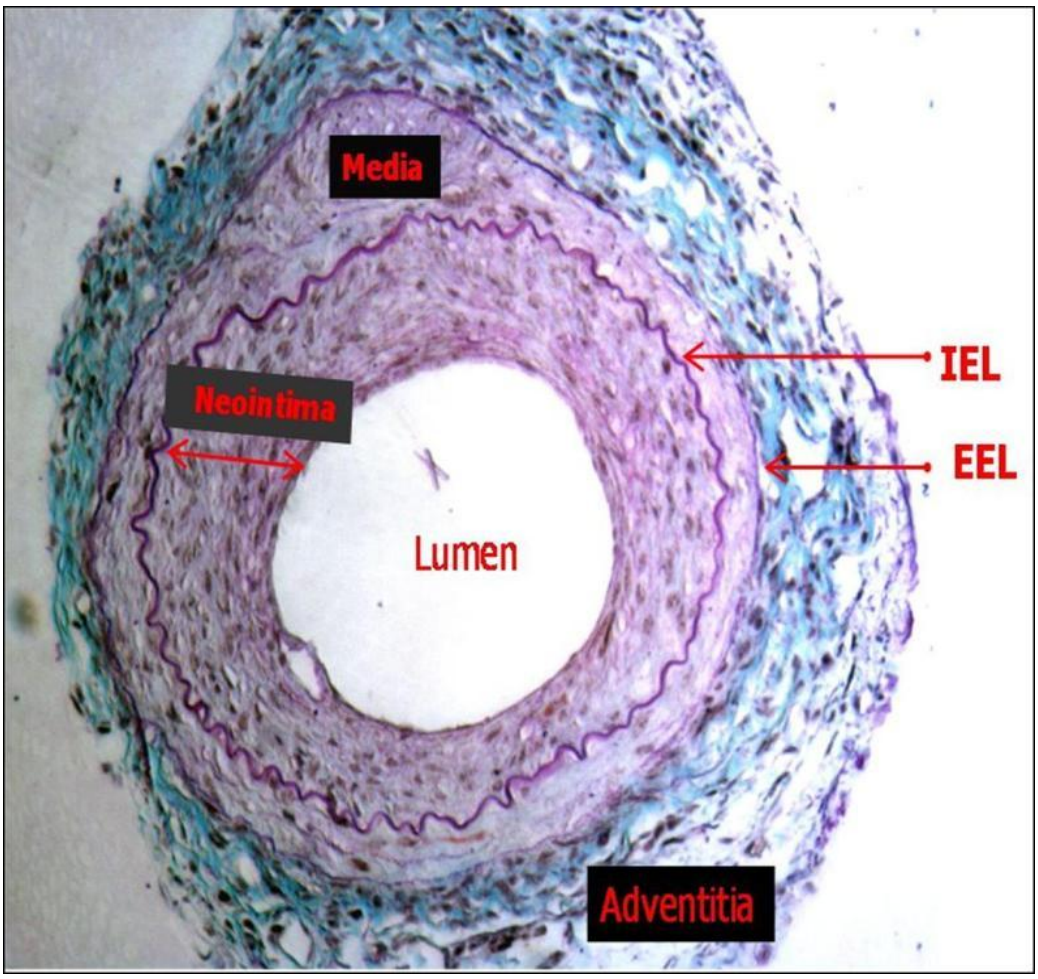
Images of stained sections were digitised using a Photometric CoolSnap camera (Tucson, U.S.A.) coupled to a light microscope (Zeiss Axioskop) via a microcolour liquid crystal turnable RGB filter (Cambridge Research and Instrumentation Inc., Woburn, U.S.A.). Microcomputer Imaging Device software (MCID Basic 7.0; 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 60μm along the length of 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 plaque or neointima (area inside IEL

- area inside lumen). The section with the largest area of plaque or neointima was chosen to represent each artery when calculating the mean lesion size in a group. All analyses were carried out under blinded conditions; slide labels were covered to ensure that there was no bias in the image analysis.

Table 2.2: Protocol for United States trichrome staining

Step	Procedure	Time
1	Deparaffinise in Xylene	2 x 5 min
2	Place in 100% ethanol	1 min
3	Place in 90% ethanol	1 min
4	Place in 70% ethanol	1 min
5	Wash in running tap water	5 min
6	Place in fresh 0.3% $KMnO_4$ in 0.3%H_2SO_4	1 min
7	Rinse in distilled water	1 min
8	Decolourise using 2% oxalic acid	5 sec
9	Wash in running tap water	5 min
10	Rinse in 70% alcohol	1 min
11	Apply filtered Gomori's aldehyde fuchsin	5-10 min
12	Rinse in 70% alcohol	1 min
13	Wash in running tap water	5 min
14	Check under microscope for elastin staining	
15	Apply filtered Weigert's iron haematoxylin	5 min
16	Blue in running tap water	5 min
17	Place in 5% aqueous phosphotungstic acid	5 min
18	Rinse in distilled water	1 min
19	Apply filtered Gomori's trichrome	20 min
20	Rinse in 0.2% acetic acid	30 sec
21	Place in 70% ethanol	1 min
22	Place in 90% ethanol	1 min
23	Place in 100% ethanol	1 min
24	Place in xylene	2 x 2 min
25	Mount cover-slips with DPX	

Figure 2.4: Section of a vessel (after wire-angioplasty) stained with UST



United States trichrome (UST) stains internal and external elastic lamina (IEL, EEL) in deep purple which allows differentiation of vascular layers and measurement of luminal, neointimal (between lumen and IEL) and medial (between IEL and EEL) areas.

2.10 Analysis of lesion composition by histology

Lesion composition was determining by staining serial sections at the point of maximum lesion cross-sectional area.

2.10.1 Picrosirius red staining for collagen

Picrosirius red is a sensitive and specific stain for collagen fibres [Dolber and Spach, 1993]. Slides were dewaxed in xylene (2 x 5 min), rehydrated through graded alcohols (100%, 90% and 70%, 1 min each) and rinsed in running tap water (5min). Slides were then immersed in staining solution, 0.1% *w/v* Direct Red 80 and 0.1% *w/v* Fast Green FCF in saturated aqueous picric acid, for 2hr. Slides were then briefly rinsed in running tap water, dehydrated through graded alcohols (70%, 90% and 1005, 1 min each) and xylene (2 x 2 min) and cover-slipped using DPX medium.

2.10.2 Immunohistochemistry for smooth muscle cells

Smooth muscle content in atherosclerotic and neointimal lesions was determined by immunohistochemistry. The protocol is in Table 2.2. Slides were deparaffinised and rehydrated, then mounted in sequenza cassettes (Thermo Shandon, U.K.). 20% goat serum in PBS was applied to sections which were then incubated with a 1:400 dilution of mouse anti-mouse monoclonal primary antibody against α-smooth muscle actin (Sigma A2547) made up in PBS/1% BSA. Goat anti-mouse IgG (Vector Labs BA-9200) diluted 1:400 in PBS/1% BSA was then used as secondary antibody. To visualise sites of antibody binding, sections were treated with Extravidin-Peroxidase LSAB reagent and developed with DAB substrate. Sections were then washed, counterstained with haematoxylin, blued in tap water, dehydrated, cleared and mounted in DPX.

Table 2.3: Protocol for immunohistochemical staining for smooth muscle cells

Step	Procedure	Duration
1	Bake sections at 55ºC	30 min
2	Deparaffinise in Xylene	2 x 5 min
3	Place in 100% ethanol	1 min
4	Place in 90% ethanol	1 min
5	Place in 70% ethanol	1 min
6	Wash in running tap water	5 min
7	Mount slides onto sequenza	
8	Wash with PBS	2 x 5 min
9	Apply 200µl 3% H_2O_2	10 min
10	Wash with PBS	2 x 5 min
11	Apply 200µl 2.5% BSA, 2.5% milk powder in PBS	1 hour
12	Apply 200µl 20% goat serum	30 min
13	Apply 200µl of 1:400 mouse anti-mouse α-SMA antibody	30 min
14	Wash with PBS	3 x 5 min
15	Apply 200µl of 1:400 goat anti-mouse IgG (2º antibody)	30 min
16	Wash with PBS	3 x 5 min
17	Apply 200µl of 1:200 Extravidin-Peroxidase LSAB reagent	30 min
18	Wash with PBS	3 x 5 min
19	Apply prepared DAB solution (Vector Labs SK-4100)	~ 3 min
20	Quench reaction with tap water	
21	Remove from sequenza and rinse in distilled water	5 min
22	Counter-stain nuclei with haematoxylin	10 sec
23	Blue in running tap water	5 min
24	Place in 70% ethanol	1 min
25	Place in 90% ethanol	1 min
26	Place in 100% ethanol	1 min
27	Place in xylene	2 x 2 min
28	Mount cover-slips with DPX	

2.10.3 Immunohistochemistry for macrophages

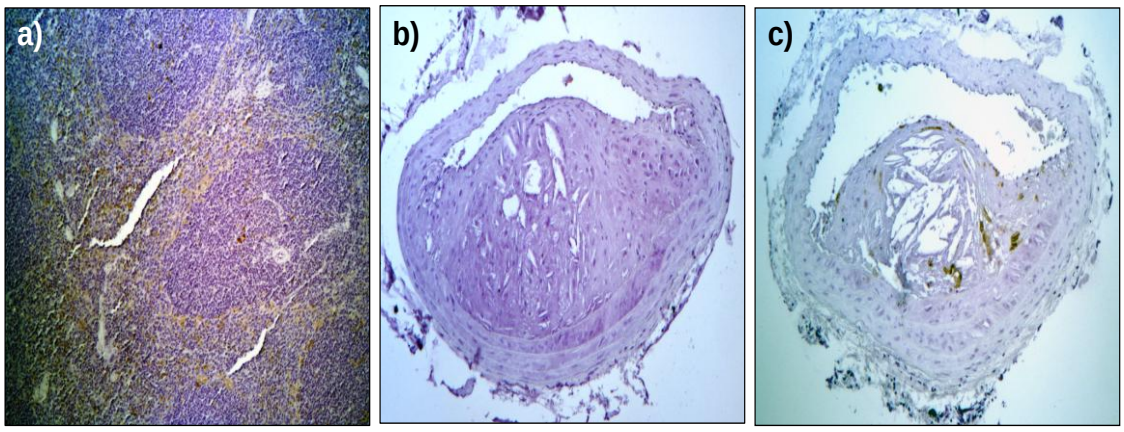
Macrophage content was assessed by immunohistochemistry for which sections were deparaffinised, blocked with normal goat serum, incubated with purified monoclonal rat anti-mouse Mac-2 antibodies (1:6000; Cedarlane, UK), followed by goat anti-rat IgG secondary antibody and Extravidin-Peroxidase LSAB reagent, developed with DAB substrate and counterstained with haematoxylin (Table 2.3).

All immunohistochemistry examinations had a positive control using tissues with definitive expression of antigen being tested and a negative control by omitting primary antibody in staining steps. The vessels wall (tunica media) was used as positive control for smooth muscle cells and spleen was used as positive control for macrophages (Figure 2.4).

Table 2.4: Protocol for immunohistochemical staining for macrophages

Step	Procedure	Duration
1	Bake sections at 55ºC	30 min
2	Deparaffinise in Xylene	2 x 5 min
3	Place in 100% ethanol	1 min
4	Place in 90% ethanol	1 min
5	Place in 70% ethanol	1 min
6	Wash in running tap water	5 min
7	Mount slides onto sequenza	
8	Wash with PBS	2 x 5 min
9	Apply 200µl 3% H_2O_2	10 min
10	Wash with PBS	2 x 5 min
11	Apply 200µl 20% goat serum	30 min
12	Apply 200µl of 1:6000 rat anti-mouse Mac-2 antibody	
13	Store slides at 4ºC	Overnight
14	Wash with PBS	3 x 10 min
15	Apply 200µl of 1:200 goat anti-rat IgG (2º antibody)	30 min
16	Wash with PBS	3 x 5 min
17	Apply 200µl of 1:200 Extravidin-Peroxidase LSAB reagent	30 min
18	Wash with PBS	3 x 5 min
19	Apply prepared DAB solution (Vector Labs SK-4100)	~ 3 min
20	Quench reaction with tap water	
21	Remove from sequenza and rinse in distilled water	5 min
22	Counter-stain nuclei with haematoxylin	10 sec
23	Blue in running tap water	5 min
24	Place in 70% ethanol	1 min
25	Place in 90% ethanol	1 min
26	Place in 100% ethanol	1 min
27	Place in xylene	2 x 2 min
28	Mount cover-slips with DPX	

Figure 2.5: Illustration of positive and negative controls for immuno-histochemsitry



+ve control (Spleen) -ve control (vessel without Ab) Vessel sample

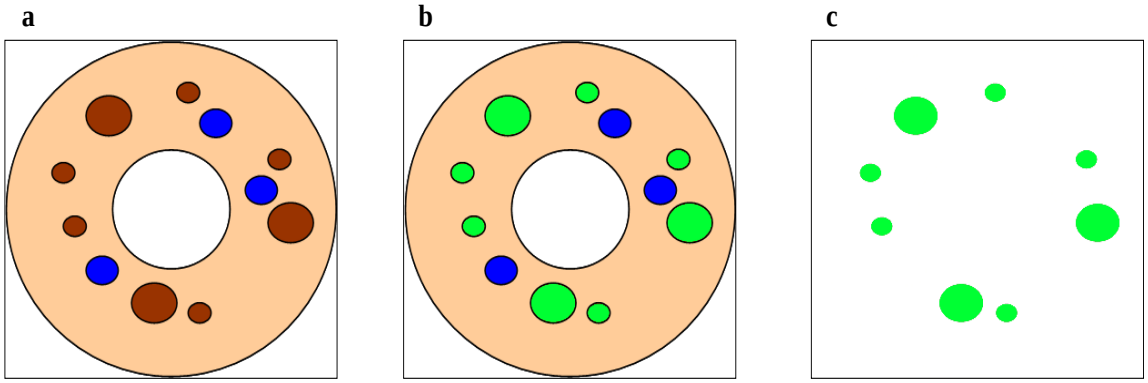
Vascular lesion composition was determined by analysing sections for immunoreactivity for Mac-2 (macrophage content) and immunoreactivity for α-smooth muscle actin (smooth muscle content). Positive controls were used to ensure that technique is working and negative controls were used to ensure specificity of primary antibodies used. Immunoreactivity for Mac-2 yielded positive staining (brown) in spleen (a), vessel sections without primary antibody (b) did not shown any staining and therefore vessel sections with primary antibody (c) show specific staining of macrophages.

2.10.4 Morphometric analysis of lesion composition

Images of sections stained histologically or immunohistochemically were digitalised (as described in section 2.10.3). Quantification of the area of a particular stain was performed using semi-automated colour de-convolution process (Figure 2.5) with Photoshop CS3 Extended (Adobe Systems Inc, USA) software. This technique has been validated in literature [Loughlin et al., 2007] and also in our deprtment (Kirkby *et al.*, unpublished data).

All analysis was carried out under blinded conditions.

Figure 2.6: Illustration of colour deconvolution technique for composition analysis of immunohistochemical stains



The colour deconvolution technique using adobe Photoshop allowed composition analysis of lesion. Sections stained for collagen, smooth muscle cells or macrophages were digitalised and colour range of a particular stain (for example brown staining in panel a) were determined from positive control slides. These colour values were then applied to sections of interest and then the area for that colour was identified and isolated (green area in panel b). Images were then segmented (panel c) according to this selected colour range and the area was calculated and expressed as a percentage of the plaque or neointimal lesion area.

2.11 Statistical analysis

Data are expressed as mean ± SEM unless stated otherwise. Student's t test was used to compare two sets of parametric data. One-way ANOVA was used to compare three or more sets of parametric data followed by *post-hoc* (Tukey or Bonferroni) tests, where appropriate. Two-way ANOVA was used to evaluate the effects of two factors on measurements. Statistical significance was assumed when $p<0.05$. All statistical testing and data analyses were performed using Prism 4.0 (GraphPad software, USA) software.

3. Role of 11β-HSD1 in atherosclerosis

3.1 Introduction

Selective inhibitors of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) are currently being developed as a potential therapeutic target for metabolic conditions [Rosenstock et al., 2010] and it is suggested that 11β-HSD1 inhibitors, by ameliorating cardiovascular risk factors, may have a role in the prevention and treatment of atherosclerosis [Hadoke et al., 2009]. In support of this notion, it has been shown that mice with transgenic deletion of 11β-HSD1 have a cardio-protective phenotype [Morton et al., 2001]

However, few studies have directly assessed the impact of these compounds on the development of atherosclerotic lesions. Non-selective inhibition of both isozymes (type 1 and type 2) of 11β-HSD with carbenoxolone reduced lesion size in obese-hyperlipidaemic LDL receptor knockout ($LDLR^{-/-}$) mice [Nuotio-Antar et al., 2007]. The impact of selective inhibitors of 11β-HSD1, however, remains unclear. A selective 11β-HSD1 inhibitor produced by Merck Laboratories (compound 544, Triazole group) produced a dramatic (~85%) reduction in aortic cholesterol content (a surrogate marker of atherosclerosis) in apolipoprotein-E deficient ($ApoE^{-/-}$) mice [Hermanowski-Vosatka et al., 2005]. In contrast, it has been suggested that another inhibitor produced by Amgen (compound 2922, Thiazolone group) does not influence atherosclerosis in $LDLR^{-/-}$ mice [Lloyd et al., 2009]. However, size of atherosclerotic lesions or composition of plaques were not analysed in these studies. Furthermore, the mechanism for the potential atheroprotective effect remains uncertain, though it is suggested that a direct alteration of inflammatory signalling in the vessel wall may account for reduced lipid incorporation [Hermanowski-Vosatka et al., 2005]. Finally, given the fact that many cardiovascular deaths are the result of plaque rupture [Zaman et al., 2000], it remains to be established whether 11β-HSD1 inhibition actually alters plaque composition to produce a more stable lesion phenotype.

The work in this chapter, therefore, addressed the hypothesis that selective inhibition of 11β-HSD1 reduces the development of atherosclerotic lesions by improvement in cardiovascular risk factors. To test this hypothesis, the specific aims of this chapter were:

- To establish whether selective inhibition of 11β-HSD1 reduces the size of plaques in atherosclerosis-prone *ApoE*$^{-/-}$ mice
- To determine the effect of selective inhibition of 11β-HSD1 on plaque composition
- To assess the effect of selective inhibition of 11β-HSD1 on systemic cardiovascular risk factors

3.2 Methods

3.2.1 Animals and materials

Adult, male *ApoE*$^{-/-}$ mice used in this study were bred in-house, caged individually and maintained under standard conditions of light (lights on 8am-8pm) and temperature (21-22^{o}C). All animal work was carried out under Home Office (UK) Licence.

All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Compound 544 (3-adamantan-1-yl-6,7,8,9-tetrahydro-5H-[1,2,4]triazolo[4,3-α]azepine), obtained from Enamine Ltd. Ukraine (as compound T5293658) was used as a selective inhibitor of 11ß-HSD1 [Hermanowski-Vosatka et al., 2005].

3.2.2 Measurement of effect of compound 544 on 11β-HSD1 activity

C57Bl/6J mice (n=6) fed on standard chow were randomised to receive either a single dose of compound 544 (30mg/kg, in 5% 2-hydroxypropyl-β-cyclodextrin) or vehicle alone by oral gavage. Mice were culled 4hr later and livers were snap-frozen. Hepatic 11β-HSD1 kinetics were determined from liver homogenate (by centrifugation, 1000g, 4°C, 15min). Protein concentration was assessed using the Bradford method. Liver homogenates (40-80µg/ml protein) were incubated (1hr, 37^{o}C) in duplicate in Krebs-Ringer buffer containing 0.2% glucose, NADP (2 mM), [^{3}H]$_{4}$-corticosterone (5nM), and unlabeled corticosterone (15nM). Steroids were separated by HPLC, using a reverse phase C_{18} Sunfire Symmetry column (Waters, Edinburgh, UK) and quantified by on-line liquid scintillation counting (2ml/min, Proflow, Meridian, Surrey, UK), as described in section 2.6.

3.2.3 Effects of compound 544 on vascular lesions and risk profile

ApoE$^{-/-}$ mice, aged 10 weeks, were fed an atherogenic western diet (D12079B, Research Diets, USA) for 6 weeks and then randomised to either control (n=6, western diet only) or inhibitor (compound 544,10 mg/kg/day, mixed with western diet; n=6) groups for further 8 weeks. Body weight was measured every week and systolic blood pressure (BP) was measured at 2-4week intervals. Fasting samples were collected for

biochemical analysis and glucose tolerance tests (section 2.5.1) performed one week before cull.

At the end of the treatment period, mice were weighed for a final time and then killed by asphyxiation in CO_2. Trunk blood was collected into EDTA tubes on wet ice and centrifuged to separate plasma which was then snap-frozen on dry ice and stored at -80ºC. Internal organs including heart, liver, kidneys, adrenal glands and thymus were removed, weighed, snap-frozen and stored at -80º C. The arterial tree, from the aortic root to the femoral arteries (including all major branches), was removed for analysis of plaque distribution. Arteries were fixed in 10% neutral buffered formalin solution for 24 hours and then stored in 70% ethanol. The brachiocephalic trunk (BCT) was isolated, dehydrated though graded alcohols, embedded in paraffin and cut into 4µm thick transverse sections along its entire length.

3.2.4 Analysis of atherosclerotic lesions

Atherosclerotic lesions in several predisposed sites were assessed semi-quantitatively by inspection of fixed vascular trees under the operating microscope and were scored using a scale of arbitrary units (AU 0 - 5). For histological analysis, sections taken at 60µm intervals were stained with the United States trichrome (section 2.9.2). Images of stained sections were analysed using a light microscope coupled to a colour camera and an image analysis system (section 2.9.3). Plaque area (μm^2) and the area inside the internal elastic lamina (IEL) were measured. These measurements were then used to calculate the percentage cross-sectional narrowing [(area of plaque / area inside IEL) x 100]. The section with the largest cross-sectional narrowing was chosen to represent each artery when calculating the mean plaque area in a group. All analyses were carried out under blinded conditions.

3.2.5 Analysis of plaque composition

Plaque composition was determined by serial sections at the site of maximum plaque size. Collagen content was assessed by staining sections with Picrosirius red (section 2.10.1). Macrophage and smooth muscle content were assessed by immunohistochemistry using monoclonal rat anti-mouse Mac-2 antibodies for

macrophages and mouse anti-mouse α-smooth muscle actin antibodies for smooth muscle cells (section 2.10.2 and 2.10.3). Extra-cellular lipids were quantified by measuring acellular clefts within plaques (blank areas in plaques in UST stained sections) [Moreno et al., 2002]. A measure of plaque stability was generated by calculating a Plaque Vulnerability Index (PVI= area of macrophage staining + area of lipid staining /area of collagen staining + area of SMC staining) [Davies, 1996;Suzuki et al., 2003;Shiomi et al., 2008;Guo et al., 2009]. All analyses were carried out under blinded conditions.

3.2.6 Biochemical Tests

GTTs were performed in fasting (6hr) mice by intra-peritoneal bolus injection of glucose (2mg/g body weight). Blood samples were taken 0, 15, 30, 60 and 90 minutes after glucose administration. Serum glucose levels were determined spectrophotometrically by the glucose hexokinase reagent and serum insulin levels were measured by ultra-sensitive mouse insulin ELISA kit (section 2.5.5). Insulin resistance (IR) was assessed by HOmeostasis Model Assessment method (HOMA-IR = Fasting insulin (μU/ml) x Fasting glucose (mmol/L) / 22.5) and QUantitative Insulin sensitivity ChecK Index (QUICKI = 1/[(log Fasting Glucose)+(log Fasting Insulin)] [Cacho et al., 2008]. Serum cholesterol levels and triglyceride levels were measured spectrophotometrically using Infinity™ reagents (section 2.5.3). Blood collected at the time of cull was used to determine plasma renin activity and angiotensinogen by radioimmunoassay and aldosterone by ELISA (kindly performed by Dr. Christopher J Kenyon as previously described [Morton et al., 2005b;Al-Dujaili et al., 2009]).

3.2.7 Statistics

Data are mean ± SEM (with n indicating the number of animals in each experimental group) and were analysed using unpaired Student's t-test or repeat measure two-way ANOVA, as appropriate. Statistical significance was assumed when $p<0.05$. All calculations were performed using GraphPad Prism software.

3.3 Results

3.3.1 Compound 544 inhibits 11β-HSD1 activity

A single dose of compound 544 inhibited dehydrogenase activity (29.2±6.8pmol/mg/min vs. 61.3±17.9pmol/mg/min, p=0.04, Figure 3.1) in hepatic homogenates. 11β-HSD1 is a predominant reductase *in-vivo* [Jamieson et al., 2000], however, in tissue preparations dehydrogenase activity predominates, which was quantified here and was nearly halved by administration of compound 544.

3.3.2 Systemic effects of 11β-HSD1 inhibition

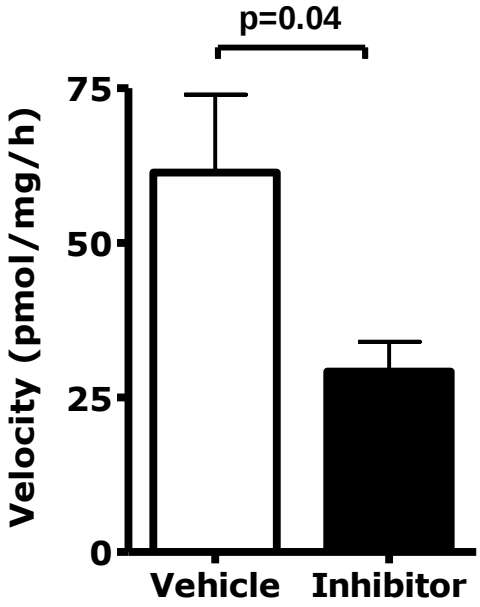
3.3.2.1 11β-HSD1 inhibition reduced weight gain

All mice gained weight on western diet but this was less pronounced in compound 544-treated mice than in controls (Figure 3.2). There was no significant difference in food intake between the two groups (Control 8.0±0.4g/day vs. Inhibitor 7.6±0.2g/day, p=0.3). There were no differences in the weights of heart, kidneys, adrenal glands, thymus or spleen but the inhibitor-treated group had lower liver weight (Table 3.1).

3.3.2.2 11β-HSD1 inhibition lowered blood pressure

Blood pressure in the two groups was similar at baseline. However, at the end of the study (8 weeks of treatment), BP was significantly lower in the inhibitor-treated mice than in the vehicle-treated controls (Figure 3.3). To explore the possible mechanism for this blood pressure reduction, activity of the renin-angiotensin system was assessed. No differences were found in plasma renin activity angiotensinogen levels or aldosterone levels between the two groups (Table 3.2).

Figure 3.1: Compound 544 inhibited dehydrogenase activity in C57Bl/6J mice



Single doses of compound 544 (30mg/kg, in 5% 2-hydroxypropyl-β-cyclodextrin) or vehicle (5% 2-hydroxypropyl-β-cyclodextrin) were given to C57Bl/6J mice via oral gavage. Mice were culled after 4 hours and hepatic dehydrogenase activity was assessed. Compound 544 reduced 11β-HSD1 dehydrogenase activity by ~50%.

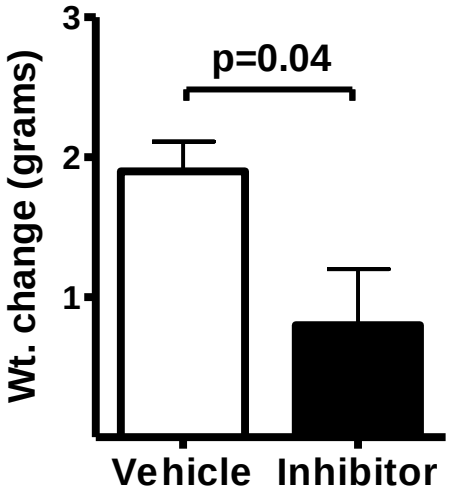
Data are mean ± SEM for n=3/group and analysed by unpaired Student's t-test.

Table 3.1: Effect of 11β-HSD1 inhibition on organ weights

Organ	Inhibitor	Control	p
Liver	48.0±1.7	53.8±1.5	0.03
Heart	5.9±0.6	6.5±0.5	0.2
Kidneys	12.4±0.6	12.6±0.7	0.8
Adrenal glands	0.11 ± 0.01	0.09±0.01	0.06
Spleen	2.89±0.18	2.88±0.11	0.9
Thymus	0.97±0.06	0.84±0.03	0.08

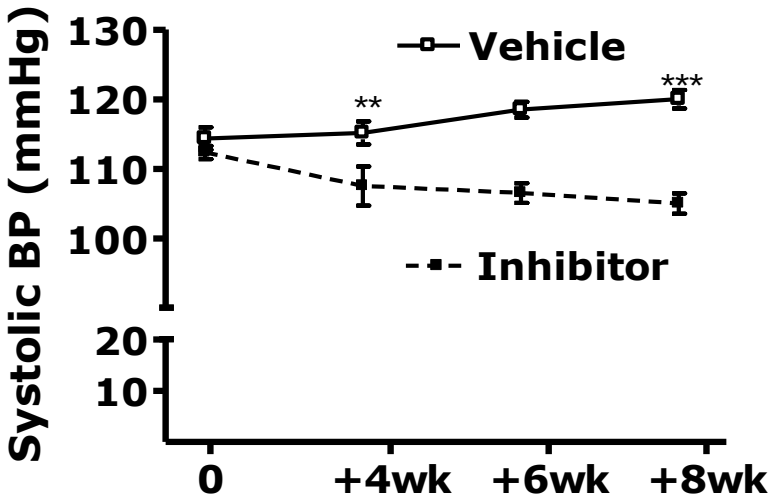
Organ weights are expressed as mg/g of body weight after 8 weeks of treatment with vehicle or 11β-HSD1 inhibitor (compound 544; 10mg/kg/day in western diet). Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

Figure 3.2: 11β-HSD1 inhibition reduced weight gain in western diet fed *ApoE*$^{-/-}$ mice



Treatment with selective 11β-HSD1 inhibitor (compound 544; 10mg/kg/day in western diet; 8weeks) reduced weight gain in apolipoprotein E deficient (*ApoE*$^{-/-}$) mice. Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test

Figure 3.3: 11β-HSD1 inhibition lowered blood pressure in *ApoE*$^{-/-}$ mice fed a western diet

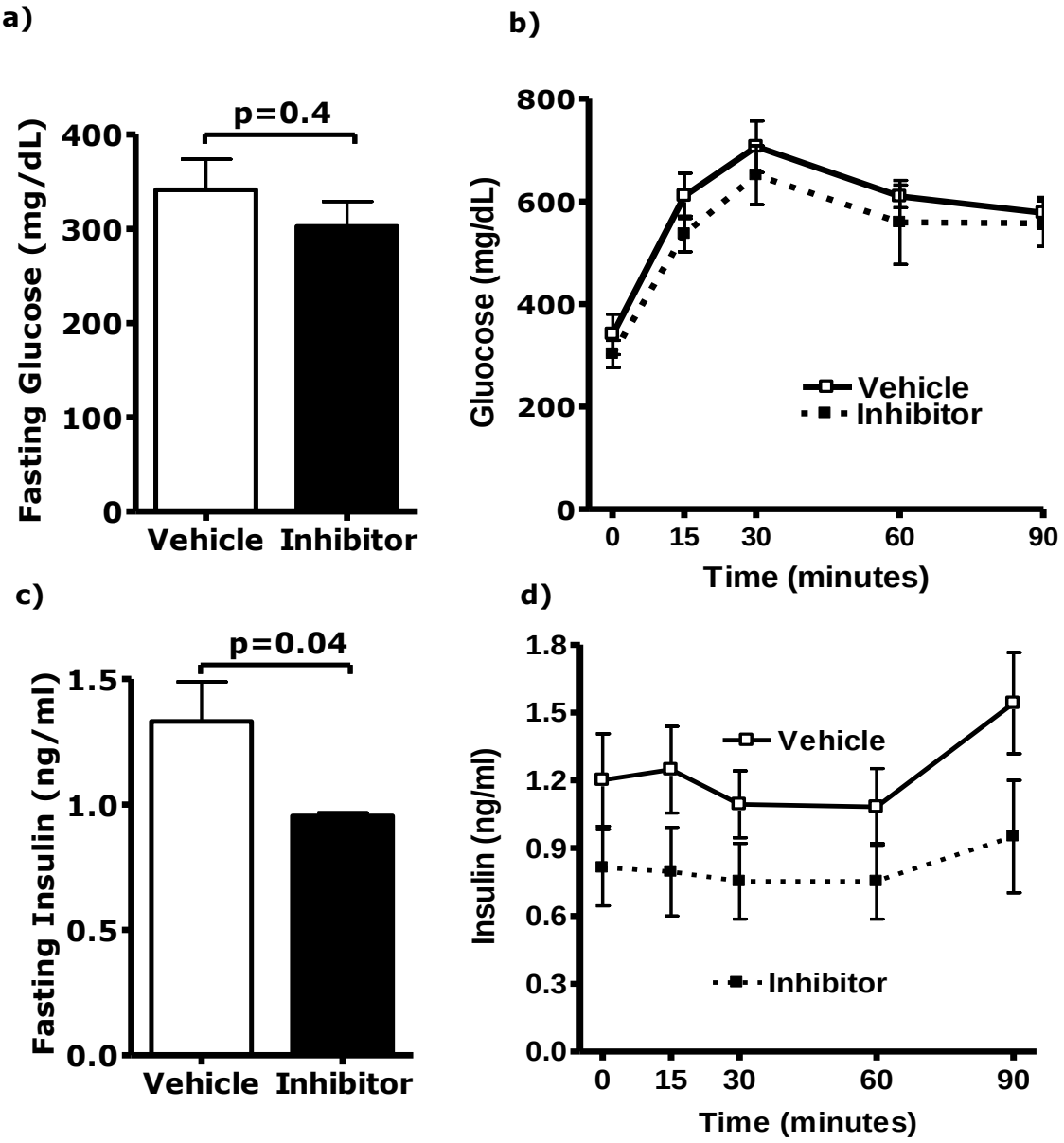


BP was similar in two groups at baseline (time 0, start of treatment). However, treatment with 11β-HSD1 inhibitor (compound 544, 10mg/kg/day in western diet; 8weeks) significantly lowered blood pressure ($p<0.001$). Bonferroni post-hoc analysis revealed a significant BP difference between the two groups after 6 (**$p<0.01$) and 8 (***$p<0.001$) weeks of treatment. Data are mean ± SEM for n=6/group.

3.3.2.3 11β-HSD1 inhibition had little effect on glycaemic and lipid profile

There were no differences in fasting total cholesterol or triglyceride levels between inhibitor and vehicle treated groups (Table 3.2). Similarly, fasting and GTT glucose levels (Figure 3.4a and 3.4b) were not influenced by inhibitor treatment and also the area under curve (AUC) for glucose on GTT was not different between vehicle and inhibitor treated groups (Table 3.2). However, fasting insulin levels were significantly lower in the inhibitor treated group (Figure 3.2c) and insulin levels did not rise during the GTT in these mice (Figure 3.4d), producing a trend towards improved insulin sensitivity, as measured by HOMA-IR or QUICKI (Table 3.2).

Figure 3.4: 11β-HSD1 inhibition did not affect glucose levels but reduced insulin levels in *ApoE*$^{-/-}$ mice fed a western diet



After 8 weeks of treatment with vehicle or 11β-HSD1 inhibitor (compound 544; 10mg/kg/day in western diet) there was no significant difference in fasting (a) and GTT (b) glucose levels. However, fasting (c) and GTT (d) insulin levels were lower in the inhibitor treated groups, suggesting an improvement in insulin sensitivity (Table 3.2). Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

Table 3.2: Effect of 11β-HSD1 inhibition on biochemical profile

Parameter	Units	Control	Inhibitor	p
Fasting Glucose (FG)	mg/dL	341±39.6	302.±26.7	0.4
$AUC_{Glucose}$ on GTT	(mg/dL).min	54630±1504	50100±4448	0.4
Fasting Insulin (F1)	ng/ml	1.33±0.16	0.95±0.024	0.04
$AUC_{Insulin}$ on GTT	(ng/ml).min	108±15	71±14	0.1
Insulin Resistance (IR)				
HOMA-IR		28.8±6.1	17.7±1.6	0.1
QUICKI		0.36±0.01	0.39±0.01	0.05
Fasting total cholesterol	mmol/L	13.2±1.5	12.0±1.6	0.2
Fasting triglycerides	mmol/L	2.0±0.1	2.1±0.1	0.3
Plasma renin activity	ng-AngI/ml/hr	626±68	637±129	0.9
Angiotensinogen	ng/ml	145±9	127±26	0.5
Aldosterone	pmol/L	2193±653	1675±518	0.5

HOMA HOmeostasis Model Assessment,

QUICKI QUantitative Insulin sensitivity ChecK Index

3.3.3 11β-HSD1 inhibition reduced the size of atherosclerotic lesions

Atherosclerotic lesions developed in all *ApoE*$^{-/-}$ mice fed on a western diet but were smaller in those treated with the 11β-HSD1 inhibitor (Figure 3.5a). Semi-quantitative assessment of atherosclerotic burden revealed that the 11β-HSD1 inhibitor reduced the size of atherosclerotic lesions in all major vessels (especially in the aortic arch, common carotid arteries and brachiocephalic trunk, Table 3.3) and, hence, reduced the global atheroma burden (Figure 3.5b).

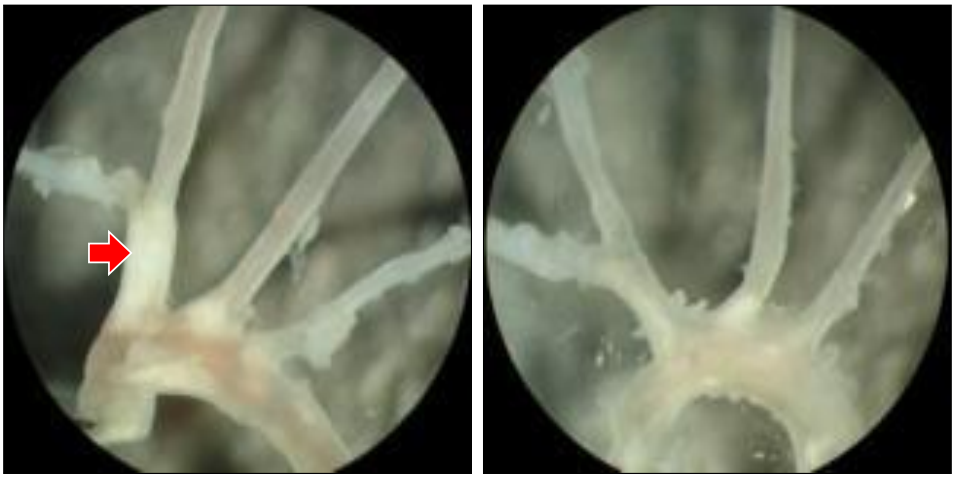
This semi-quantitative assessment was confirmed by the histological data from UST-stained sections of BCT showing that the maximum cross-sectional narrowing (CSN) was reduced by 26% following 11β-HSD1 inhibitor administration (Figure 3.6).

3.3.4 11β-HSD1 inhibition improved markers of plaque stability

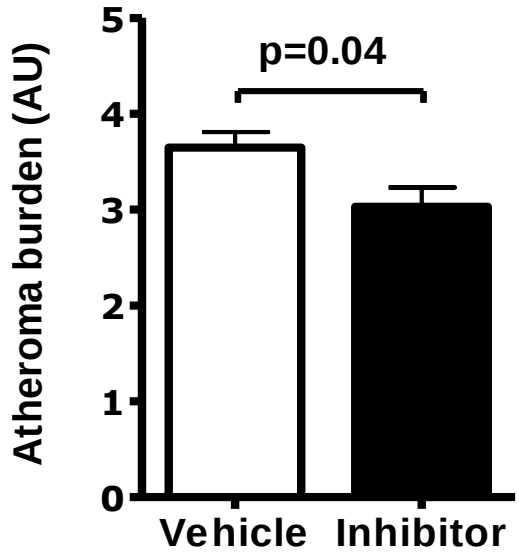
11β-HSD1 inhibition improved markers of stability by changing plaques from a lipid-rich to a fibrous composition. Compared with vehicle-treated controls, extracellular lipid content was significantly lower in lesions from mice exposed to the 11β-HSD1 inhibitor (Figure 3.6). Although there were no differences in macrophage (Figure 3.7a) or smooth muscle cell (Figure 3.7b) content between groups, collagen content was increased (Figure 3.8) in the treated group. The plaque vulnerability index, calculated by incorporating these four parameters, was reduced by 52% in the inhibitor treated group (Figure 3.9).

Figure 3.5: Selective inhibition of 11β-HSD1 reduced lesion development throughout the vasculature of *ApoE*$^{-/-}$ mice

a)



b)



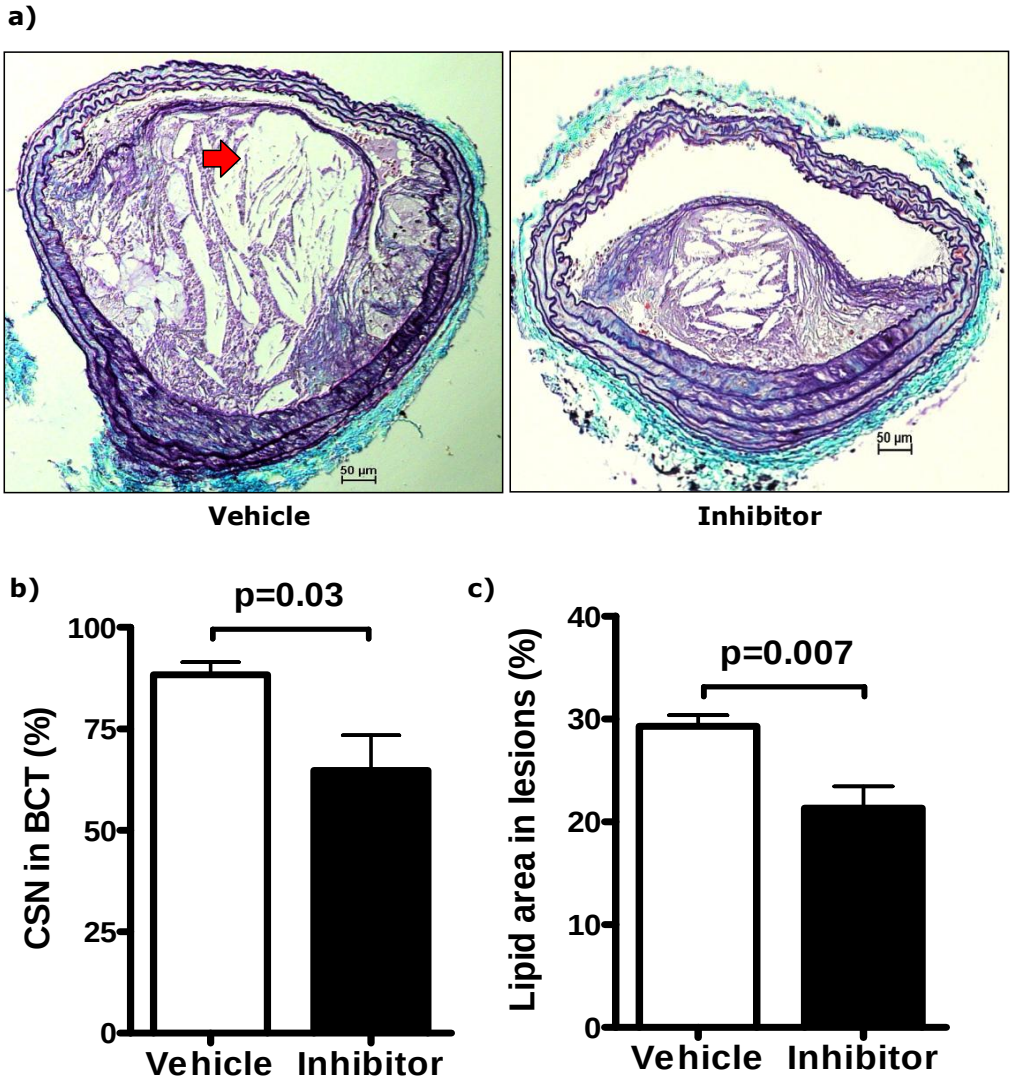
Morphological examination indicated reduced lesion size in the aorta and its major branches from apolipoprotein E deficient (*ApoE*$^{-/-}$) mice treated with the selective 11β-HSD1 inhibitor (compound 544; 10mg/kg/day in western diet; 8weeks). An almost occlusive lesion is evident (arrow-head) in the BCT of a mouse from the vehicle-treated group (a). Semi-quantitative assessment revealed a reduction in global atheroma burden (measured in arbitrary units) in inhibitor treated mice as compared with vehicle treated group (b).
Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

Table 3.3: Effect of 11β-HSD1 inhibition on plaque burden in *ApoE*$^{-/-}$ mice

Vascular territory	Control (AU) (n=6)	Inhibitor (AU) (n=6)	p
Aortic Arch	3.2±0.2	2.1±0.3	0.01
Common carotid	3.0±0.3	2.2±0.2	0.02
BCT	4.7±0.2	3.7±0.3	0.04
Iliac arteries	2.7±0.2	1.8±0.3	0.04
Global (average of all territories)	3.6±0.9	3.0±1.0	0.03

Lesion development was scored semi-quantitatively based on the following scale: AU 1, 0-20% luminal narrowing; AU 2, 21-40% luminal narrowing; AU 3, 41-60% luminal narrowing; AU 4, 61-80% luminal narrowing; AU 5, 81-100% luminal narrowing. Data are mean±SEM and were analysed by un-paired Student's t-test.
AU, arbitrary units; BCT, brachiocephalic trunk

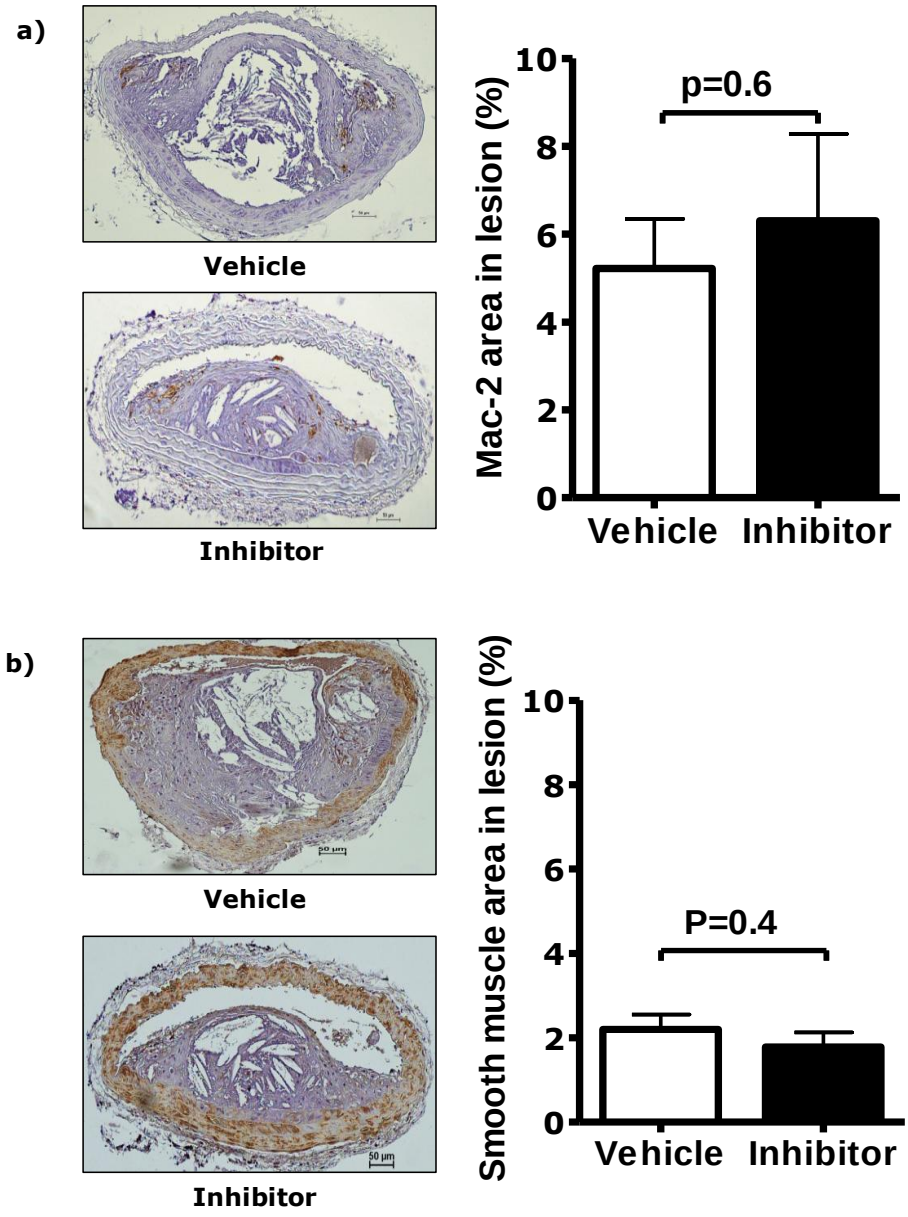
Figure 3.6: Selective inhibition of 11β-HSD1 reduced cross-sectional narrowing and lipid content of plaques



United States trichrome staining of brachiocephalic trunk (BCT) cross-sections (a) from Apolipoprotein E deficient (*ApoE*$^{-/-}$) mouse allowed measurement of lesion area using image analysis. It also revealed acellular clefts (arrow-head) which represent areas of washed out extracellular lipid crystals. The analysis confirmed that lesions in inhibitor-treated (compound 544; 10mg/kg/day in western diet; 8weeks) mice were smaller than those in vehicle-treated controls, with a significant reduction in cross-sectional narrowing (CSN, (b)). Quantification of extracellular lipid areas confirmed that plaques in inhibitor-treated mice had significantly less lipids than those in vehicle-treated controls (c).

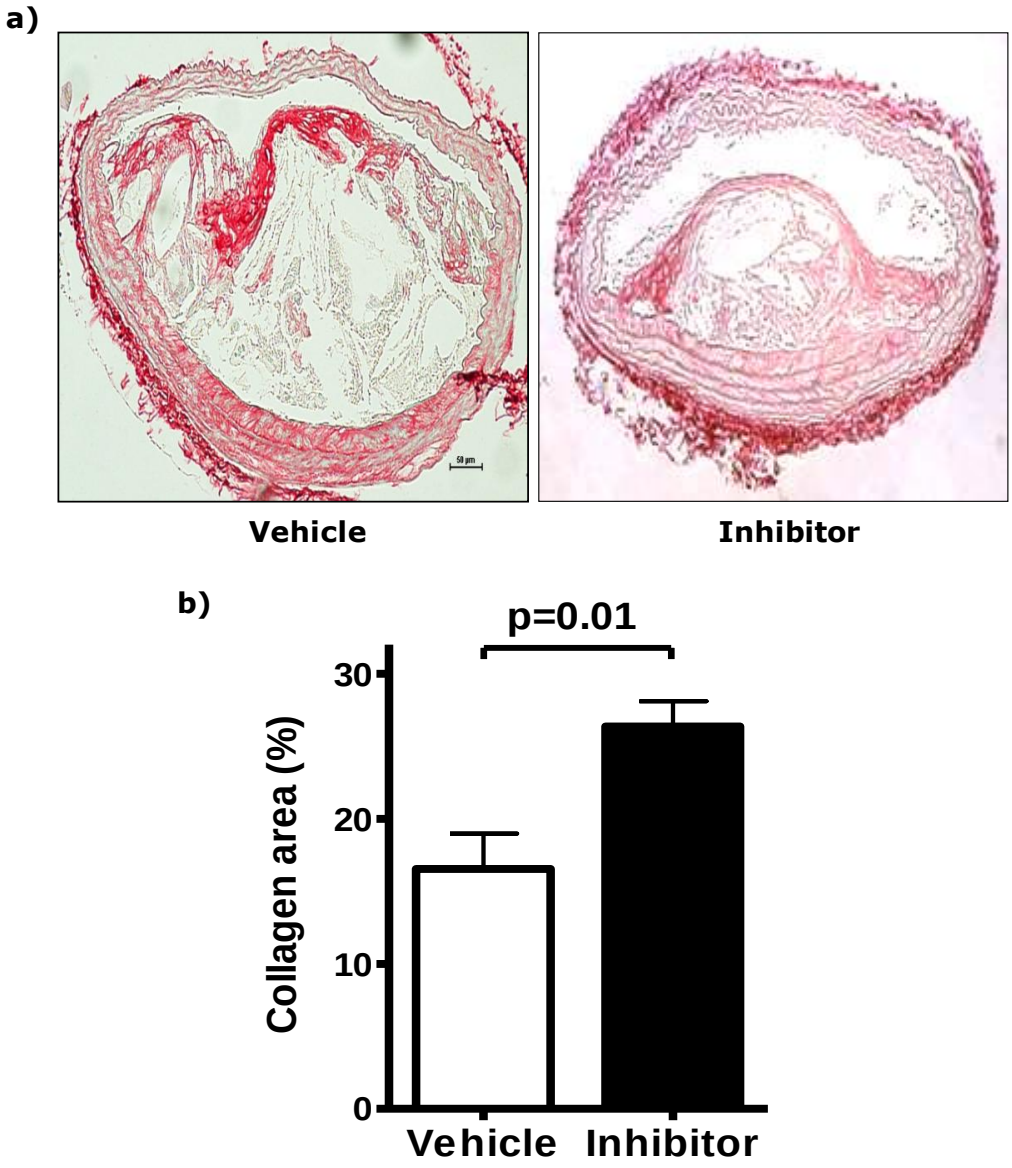
Data are mean ± SEM for n=6/group and were analysed using unpaired Student's t-test.

Figure 3.7: Selective inhibition of 11β-HSD1 did not alter the macrophage or smooth muscle content of lesions



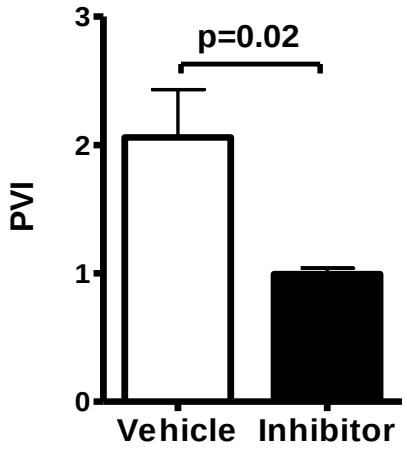
Plaque composition was assessed by determining immunoreactivity for MAC-2 (macrophage content) and immunoreactivity for α-smooth muscle actin (smooth muscle content) which appear brown on developing colour with DAB reagent. Immunohistochemistry confirmed that selective inhibition of 11β-HSD1 (compound 544; 10mg/kg/day in western diet; 8weeks) did not affect macrophage (a) or smooth muscle (b) content of plaques (expressed as percentage area of plaque).
Data are mean ± SEM for n=6/group and analysed using unpaired Student's t-test.

Figure 3.8: Selective inhibition of 11β-HSD1 was associated with increased collagen content of lesions



Picrosirius red staining (a) of sections from brachiocephalic trunk (BCT) of apolipoprotein E (*ApoE*$^{-/-}$) allowed analysis of collagen content of plaques. This confirmed that plaques in inhibitor-treated (compound 544; 10mg/kg/day in western diet; 8weeks) mice had more collagen content than those in vehicle-treated controls (b). Data are mean±SEM for n=6/group and analysed by unpaired Student's t-test.

Figure 3.9: 11β-HSD1 inhibition reduced plaque vulnerability index in *ApoE*$^{-/-}$ mice



Analysis of plaque composition allowed calculation of a plaque vulnerability index (PVI = area of macrophage staining + area of lipid staining / area of collagen staining + area of SMC staining). Treatment with a selective 11β-HSD1 inhibitor (compound 544; 10mg/kg/day in western diet; 8weeks) produced a significant reduction in vulnerability index of lesions in *ApoE*$^{-/-}$ mice.
Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

3.4 Discussion

The data in this chapter show that selective 11β-HSD1 inhibition reduces the size of atherosclerotic lesions with a corresponding increase in area of patent lumen. Furthermore, the reduction in lipid content of plaque, combined with increased collagen deposition (but unchanged smooth muscle and macrophage content), gives the lesions a more stable appearance. This is associated with a reduction in weight gain and blood pressure and a trend towards improved insulin sensitivity but without any effect on glucose or cholesterol levels.

3.4.1 Selective 11β-HSD1 inhibition reduces atherosclerosis

Semi-quantitative assessment revealed a reduction in atherosclerotic burden at several sites throughout the arterial tree indicating that the beneficial effect of 11β-HSD1 inhibition is not limited to any specific vascular territory. The BCT was chosen for detailed analyses as it develops extensive, complex lesions and has been used widely in investigations of atherogenesis [Williams et al., 2002]. The demonstration that lesion cross-sectional area in the BCT is reduced following 11β-HSD1 inhibition is consistent with a previous investigation using *ApoE*$^{-/-}$ mice in which compound 544 substantially reduced aortic cholesterol (which was used as a marker for atherosclerosis) content [Hermanowski-Vosatka et al., 2005]. In another study non-selective inhibition of 11β-HSD with carbenoxolone showed an atheroprotective effect in obese LDLR$^{-/-}$ mice [Nuotio-Antar et al., 2007]. However, in mice with LDL receptor deficiency combined with severe dyslipidaemia and obesity due to *ApoB* and leptin deficiency, Amgen compound 2922 reduced lipid content (by en face staining with Sudan IV) without reducing the atherosclerotic burden [Lloyd et al., 2009]. The extent of reduction in atherosclerotic burden in the current study was less than the dramatic (~85%) reduction reported previously with compound 544 [Hermanowski-Vosatka et al., 2005]. This difference could be attributed to the methods employed for assessment of lesion burden. Whilst Hermanowski-Vosatka et al. used qualitative gross morphology combined with extraction and quantification of lipids from isolated aortas, the current study combined a semi-quantitative assessment of lesion burden throughout the vasculature with a detailed

histological analysis of lesions in the BCT with direct measurement of lesion and lumen sizes. It is possible that measurement of lipid incorporation could over-estimate the impact of treatment on reducing arterial narrowing. Consistent with this argument, results obtained using histological analysis in the current study were closer to the direct measurement of lesion size in the aortic root after treatment with carbenoxolone [Nuotio-Antar et al., 2007]. The lack of effect of Amgen 2922 on atheroma burden in $LDLR^{-/-}$ $ApoB^{-/-}$ ob/ob mice [Lloyd et al., 2009] may reflect both the simplicity of the assessment (en face Sudan IV staining) and the severity of the metabolic abnormality in these mice.

3.4.2 Selective 11β-HSD1 inhibition improves plaque stability

11β-HSD1 inhibition lowered the accumulation of extracellular lipids and increased the fibrous collagen component of plaques. Plaques which are rich in extracellular lipids and macrophages, and poor in fibro-muscular components are unstable and prone to rupture [Davies, 1996;Naghavi et al., 2003a;Naghavi et al., 2003]. Therefore, the present findings suggest that plaques were changed beneficially to a more stable form of lesion by treatment with selective 11β-HSD1 inhibitor.

The reduction in the lipid content of atherosclerotic plaques seen in this study was without any significant effect on serum cholesterol. This is in contrast with data reported by Hermanowski-Vosatka *et al.*, which showed that both serum cholesterol and aortic cholesterol were reduced by 11β-HSD1 inhibition, however, the reduction in aortic cholesterol was more pronounced than the improvement in serum lipid profile [Hermanowski-Vosatka et al., 2005]. The mechanism responsible for the reduction in cholesterol, however, is unclear. It has been shown previously that certain pharmacological agents (e.g. captopril, an ACE inhibitors) may reduce arterial cholesterol content without any significant effect on serum cholesterol [Aberg and Ferrer, 1990]. Therefore, the results presented here suggest that reduced lesion formation in response to 11β-HSD1 inhibition is not due to a reduction in circulating lipids.

Given the anti-inflammatory, anti-proliferative [Longenecker et al., 1982;Longenecker et al., 1984] and anti-migratory [Goncharova et al., 2003] properties of glucocorticoids, it was a concern that 11β-HSD1 inhibition might increase inflammation and reduce

smooth muscle proliferation in the atherosclerotic plaques, hence changing plaques to a less stable phenotype. However, this study did not show any difference in SMC or macrophage content of plaques in two groups. Indeed, the increase in collagen content within the plaque, coupled with reduction in extracellular lipids, point towards a more stable phenotype. Although atherosclerotic plaques in *ApoE*$^{-/-}$ mice on a C57Bl/6J background are remarkably stable and the mice do not, as a rule, die from plaque rupture [Williams et al., 2002], this change in characteristics of plaques, if translated in humans, could help in prevention of plaque rupture potentially leading to reduction in acute cardiovascular events.

3.4.3 Mechanism of atheroprotective effect of 11β-HSD1 inhibition

Reduction in atherosclerosis in this study was associated with a significant reduction in blood pressure and modest improvement in insulin resistance and body weight, but independent of any change in circulating glucose or cholesterol levels.

Western diet fed *ApoE*$^{-/-}$ mice treated with 11β-HSD1 inhibitor gained less weight than the control group. This is consistent with previous data showing that 11β-HSD$^{-/-}$ mice do not gain weight on a high fat diet [Morton et al., 2004]. However, in this study, the anti-obesity action was not secondary to a change in food intake. Reduction in weight gain can attenuate atherogenesis and it has been shown that early atherosclerotic changes seen in obese humans can be attenuated by reducing body weight [Mavri et al., 2001].

Insulin resistance and diabetes mellitus are independent risk factors for atherosclerosis [Despres et al., 1996] and reducing insulin resistance can improve endothelial dysfunction leading to reduction in atherosclerosis [Kim et al., 2006]. Excess glucocorticoid action in target tissues may contribute to insulin resistance and impaired glucose tolerance [Stewart et al., 1995;Masuzaki et al., 2001;Masuzaki et al., 2003;Paterson et al., 2004]. Detailed metabolic studies have demonstrated that 11β-HSD1 deletion produces hepatic insulin sensitization [Morton et al., 2001] and selective inhibition reduced plasma insulin and improved glucose tolerance in genetically-obese and fat-fed mice [Hermanowski-Vosatka et al., 2005;Lloyd et al., 2009]. This contrasts with the data presented here, which showed little or no improvement in glucose tolerance and a modest trend towards improvement in insulin sensitivity. One possible

explanation for the lack of effect on glycaemic profile in this study may be the fact that inhibitor was administered by mixing in food so that, during the fasting period (6-8 hours for GTT) mice did not receive any inhibitor. Hermanowski-Vosatka *et al*. have shown that inhibition of 11β-HSD1 activity in the liver had nearly disappeared 6 hours after oral administration [Hermanowski-Vosatka et al., 2005]. The demonstration of an effect of the inhibitor on fasting biochemical profile would necessitate the design of separate experiments, to recapitulate results of Hermanowski-Vosatka *et al.* (2005). This would involve administration of the inhibitor by mixing in drinking water or sub-cut implants. It is also notable, however, that in the studies showing improvements in metabolic profile mice were either fed on chow or on an obesogenic diet [Hermanowski-Vosatka et al., 2005;Nuotio-Antar et al., 2007;Lloyd et al., 2009], rather than the atherogenic western diet used here. Furthermore, whilst carbenoxolone reduced fasting insulin in genetically-obese $LDLR^{-/-}$, no such improvement was observed in non-obese $LDLR^{-/-}$ mice fed a western diet [Nuotio-Antar et al., 2007]. This suggests that the effect of 11β-HSD1 inhibitors on glycaemic profile in rodents may actually depend on diet, genotype and the cause of obesity.

This study has also shown, for the first time, a reduction in BP with administration of compound 544 in $ApoE^{-/-}$ mice fed a western diet. Hypertension is a recognised feature of glucocorticoid excess, but the mechanisms of glucocorticoid-induced hypertension are complex and probably involve both central actions in the CNS and peripheral actions on vascular tone and sodium balance [Whitworth et al., 1997]. Neither deletion nor selective inhibition of 11β-HSD1 in C57Bl/6J mice, however, have previously been shown to lower BP [Kotelevtsev et al., 1997]. BP has only been mentioned in one previous study with 11β-HSD1 inhibition, which found no effect of Amgen compound 2922, but data were not shown [Lloyd et al., 2009]. Transgenic over-expression of 11β-HSD1 in the liver or adipose tissue causes hypertension, putatively due to increased local angiotensinogen expression and hence activation of the renin-angiotensin system [Masuzaki et al., 2003;Paterson et al., 2004]. However, measurement of angiotensinogen, aldosterone and plasma renin activity suggested that this did not account for the anti-hypertensive effect of compound 544 and the mechanism remains uncertain. Whether the atheroprotection associated with 11β-HSD1 inhibition is a

consequence of reduced blood pressure remains to be established. Clinical studies have consistently correlated hypertension and atherosclerotic coronary artery disease [Kuulasmaa et al., 2000;Neaton et al., 1984] and hypertension is a recognised target in prevention and treatment of atherosclerosis [Weiss and Taylor, 2008;Irace et al., 2005;Agmon et al., 2000]. However, changes in blood pressure are not invariably linked to lesion development; for example, normalising blood pressure (by hydralazine administration) in hypertensive atherosclerosis-prone eNOS knockout *ApoE*$^{-/-}$ mice had no effect on atherogenesis [Chen et al., 2001]. Therefore, it is possible that compound 544 is reducing lesion formation by both blood pressure-dependent and -independent mechanisms.

3.5 Conclusion

In conclusion, data in this chapter support the hypothesis that selective inhibition of 11β-HSD1 represents a potential new therapeutic option for prevention of atherosclerosis. The novel effect shown on markers of plaque stability is of clinical importance given that the acute cardiovascular events occur due to plaque disruption. This has implications for the development of novel anti-atherosclerotic agents but also provides evidence that use of such compounds in the treatment of diabetes mellitus will also lead to a reduction in arterial lesion formation.

4. Role of 11β-HSD1 in neointimal proliferation

4.1 Introduction

Inhibition of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) improves cardiovascular risk profile [Hermanowski-Vosatka et al., 2005] and reduces atherosclerosis (chapter 3). Emerging data from clinical trials investigating effects of 11β-HSD1 inhibition on features of metabolic syndrome look very promising, with the demonstration in a recently reported Phase-IIb trial that treatment with a selective 11β-HSD1 inhibitor significantly improved glycaemic control, insulin sensitivity and total-cholesterol levels [Rosenstock et al., 2010] Many pharmaceutical companies and research organisations are, therefore, actively developing 11β-HSD1 inhibitors as a potential treatment for metabolic syndrome and currently there are nearly 100 patents filed [Hughes et al., 2008].

Morbidity and mortality in metabolic syndrome are largely due to the development of atherosclerotic lesions [Wang et al., 2007], which often require treatment with percutaneous revascularisation procedures (angioplasty ± stenting). However, post angioplasty restenosis due to neointimal hyperplasia, which represents an inflammatory and proliferative response to acute vascular injury, remains a significant problem [Al et al., 2000]. Glucocorticoids have anti-inflammatory and anti-proliferative properties suggesting that they may prevent neointimal proliferation after angioplasty. Studies in animals have generally supported this hypothesis. Systemic or local glucocorticoid administration inhibited neointimal lesion formation in rats [Villa et al., 1994;Guzman et al., 1996], rabbits [Van Put et al., 1995;Poon et al., 2001;Ribichini et al., 2007] and dogs [Strecker et al., 1998]. However, not all studies in animals have yielded positive results: dexamethasone treatment did not reduce neointimal hyperplasia after angioplasty in rabbits [Karim et al., 1997] or pigs [Lincoff et al., 1997]. Similarly clinical trials in humans have produced variable results [Liu et al., 2004]. However, there are on-going studies to explore the role of systemic and local administration of steroids (e.g. STARS Trial, ISRCTN05886349). Given the potential beneficial effect of steroids in reducing neointimal proliferation, there is a concern that the use of 11β-HSD1 inhibitors at times of revascularisation may increase neointimal proliferation by reducing the availability of active glucocorticoids within the cells. This study, therefore, addressed the hypothesis that 11β-HSD1 inhibition / deletion increases neointimal proliferation.

4.2 Materials and methods

4.2.1 Animals

C57Bl/6J mice were purchased from Harlan Laboratories (Oxfordshire, UK) and *ApoE*$^{-/-}$ mice from Charles River Laboratories (Kent, UK). 11β-HSD1$^{-/-}$ mice, homozygous for a disrupted hsd11b1 allele and congenic on a C57Bl/6J background were bred in-house as reported previously [Kotelevtsev et al., 1997]. *ApoE*$^{-/-}$ and 11β-HSD1$^{-/-}$ mice, both lines congenic on a C57Bl/6J background, were crossed to produce 11β-HSD1$^{-/-}$, *ApoE*$^{-/-}$ double knockout (DKO) mice. All animals were maintained under standard conditions of light (lights on 8am-8pm) and temperature (21-22^{o}C).

4.2.2 Materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich, UK. Compound 544 (3-(1-adamantyl)-6,7,8,9-tetrahydro-5 H -[1,2,4] triazolo[4,3-α]azepine), a potent and selective inhibitor of 11β-HSD1 [Hermanowski-Vosatka et al., 2005] was obtained (as compound T5293658) from Enamine Ltd. (Kiev, Ukraine). Standard chow (RM1) was obtained from Special Diet Services (Witham, UK) and western diet (OpenSource^{TM} D12079B) was obtained from Research Diets Inc. (Brunswick, USA).

4.2.3 Experimental protocol

Adult male C57Bl/6J and *ApoE*$^{-/-}$ mice were divided into treatment (compound 544, 30/mg/kg/day; n=6/group) and control (vehicle, n=6/group) groups. All mice underwent wire-angioplasty of left femoral arteries (section 2.7) one week later. Body weights were recorded at weekly intervals, blood pressure was measured fortnightly (by tail-cuff photo-plethysmography, section 2.4) and biochemical tests were performed one week before cull. Serum glucose, cholesterol and triglyceride levels were measured spectrophotometrically by using commercially available Infinity^{TM} kits (section 2.5). Mice were culled three weeks after surgery by terminal anaesthesia with phenobarbitone and trans-cardiac perfusion fixation. Left femoral arteries were excised from the bifurcation of the iliac artery to the branch point of the popliteal artery and processed for 3-dimensional optical projection tomography (OPT, section 2.8) to analyse lesion

volume and subsequently for standard histology to measure luminal area and area inside the internal elastic lamina (IEL) as described (section 2.9). These measurements were then used to calculate the neointimal area (area inside IEL - luminal area) and cross-sectional stenosis (neointimal area/area inside IEL x 100).

Lesion composition was determined by staining sections at the point of maximum neointimal proliferation (section 2.10). Collagen content was indentified by staining with Picrosirius red. Macrophage and smooth muscle content were assessed by immunohistochemistry using purified monoclonal rat anti-mouse Mac-2 antibody for macrophages (1:6000; Cedarlane, UK) and monoclonal mouse anti-mouse α-smooth muscle actin antibody for smooth muscle cells (1:400; Sigma-Aldrich, UK). Quantification of stained areas was performed using a semi-automated colour deconvolution process with Photoshop CS3 Extended software.

All analyses were carried out under blinded conditions.

4.2.4 Statistical analysis

Data are expressed as mean ± SEM (with n indicating the number of animals in each experimental group) and were analysed using unpaired Student's t test or two-way ANOVA, as appropriate. Statistical significance was assumed when $p<0.05$. All calculations were performed using GraphPad prism (GraphPad Software Inc, California, USA).

4.3 Results

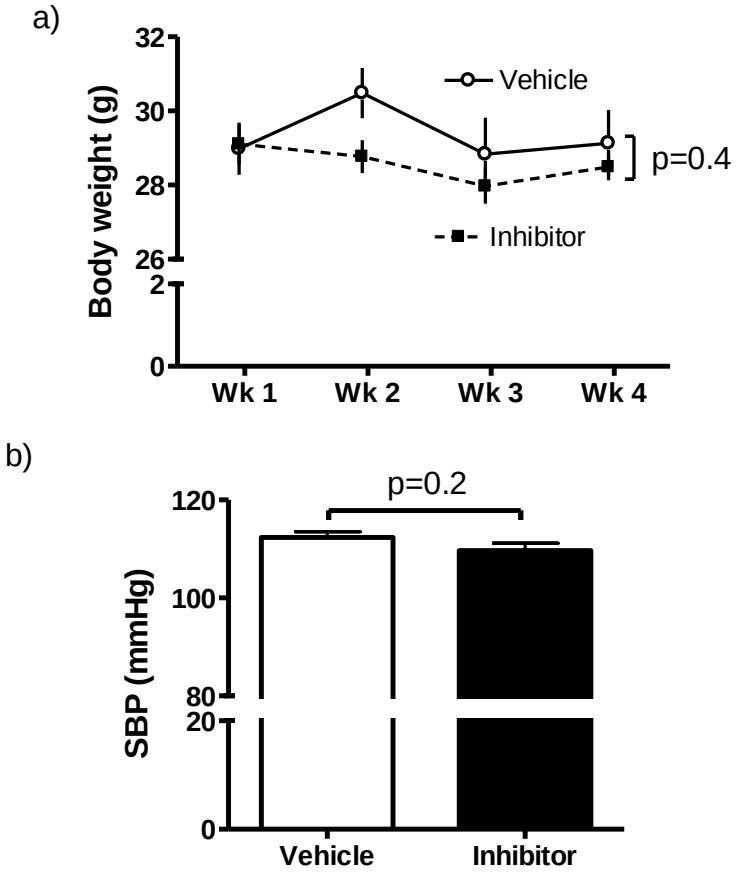
4.3.1 Effect of 11β-HSD1 inhibition in C57Bl/6J mice

Pharmacological inhibition of 11β-HSD1 did not have any significant effect on body weight of C57Bl/6J mice (Figure 4.1a). There was also no effect of 11β-HSD1 inhibition on systolic blood pressure (Figure 4.1b).

Wire-angioplasty stimulated development of fibro-cellular neointimal lesions in all mice (Figure 4.2a). There was no significant effect of 11β-HSD1 inhibition on neointimal proliferation and consequent cross-sectional stenosis in angioplastied femoral arteries (Figure 4.2b).

11β-HSD1 inhibition had no effect on macrophage (Figure 4.3a) or smooth muscle cell (Figure 4.3b) content of neointimal lesions and a trend to increase collagen content of lesions was not statistically significant (Figure 4.3c).

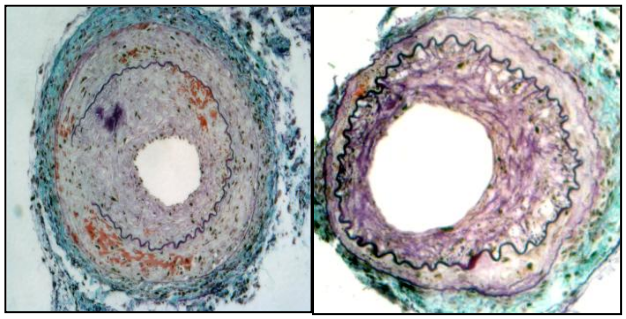
Figure 4.1: 11β-HSD1 inhibition had no effect on body weight and blood pressure in C57Bl/6J mice



C57Bl/6J mice (n=6/group) were treated with a selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in standard chow; 4weeks) or vehicle, starting one week before wire-angioplasty. 11β-HSD1 inhibition had no effect on body weight (a). Data are mean ± SEM and were analysed by repeat measures two-way ANOVA.
Systolic blood pressure (SBP) in C57Bl/6J mice was no different after 4 weeks treatment with the vehicle or 11β-HSD1 inhibitor (b). Data are mean ± SEM and were analysed by unpaired Student's t-test.

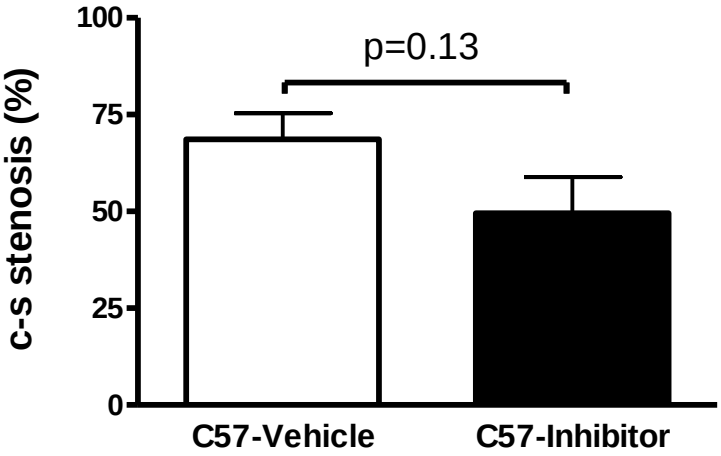
Figure 4.2: 11β-HSD1 inhibition had no effect on neointimal proliferation in C57Bl/6J mice

a)



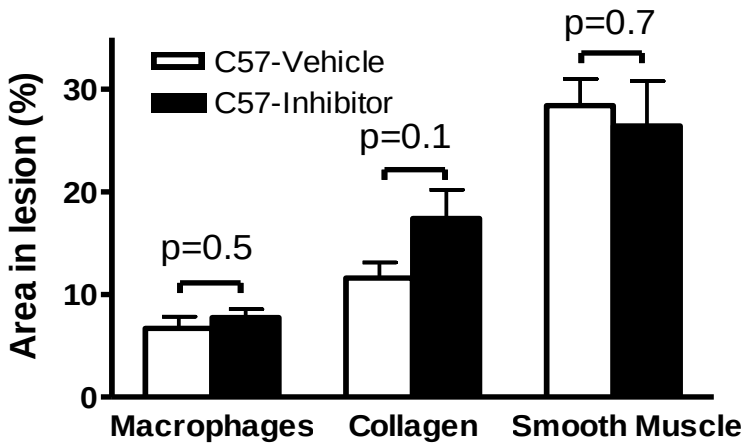
C57-Vehicle C57-Inhibitor

b)



C57Bl/6J mice (n=6/group) were treated with a selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in standard chow; 4weeks) or vehicle, starting one week before wire-angioplasty. Wire-angioplasty produced fibro-cellular neointimal lesions (a) in both groups (x 20 magnification). Administration of the selective 11β-HSD1 inhibitor produced an apparent reduction in lesion size in C57Bl/6J mice but this did not achieve significance (b).
Data are mean ± SEM for n=6/group and were analysed by unpaired Student's t-test.

Figure 4.3: 11β-HSD1 inhibition had no significant effect on composition of neointimal lesions in C57Bl/6J mice



Neointimal lesion composition was determined by analysing sections for Picrosirius red staining (collagen content), immunoreactivity for Mac-2 (macrophage content) and immunoreactivity for α-smooth muscle actin (smooth muscle content). Quantification of stained areas showed no effect of the selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in standard chow; 4weeks) on macrophage or smooth muscle content. An apparent increase in collagen content did not achieve significance.
Data are mean ± SEM for n=6/group and were analysed using unpaired Student's t-test.

4.3.2 Effect of 11β-HSD1 inhibition in *ApoE*$^{-/-}$ mice

ApoE$^{-/-}$ mice were fed a western diet mixed with vehicle (n=6) or 11β-HSD1 inhibitor (compound 544, 30/mg/kg/day; n=6) from one week before femoral artery surgery to three weeks after the surgery.

4.3.2.1 Systemic effects of 11β-HSD1 inhibition

Body weights were similar at the start of experiment; however, during the 4 weeks of treatment, the inhibitor treated group gained less weight compared with vehicle-treated controls (Figure 4.4a). Four weeks treatment with the 11β-HSD1 inhibitor resulted in a trend towards reduction in blood pressure; however, it did not achieve statistical significance (Figure 4.4b).

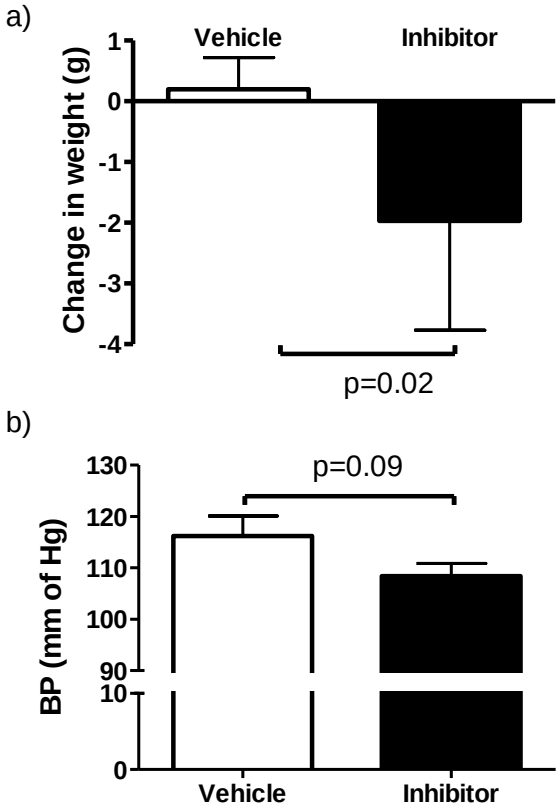
11β-HSD1 inhibition produced modest reduction in fasting glucose levels (Figure 4.5a) but had no effect on fasting total cholesterol (Figure 4.5b) or fasting triglyceride levels (Figure 5c) as compared with vehicle treated control group.

4.3.2.2 Effect on neointimal lesions

Optical projection tomography (OPT) allowed 3-dimensional reconstruction of lesions and quantification of lesion volume (Figure 4.6a). Systemic inhibition of 11β-HSD1 in *ApoE*$^{-/-}$ mice significantly reduced neointimal lesion volume (Figure 4.6b). Histological analysis confirmed the development of extensive neointimal lesions in all mice (Figure 4.7a). These lesions contained a large number of foam cells and hence looked different from lesions seen in C57Bl/6J mice. The maximum cross-sectional stenosis was significantly reduced in the inhibitor treated group as compared with the control group (Figure 4.7b).

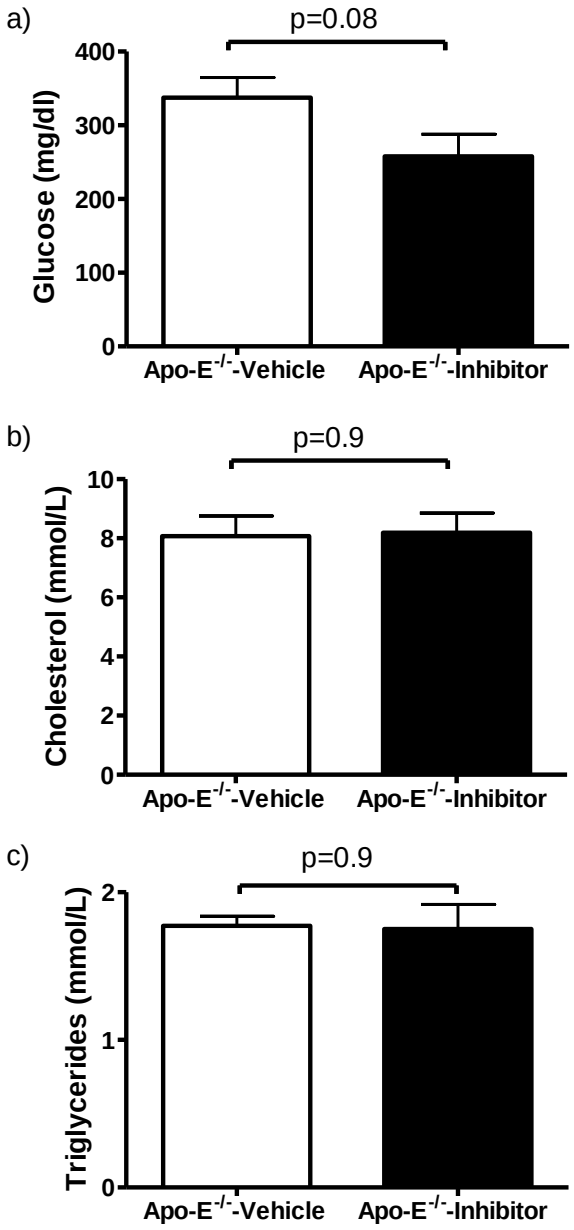
11β-HSD1 inhibition had no effect on smooth muscle content of the plaque; however, it reduced the macrophage and increased the collagen content of plaques compared with vehicle treated control mice (Figure 4.8).

Figure 4.4: 11β-HSD1 inhibition reduced body weight and tended to lower blood pressure in *ApoE*$^{-/-}$ mice



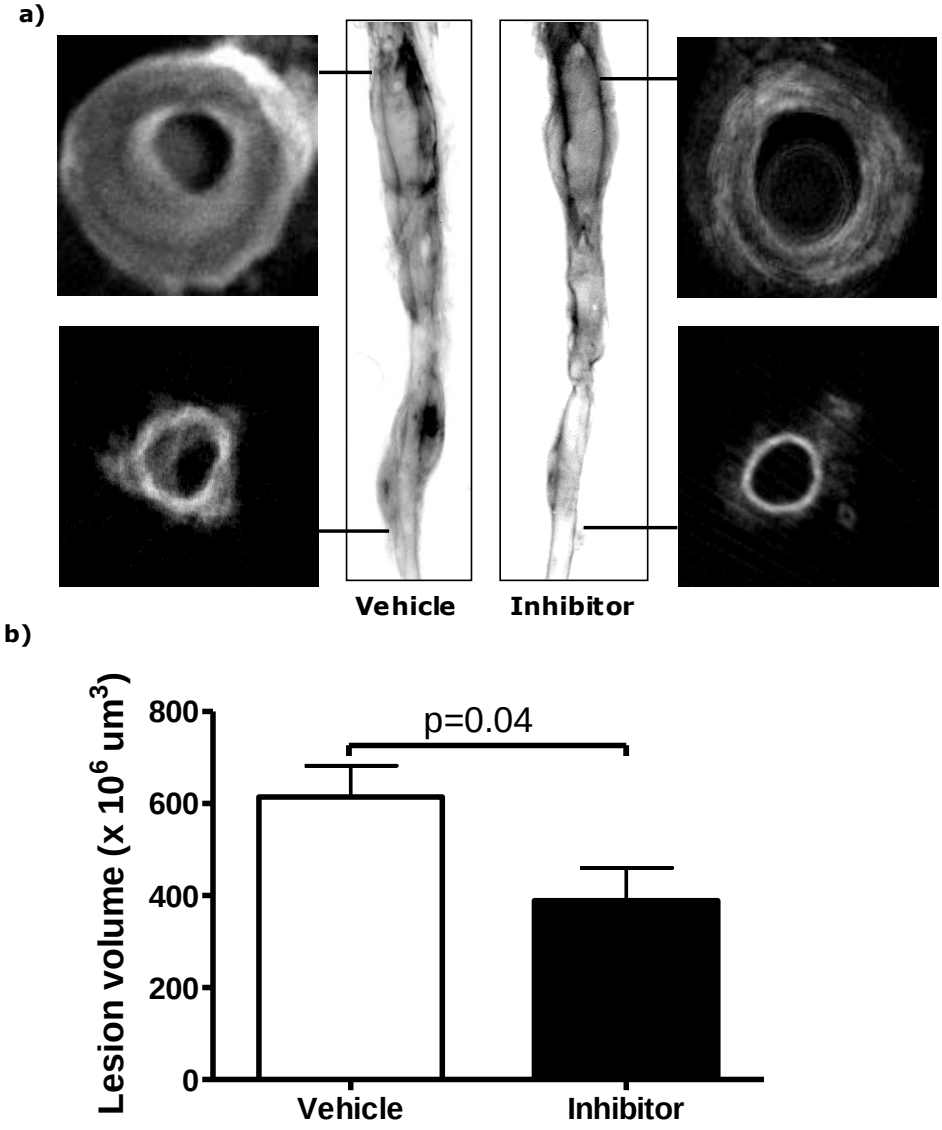
There was a significant difference in change in weight of *ApoE*$^{-/-}$ mice treated with a selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in western diet; 4weeks) compared with vehicle treated mice (a). 11β-HSD1 inhibition also produced a non-significant trend towards reduction in systolic blood pressure in *ApoE*$^{-/-}$ mice (b). Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

Figure 4.5: 11β-HSD1 inhibition tended to lower plasma glucose but had no effect on lipid profile in *ApoE*$^{-/-}$ mice



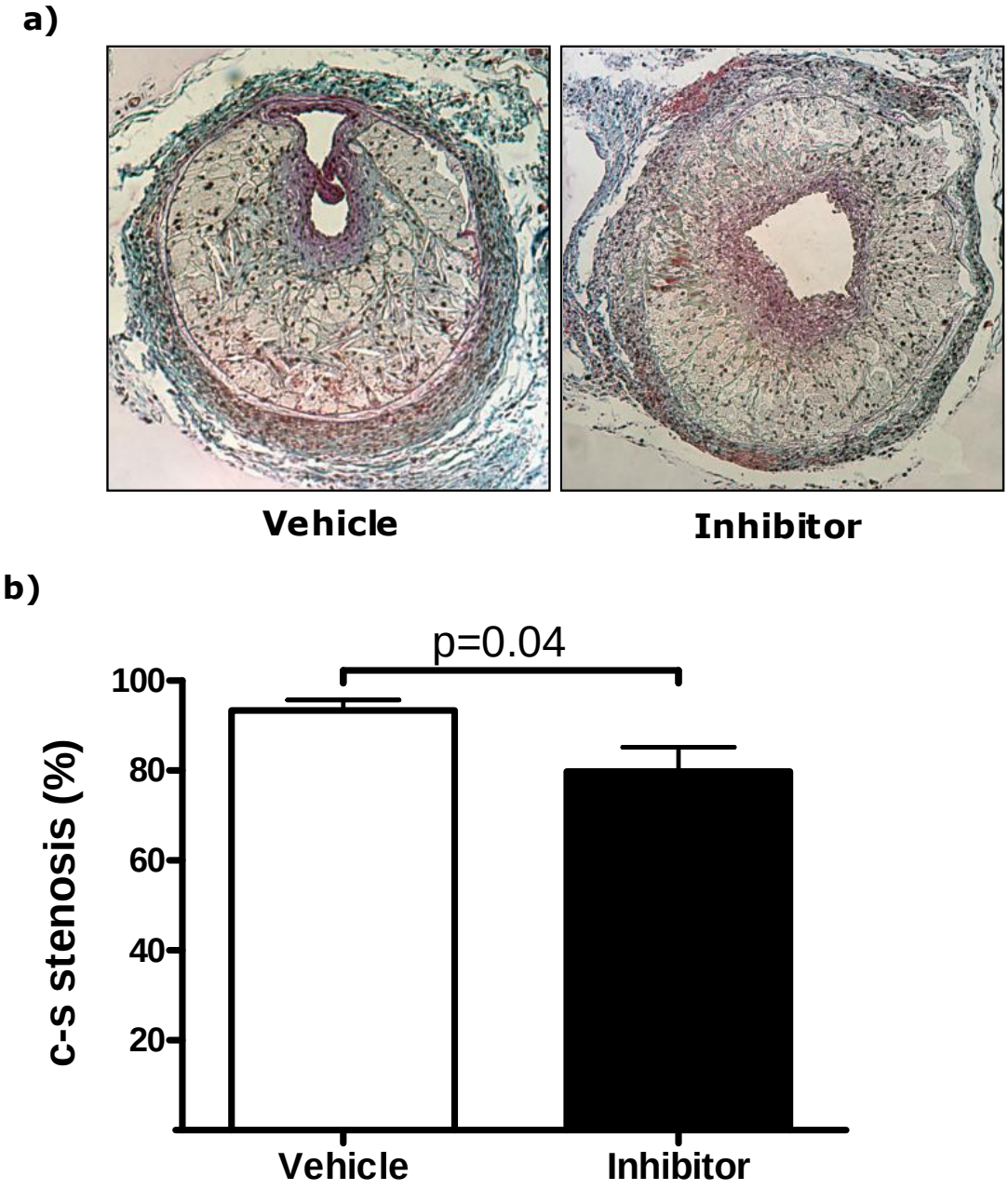
Selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in western diet, 4 weeks) produced an apparent reduction in fasting glucose levels, but it did not achieve statistical significance (a). 11β-HSD1 also had no effect on fasting cholesterol (b) or triglyceride levels (c).
Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

Figure 4.6: 11β-HSD1 inhibition reduced neointimal lesion volume in *ApoE*$^{-/-}$ mice



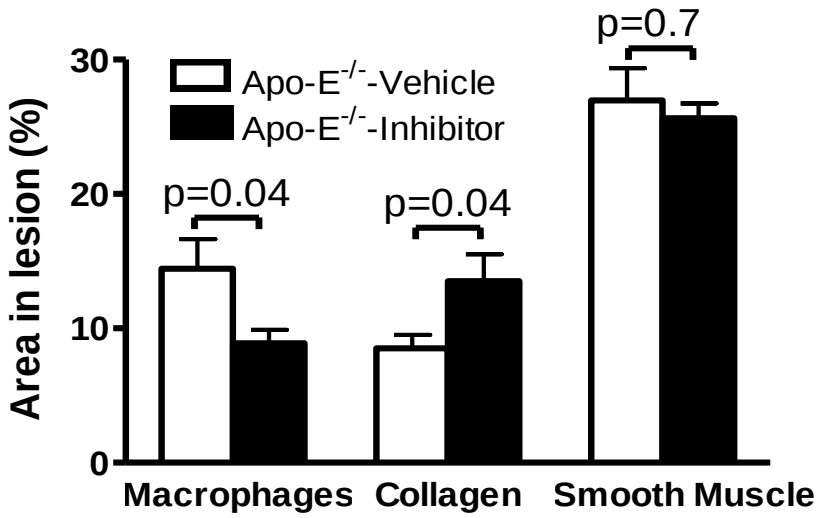
Optical Projection Tomography (OPT) allowed acquisition of 3dimensional images (a) to calculate neointimal lesion volume after wire angioplasty. Quantification of lesion volume revealed a significant reduction in neointimal lesion size in *ApoE*$^{-/-}$ mice treated with the selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in western diet; 4weeks) compared with vehicle-treated *ApoE*$^{-/-}$ mice (b).
Data are mean ± SEM for n=6/group and analysed by unpaired Student's t-test.

Figure 4.7: 11β-HSD1 inhibition reduced neointimal proliferation in *ApoE*$^{-/-}$ mice



Wire-angioplasty produced fibro-cellular neointimal lesions (a) in both groups (20 x magnifications). *ApoE*$^{-/-}$ mice treated with selective 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in western diet; 4weeks) had significantly smaller neointimal lesions and hence less cross-sectional stenosis (b) than vehicle treated group.
Data are mean ± SEM for n=6/group and were analysed by unpaired Student's t-test.

Figure 4.8: 11β-HSD1 inhibition altered macrophage and collagen, but not the smooth muscle, content of neointimal lesions in *ApoE*$^{-/-}$ mice



Neointimal lesion composition was determined by analysing sections for Picrosirius red staining (collagen content), immunoreactivity for Mac-2 (macrophage content) and immunoreactivity for α-smooth muscle actin (smooth muscle content). Quantification of stained areas showed a significant reduction in macrophage content and an increase in collagen content of lesions in the 11β-HSD1 inhibitor (compound 544; 30mg/kg/day in western diet; 4weeks) treated group, compared with vehicle-treated controls. In contrast, 11β-HSD1 inhibition did not alter smooth muscle content of lesions.
Data are mean ± SEM for n=6/group and analysed using unpaired Student's t-test.

4.3.3 Effect of 11β-HSD1 deletion in *Apo-E*$^{-/-}$ mice

To test whether the results seen with compound 544 represent effects of selective 11β-HSD1 inhibition, post-angioplasty neointimal proliferation was also studied in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$,11β-HSD1$^{-/-}$ (DKO) mice (n=8/group) fed on western diet for four weeks, starting one week before wire-angioplasty.

4.3.3.1 Systemic effects of 11β-HSD1 deletion

DKO mice weighed less than age-matched *ApoE*$^{-/-}$ mice at the start of the study. However, mice in both groups gained weight equally during the four weeks' exposure to western diet (Figure 4.9a). DKO mice had lower blood pressure at baseline and their blood pressure remained lower than *ApoE*$^{-/-}$ mice during four weeks of western diet feeding (Figure 4.9b).

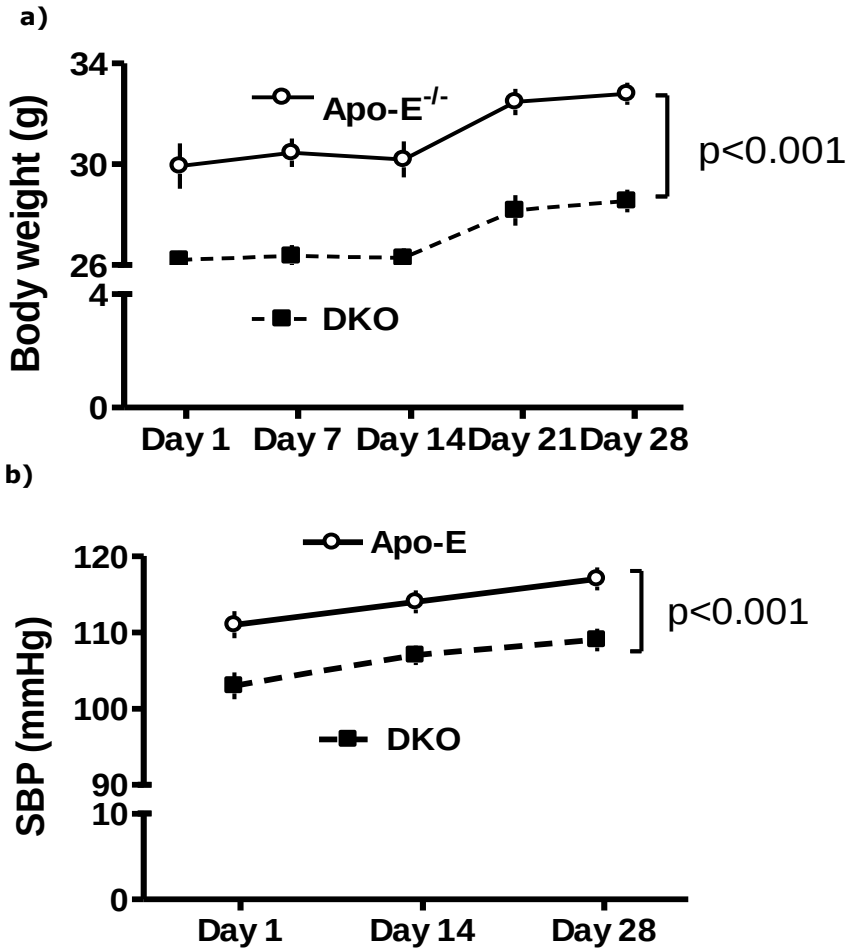
DKO mice had lower fasting glucose levels than *ApoE*$^{-/-}$ mice (Figure 10a). There was no effect of 11β-HSD1 deletion (Figure 10b) on fasting total plasma cholesterol as compared with controls. Similarly, fasting plasma triglyceride levels also remain unaffected by 11β-HSD1 deletion (Figure 4.10c).

4.3.3.2 Effects on neointimal lesions

Transgenic deletion of 11β-HSD1 in *ApoE*$^{-/-}$ mice significantly reduced neointimal lesion volume (456±48μm3 vs. 700±96μm3, p=0.04), as assessed by OPT. Histological analysis of lesions revealed that the maximum cross-sectional stenosis was significantly reduced in DKO mice compared with control group (Figure 4.11).

11β-HSD1 deletion had no effect on smooth muscle content of the plaques (25±1% vs. 25±3%, p=1); however, it reduced the macrophage (11±1% vs. 16±2%, p=0.02) and increased the collagen (40±3% vs. 30±3%, p=0.05) content of plaques compared with control mice.

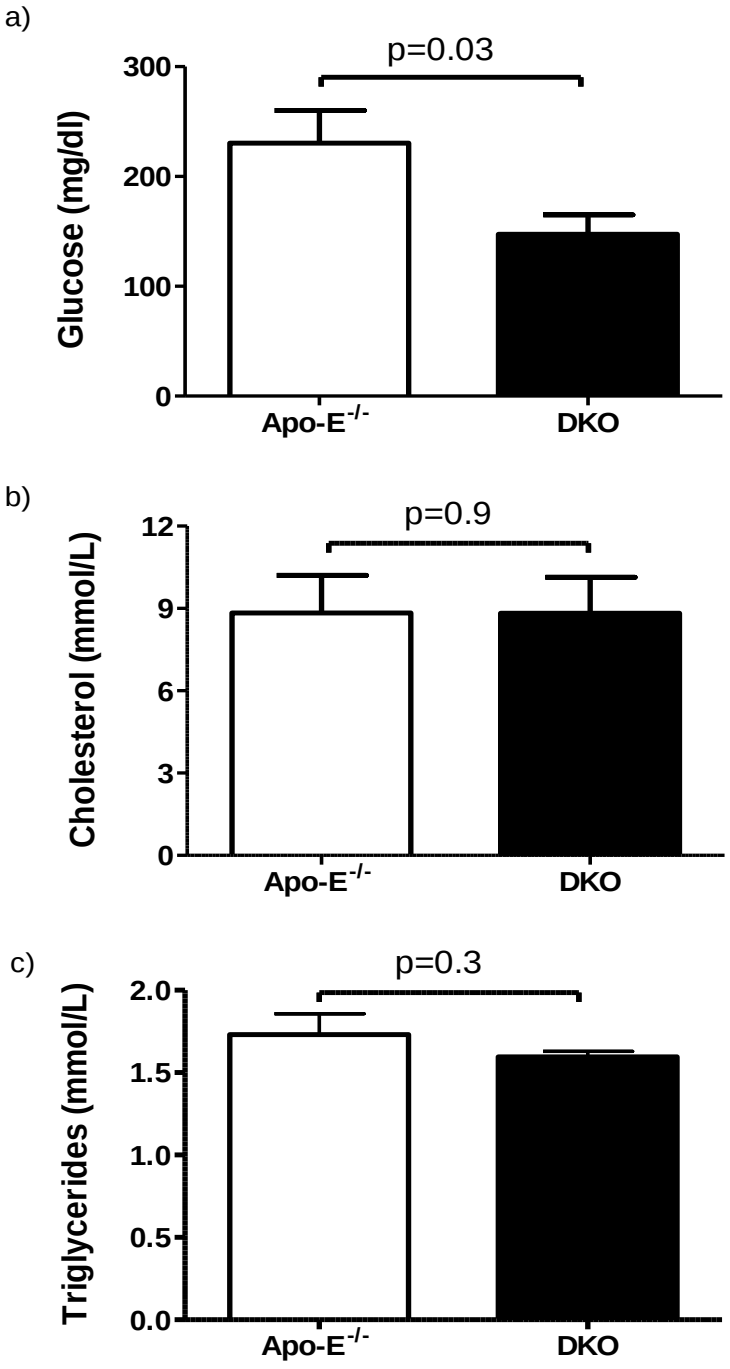
Figure 4.9: 11β-HSD1 deletion reduced body weight and blood pressure in *ApoE*$^{-/-}$ mice



Mice with transgenic deletion of *ApoE* and 11β-HSD1 (double knockout, DKO) had significantly lower body weight than ApoE$^{-/-}$ controls (a). DKO mice also had lower systolic blood pressure than age-matched ApoE$^{-/-}$ controls (b). Bonferroni post-hoc analysis revealed a significant BP difference between the two groups ($p<0.05$) at day 1 and day 18 (from start of experiment and angioplasty on day 7) but not at day 14 ($p>0.05$).

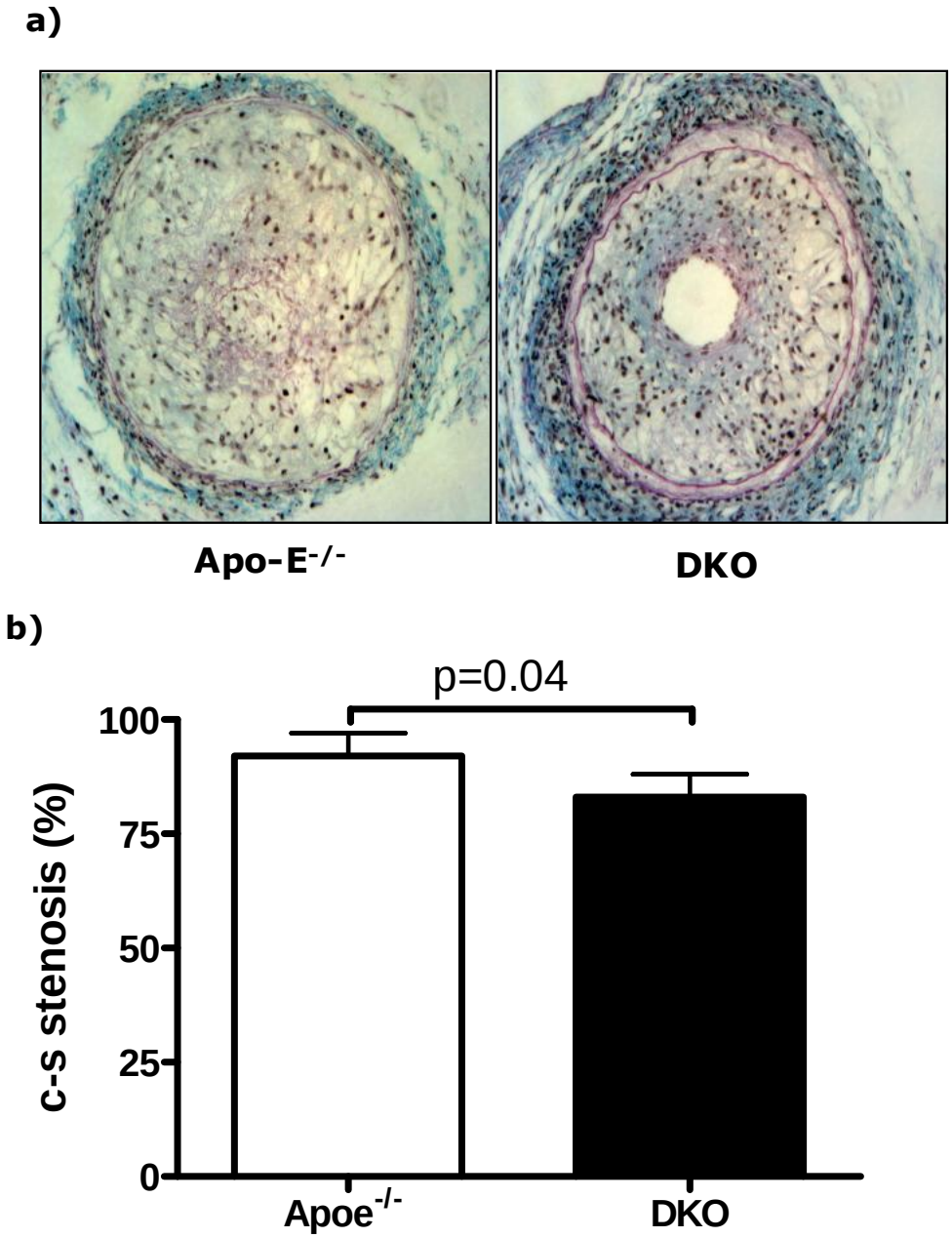
Data are mean ± SEM for n=7-8/group and analysed by repeat measures two-way ANOVA.

Figure 4.10: 11β-HSD1 deletion reduced fasting plasma glucose but had no effect on lipid profile in *ApoE*$^{-/-}$ mice



Mice with transgenic deletion of *ApoE* and 11β-HSD1 (double knockout, DKO) had significantly lower fasting plasma glucose levels than control ApoE knockout mice (a). However, there was no difference in fasting total plasma cholesterol (b) or triglyceride (c) levels between the two groups.
Data are mean ± SEM for n=7-8/group and analysed unpaired Student's t-test.

Figure 4.11: 11β-HSD1 deletion reduced neointimal proliferation in *ApoE*$^{-/-}$ mice



Wire-angioplasty produced fibro-cellular neointimal lesions in both groups (x20 magnification, a). Mice with transgenic deletion of ApoE and11β-HSD1 (double knockout, DKO) had significantly lower neointimal proliferation and consequent cross-sectional (c-s) stenosis as compared with *ApoE*$^{-/-}$ mice. Data are mean ± SEM for n=7-8/group and were analysed by unpaired Student's t-test.

4.4 Discussion

This study, contrary to the original hypothesis (endogenous glucocorticoid regeneration increases neointimal proliferation) suggests that 11β-HSD1 inhibition does not increase neointimal proliferation after wire-angioplasty and, indeed, reduces the size of neointimal lesions in western diet fed *ApoE*$^{-/-}$ mice. The reduction in neointimal lesions seen here is associated with improvements in systemic cardiovascular risk factors (body weight, blood pressure and glucose levels) and reduction in vascular inflammation, but without any effect on plasma lipid profile or VSMC proliferation.

The experiments described in this chapter evaluated the influence of pharmacological inhibition of 11β-HSD1 in chow-fed C57Bl/6J mice and western diet fed *ApoE*$^{-/-}$ mice. There was a concern that the reduction in blood pressure and vascular lesions may be due to off-target effects of compound 544. Therefore, the results were confirmed using *ApoE*$^{-/-}$ mice with transgenic deletion of 11β-HSD1 which also produced similar effects on blood pressure, lesion volume and lesion areas, suggesting that the effect of compound 544, indeed, represents effects of selective 11β-HSD1 inhibition.

4.4.1 Effect of 11β-HSD1 inhibition on metabolic profile

11β-HSD1 inhibition produced differential metabolic effects in chow-fed C57Bl/6J mice and western diet fed *ApoE*$^{-/-}$ mice. C57Bl/6J mice fed on standard chow are known to have metabolically normal phenotype with no obesity, hyperglycaemia or dyslipidaemia [Ellis et al., 2008]. *ApoE*$^{-/-}$ mice fed on western diet, on the other hand, develop adverse lipid and glycaemic profile [Zhang et al., 1992]. In this study, administration of a selective 11β-HSD1 inhibitor had no effect on body weight and blood pressure in C57BL/6J mice but reduced weight gain and blood pressure in *ApoE*$^{-/-}$ mice. It has been shown previously that the effect of 11β-HSD1 inhibition on body weight varies in different rodent models (e.g. compound 544 reduced weight gain in a model of diet induced obesity but not in a model of streptozotocin induced diabetes [Hermanowski-Vosatka et al., 2005]). 11β-HSD1 deletion in *ApoE*$^{-/-}$ mice also reduced fasting plasma glucose. Pharmacological inhibition of 11β-HSD1 also produced a similar trend,

however, did not achieve statistical significance probably due to methodological limitations (as discussed in chapter 3).

The data presented in this chapter have also shown for the first time that 11β-HSD1 deletion in *ApoE*$^{-/-}$ mice lowers systolic blood pressure. These findings are consistent with the effect of 11β-HSD1 inhibition described in Chapter 3. However, there was no effect of 11β-HSD1 inhibition on blood pressure in C57Bl/6J mice which contrasts with the blood pressure lowering effect seen in *ApoE*$^{-/-}$ mice fed on western diet, but is consistent with previous studies where transgenic deletion of 11β-HSD1 in C57Bl/6J mice did not affect blood pressure [Kotelevtsev et al., 1997]. One possible explanation for this difference may be that the metabolic effects of 11β-HSD1 inhibition are minimal when administered to mice without any significant metabolic derangements.

11β-HSD1 deletion or pharmacological inhibition in *ApoE*$^{-/-}$ mice had no effect on plasma total cholesterol and triglyceride levels. The lack of effect on lipid profile seen here is not consistent with previously reported beneficial effects of 11β-HSD1 deletion on lipid profile in C57Bl/6J mice [Morton et al., 2001]. Unfortunately, fasting lipid levels were not checked in C57Bl/6J mice in the current experiment and, therefore, a direct comparison is not possible. However, lack of effect of 11β-HSD1 deletion in *ApoE*$^{-/-}$ mice on cholesterol and triglyceride levels suggests that these mice have severe dyslipidaemia which may not be ameliorated by modest effects of blocking 11β-HSD1 activity. Finally, various lipid fractions (such as LDL, VLDL, NEFA) were not analysed in this study (due to small volume of blood samples) and, therefore, an effect on these lipid subtypes can't be excluded.

4.4.2 Effect of 11β-HSD1 inhibition/deletion on neointimal lesions

Since it was hypothesised that endogenous glucocorticoids inhibit neointimal proliferation, attenuation of glucocorticoid regeneration by inhibition of 11β-HSD1 would be expected to increase neointimal proliferation after angioplasty. Experimental studies using pharmacological doses of glucocorticoids have been shown to reduce neointimal lesions [Villa et al., 1994;Van Put et al., 1995;Poon et al., 2001;Ribichini et al., 2007]. The beneficial effect of glucocorticoids on neointimal proliferation probably originates from their effect on VSMC proliferation and inflammation [Celik et al.,

2009]. However, systemic glucocorticoid administration may also exacerbate cardiovascular risk factors [Walker, 2007] with potential secondary increase in neointimal proliferation. Glucocorticoids can also possibly influence neointimal proliferation via activating mineralocorticoid receptors in the vascular wall if these receptors are not being protected by 11β-HSD2 (discussed in chapter 5). Therefore, the net effect will be determined by the balance between these factors.

The data presented in this chapter indicate that loss of 11β-HSD1 activity does not increase neointimal proliferation and, on the contrary, may reduce neointimal proliferation (at least in western diet fed *ApoE*$^{-/-}$ mice). There was also an apparent reduction in neointimal lesion size in C57Bl/6J mice, however, it did not reach statistical significance (p=0.13). Due to a relatively small sample size (n=6/group) and variability in lesion size, the statistical power of the experiment (calculated retrospectively) was only 33%. If there was more time and resources available, it would have been useful to increase the sample size (~n=10/group) to obtain adequate statistical power to ensure that the lack of effect observed in C57Bl/6J mice is indeed due to differential effect of 11β-HSD1 inhibition in the two genotypes. However, in support of the notion that loss of 11β-HSD1 activity in C57Bl/6J mice does not influence the vascular response to injury, it is worth noting that in a previous experiment transgenic deletion of 11β-HSD1 in C57Bl/6J mice also did not influence post-angioplasty neointimal lesion formation (LJ Macdonald, unpublished data).

ApoE$^{-/-}$ mice fed on western diet had more pronounced neointimal proliferation than chow-fed C57Bl/6J mice. The lesions also appeared different in C57Bl/6J and *ApoE*$^{-/-}$ mice with the presence of a large number of foam cells in latter. This may reflect the hyperlipidaemic state of western diet fed *ApoE*$^{-/-}$ mice and consequent accumulation of cholesterol in macrophages. Loss of whole-body 11β-HSD1 activity by systemic inhibition or transgenic deletion attenuated neointimal lesion development in *ApoE*$^{-/-}$ mice. The absolute reduction in the cross-sectional stenosis (~10%), though statistically significant, is relatively modest in-terms of clinical outcomes; however the reduction in the volume of lesions (~35%) may also be clinically relevant, given that the currently used drug eluting stents produce 40-70% reduction in neointimal proliferation [Heldman et al., 2001;Weissman et al., 2005].

ApoE$^{-/-}$ mice had significantly higher macrophage content in neointimal lesions than C57Bl/6J. Whilst 11β-HSD1 inhibition had no effect on macrophage content of lesions in C57Bl/6J mice, it significantly reduced macrophage content in neointimal lesions of *ApoE*$^{-/-}$ mice suggesting that the role of 11β-HSD1 in modulating inflammation may be more important in the presence of a continuous inflammatory stimulus like feeding a western or high fat diet [Baer et al., 2004;Esposito and Giugliano, 2006]. 11β-HSD1 is expressed in macrophages [Thieringer et al., 2001] and may regulate immune and inflammatory processes [Chapman et al., 2006]. However, the precise mechanism of how 11β-HSD1 interacts with inflammatory cells remains unknown and merits future studies (discussed in section 6.2). Furthermore, although glucocorticoids inhibit proliferation of VSMC *in-vitro;* they may also promote VSMC proliferation *in-vivo* by increasing ACE activity in vascular cells [Mendelsohn et al., 1982;Fishel et al., 1995], up-regulating endothelin-1 expression [Morin et al., 1998] or decreasing the activity of nitric oxide [Mangos et al., 2000]. These opposing effects may have resulted in no net effect on VSMC content of neointimal lesions.

The reduction in neointimal proliferation in *ApoE*$^{-/-}$ mice was associated with a reduction in plasma glucose, systolic blood pressure and macrophage content of neointimal lesions but without any effect on plasma cholesterol or VSMC proliferation. Glucocorticoids have been shown to promote foam cell formation by increasing the accumulation of cholesterol esters, by enhancing acyl coenzyme A cholesterol acyltransferase 1 (ACAT-1) expression [Yang et al., 2004]. Loss of 11β-HSD1 activity reduced the number of foam cells in lesions without affecting systemic cholesterol levels, suggesting a direct effect on cholesterol uptake or efflux in macrophages. Diabetes mellitus is also a well recognised risk factor for development of restenosis [Stein et al., 1995;Hong et al., 2006] due to increased neointimal proliferation [Kornowski et al., 1997] as well as constrictive remodelling [Van et al., 1997]. Optimum glycaemic control has been shown to reduce the incidence of post angioplasty restenosis [Mazeika et al., 2003;Corpus et al., 2004]. Hypertension has also been linked to the development of restenosis [Klugherz et al., 2000;G.Tocci et al., 2010] and lowering blood pressure along with improvements in glycaemic profile may have contributed towards beneficial effect of 11β-HSD1 inhibition on neointimal proliferation. The data presented here, therefore, suggest that

regulating intra-cellular pre-receptor metabolism of physiological concentrations of endogenous glucocorticoids in metabolically normal mice does not affect metabolic profile or VSMC proliferation and, hence, has no influence on neointimal lesion formation. However, in *ApoE*$^{-/-}$ mice, it seems plausible that 11β-HSD1 inhibition may have reduced neointimal proliferation by improving systemic risk factors and reducing vascular inflammation, but without affecting VSMC proliferation.

4.4.3 Potential of OPT in assessment of vascular lesions

Finally, these experiments also highlight the potential of OPT in vascular lesion analysis. OPT is a relatively new technique for vascular lesion assessment and has been validated by NS Kirkby *et al.* (unpublished data). It has been shown to correlate well with standard histology and additionally provides a three dimensional volumetric assessment of the lesions. OPT is a non-destructive technique and tissues can be processed for standard histology and immunohistochemistry afterwards. Therefore, OPT can complement histological analysis in pre-clinical work. Both the extent (volume determined by OPT) and severity (maximum cross-sectional stenosis determined by OPT/histology) are important in clinical practice to determine the management and prognosis of vascular lesions in humans.

4.5 Conclusion

In conclusion, work presented in this chapter has shown that selective inhibition of 11β-HSD1 does not increase post-angioplasty neointimal proliferation in mice and may indeed reduce neointimal lesions in western diet fed $ApoE^{-/-}$ mice. These results suggest that 11β-HSD1 inhibition may reduce neointimal proliferation in the presence of metabolic syndrome or cardiovascular risk factors but may have no effect in metabolically normal individuals. These data also suggest that 11β-HSD1 inhibitors can be initiated or continued for treatment of diabetes mellitus or metabolic syndrome in patients undergoing angioplasty procedures. This may also have implications for the development of novel anti-restenotic agents, though the magnitude of reduction in lesion size (and hence likelihood of any clinical benefit in the era of stents) needs to be assessed in future studies.

5. Role of 11β-HSD2 and MR in neointimal proliferation

5.1 Introduction

Investigations into the effect of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) inhibition on neointimal proliferation (Chapter 4) suggested that regulating pre-receptor metabolism of endogenous glucocorticoids alters arterial remodelling by modulating systemic risk factors and local inflammatory response. It is logical, therefore, that the activity of the type 2 isozyme of 11β-HSD (which converts glucocorticoids into their inactive form and protects MR from glucocorticoid-mediated activation) may also influence vascular remodelling and neointimal proliferation.

MR activation has been suggested to play a key role in cardiovascular remodelling (in humans and in experimental animals) by influencing inflammation and VSMC proliferation [Ishizawa et al., 2005b;Suzuki et al., 2006c]. MR antagonism improves cardiovascular outcome in patients with heart failure [Pitt et al., 1999d;Pitt et al., 2001d] and also attenuates post-angioplasty neointimal proliferation in rabbits [Van et al., 1995d] and pigs [Ward et al., 2001i]. MR bind mineralocorticoids and glucocorticoids with equal affinity but the circulating levels of glucocorticoids are 100-1000 fold greater than those of aldosterone [Arriza et al., 1987;Funder et al., 1988]. Mineralocorticoid selectivity of MR in aldosterone target tissues is conferred by 11β-HSD2. 11β-HSD2 converts cortisol (corticosterone in rodents) to its inert 11-keto metabolite, cortisone, (11-dehydrocorticosterone) which has negligible affinity for MR. Thus the presence of 11β-HSD2 effectively excludes cortisol from MR and allows aldosterone to occupy and activate MR [Edwards et al., 1988;Funder et al., 1988]. Pharmacological inhibition or genetic deletion of 11β-HSD2, therefore, can lead to activation of MR by glucocorticoids, producing the glucocorticoid-mediated 'syndrome of apparent mineralocorticoid excess' [Stewart et al., 1988].

Whilst 11β-HSD2 is expressed predominantly in mineralocorticoid target tissues (e.g., kidneys, colon) it is also present in the cells of the vascular wall; especially in endothelial cells [Walker et al., 1991;Christy et al., 2003]. However, any role for vascular 11β-HSD2 in modulating neointimal lesion development remains unknown. The intriguing findings that MR blockade improves cardiovascular outcome in the face of normal circulating aldosterone levels [Pitt et al., 1999;Pitt et al., 2001] and reduces

neointimal proliferation even though aldosterone administration itself has a limited effect [Ward et al., 2001] led to the hypothesis that glucocorticoids may modulate vascular remodelling by acting as MR ligands. If this hypothesis is correct, it predicts that the effects of MR on neointimal proliferation are not influenced by 11β-HSD2. This was addressed by studying vascular remodelling in mice with targeted deletion of hsd11b2 and further exploring the effect of MR blockade in the presence and absence of 11β-HSD2. The specific objectives of studies in this chapter, therefore, included:

- To determine the effect of 11β-HSD2 deletion on vascular remodelling
- To determine the effect of MR blockade in the presence or absence of 11β-HSD2

5.2 Materials and methods

5.2.1 Animals

C57Bl/6J mice (Harlan Laboratories, UK) and 11β-HSD2$^{-/-}$ mice, homozygous for a disrupted hsd11b2 allele and congenic on a C57Bl/6J background [Kotelevtsev et al., 1999;Bailey et al., 2008], were used in this study (genotype confirmed at weaning by Dr. Bailey). Animals were maintained under standard conditions of light (lights on 8am-8pm) and temperature (21-22^{o}C).

5.2.2 Experimental protocol

Adult (12 week old) male C57Bl/6J and 11β-HSD2$^{-/-}$ mice (n=6/group) underwent wire-angioplasty of left femoral arteries, as described (section 2.7). Three weeks later vascular remodelling was studied in uninjured right femoral arteries and angioplastied left femoral arteries. In a further experiment, groups of mice (n=6) of each genotype received vehicle or the MR antagonist eplerenone (Merck & Co., New Jersey, USA) mixed with chow (1.7mg/g) to achieve a daily dose of eplerenone ~ 200mg/kg body weight, for one week before and three weeks after wire-angioplasty. Mice were housed singly and their body weights measured weekly. Systolic blood pressure (sBP) was measured weekly in conscious, warmed, restrained mice using tail-cuff photo-plethysmography (section 2.4).

Three weeks after the femoral angioplasty mice were killed by either asphyxiation (non-drug experiment) or terminal anaesthesia with phenobarbitone and trans-cardiac perfusion fixation (eplerenone experiment). Left femoral arteries were excised from the bifurcation of the iliac artery to the branch point of the popliteal artery. Arteries were fixed in 10% formalin for 24hr, and then stored in 70% ethanol. Arteries were then processed for histology and embedded in paraffin. Transverse sections (4 μm) were cut along the length of the vessel.

5.2.3 Lesion analysis

Sections of femoral artery (taken at 60μm intervals) were stained with United States trichrome (section 2.9.2). Images of stained sections were digitised and used for analysis

of sections as described (section 2.9.3). Mean areas (μm^2) were measured inside the external elastic lamina (EEL, for vessel area), internal elastic lamina (IEL) and lumen. Medial area was calculated as (area inside EEL - area inside IEL) and neointimal area as (area inside IEL - luminal area).

Lesion composition was determined by staining sections at the point of maximum neointimal proliferation. Collagen content was quantified by staining with Picrosirius red (section 2.10.1). Macrophage and smooth muscle content were assessed by immunohistochemistry (section 2.10.2) using purified monoclonal rat anti-mouse Mac2 antibodies for macrophages (1:6000) and monoclonal mouse anti-mouse α-smooth muscle actin antibodies for smooth muscle cells (1:400). Photomicrographs of stained sections were acquired and quantification of the stained areas performed using a semi-automated colour deconvolution process with Adobe Photoshop CS3 Extended software (chapter 2.10.4). All lesion analyses were carried out under blinded conditions.

5.2.4 Statistical analysis

Data are expressed as mean ± SEM, with n indicating the number of different animals in each group. Student's unpaired t-test was used to compare variables from two groups. Two-way ANOVA was used to investigate effects of genotype and drug treatment for the four groups. Statistical analyses were performed using GraphPad prism software.

5.3 Results

5.3.1 Influence of 11β-HSD2 deletion on arterial remodelling

5.3.1.1 Systemic effects of 11β-HSD2 deletion

11β-HSD2 deletion did not have any effect on body weight. There was no difference in body weights between 11β-HSD2$^{-/-}$ and C57Bl/6J mice, either pre-operatively (baseline) or 3 weeks after angioplasty (Table 5.1). All mice lost some weight after surgery, but this was regained over the next 3 weeks of the study. There were no obvious differences in wound healing and fur re-growth. The weights of most organs retrieved at cull were similar in 11β-HSD2$^{-/-}$ and C57Bl/6J (Table 5.1). However, the thymus was significantly heavier in 11β-HSD2$^{-/-}$ mice and a trend towards higher spleen weight in these mice did not reach significance (Table 5.1)

5.3.1.2 Influence on blood pressure and arterial structure

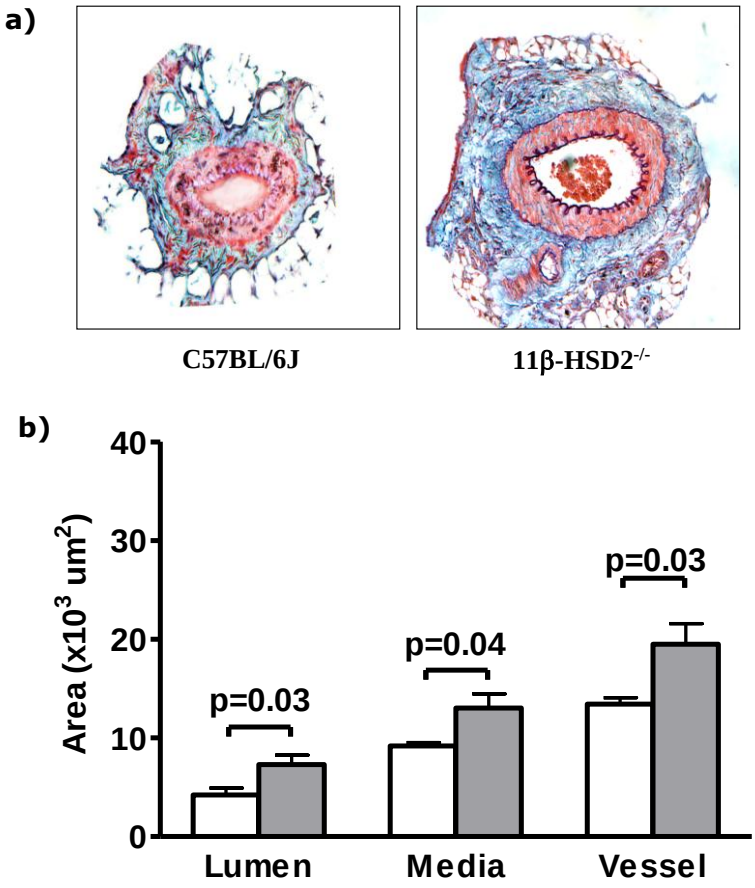
11β-HSD2$^{-/-}$ mice had substantially higher systolic blood pressure (129±3mmHg vs. 112±2mmHg, $p<0.001$) than age-matched C57Bl/6J mice.

Uninjured right femoral arteries in 11β-HSD2$^{-/-}$ mice had 31% larger vessel calibre due to bigger luminal and medial area compared with C57Bl/6J controls (Figure 5.1)

Table 5.1: Effect of 11β-HSD2 deletion on body/organ weights

Organ	11β-HSD2$^{-/-}$	C57Bl/6J	p
Body wt (baseline)	27.0±0.7	28.2±0.8	0.3
Body wt (final)	27.5±0.7	28.6±0.3	0.2
Liver (mg/g of body wt)	45.5±1.1	47.5±0.6	0.1
Heart (mg/g of body wt)	5.5±0.3	5.0±0.1	0.1
Kidneys (mg/g of body wt)	13.2±0.3	12.8±0.2	0.3
Spleen (mg/g of body wt)	2.64±0.1	2.26±0.2	0.07
Thymus (mg/g of body wt)	1.69±0.06	1.28±0.06	0.001
Adrenal glands (mg/g of body wt)	0.12±0.01	0.13 ± 0.01	0.3

Figure 5.1: 11β-HSD2 deletion produces an outward remodelling in mouse femoral artery



Sections of uninjured vessels stained with United States trichrome (20x magnification) illustrate the difference in size of vascular layers (a). 11β-HSD2$^{-/-}$ mice (grey columns) had larger calibre arteries than C57Bl/6J controls (white columns) with significantly bigger medial and luminal cross-sectional areas (b).

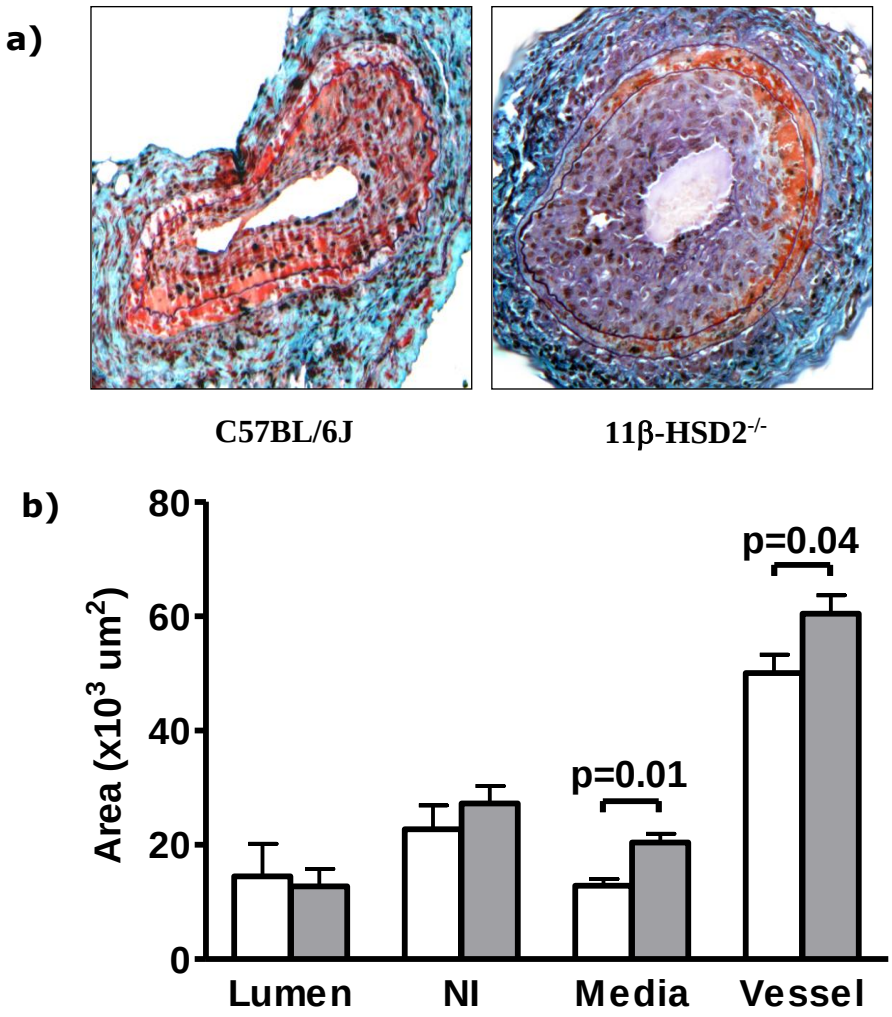
Data are mean ± SEM for n=6/group and were analysed by un-paired Student's t-test.

5.3.1.3 Vascular remodelling after wire-angioplasty

Wire-angioplasty produced fibro-cellular neointimal lesions in all mice (Figure 5.2a) and resulted in a significant outward remodelling with an approximately three fold increase in the total vessel area in the angioplastied arteries compared with uninjured vessels. After wire-angioplasty, the size of neointimal lesions was similar in 11β-HSD2$^{-/-}$ mice and C57Bl/6J controls. Furthermore, there was no difference in the size of patent lumen, although differences in the area of the media and total vessel persisted between the two groups (Figure 5.2b).

Neointimal lesions in the 11β-HSD2$^{-/-}$ group contained more macrophages (13.5±1.2% vs. 9.9±0.8%, p=0.02) and tended to contain less collagen (23±3% vs. 32±4%, p=0.08) but there was no difference in smooth muscle content (14.0±1.9% vs. 11.7±1.3%, p=0.3).

Figure 5.2: 11β-HSD2 deletion does not influence neointimal proliferation after wire angioplasty



Wire-angioplasty produced fibro-cellular neointimal lesions and an outward remodelling in both groups (x 20 magnification, (a)). 11β-HSD2 deletion had no effect on the size of lumen or neointimal lesion in angioplastied arteries. However, wire-angioplasty caused significantly greater outward remodelling in 11β-HSD2$^{-/-}$ mice, mainly due to an increase in the medial area (b).
Data are mean ± SEM for n=6/group and were analysed by unpaired Student's t-test.

5.3.2 Effect of mineralocorticoid receptor antagonism on neointimal remodelling in C57Bl/6J and 11β-HSD2$^{-/-}$ mice

5.3.2.1 Systemic effects

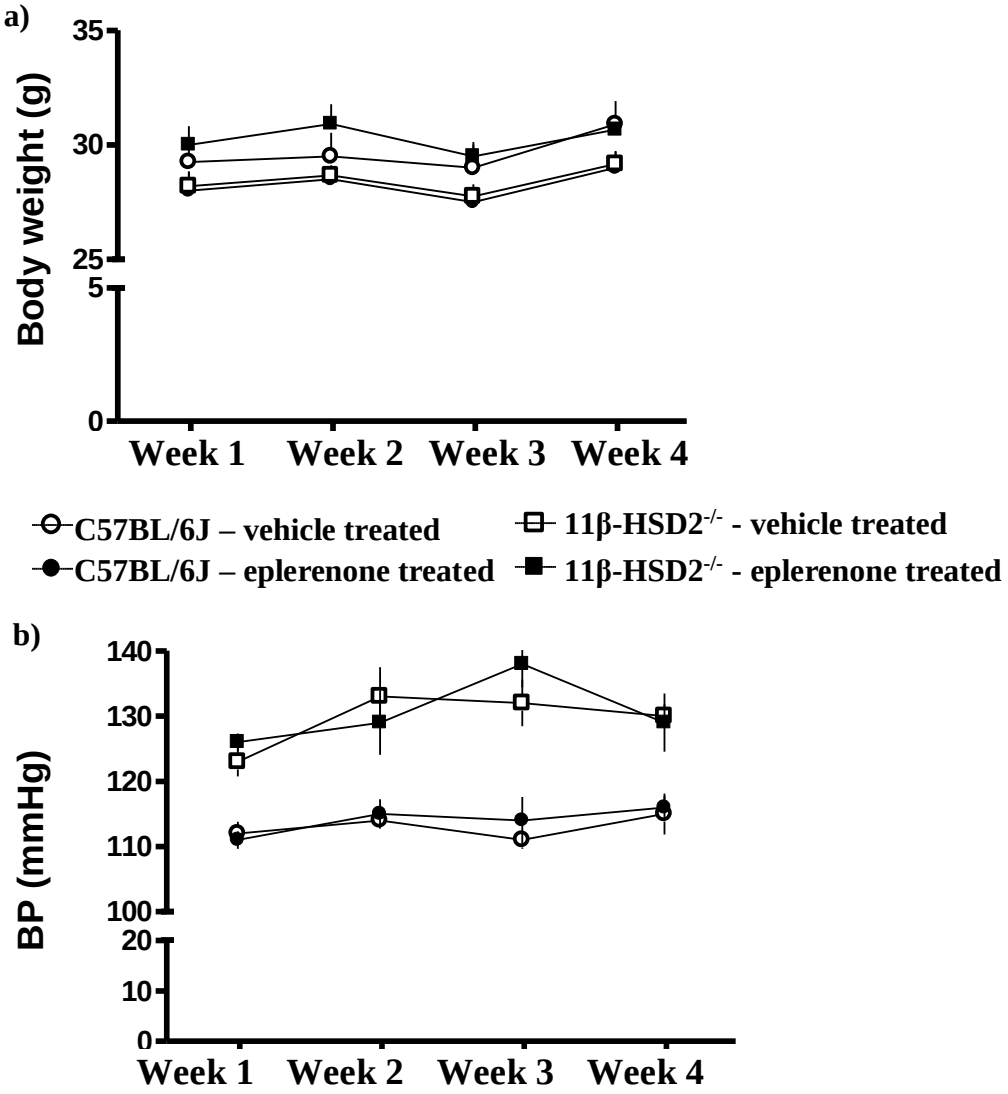
Neither eplerenone administration nor 11β-HSD2 deletion altered body weight (Figure 5.3a). Eplerenone also had no effect on blood pressure in either normotensive C57Bl/6J or hypertensive 11β-HSD2$^{-/-}$ mice (Figure 5.3b).

5.3.2.2 Effect on post-angioplasty vascular remodelling

Wire-angioplasty resulted in fibro-cellular neointimal lesion formation in all four groups (Figure 5.4). Eplerenone administration reduced neointimal lesion area in both C57Bl/6J and 11β-HSD2$^{-/-}$ mice but had no effect on lumen, media or vessel area (Figure 5.5). There was no significant interaction between eplerenone treatment and 11β-HSD2 deletion.

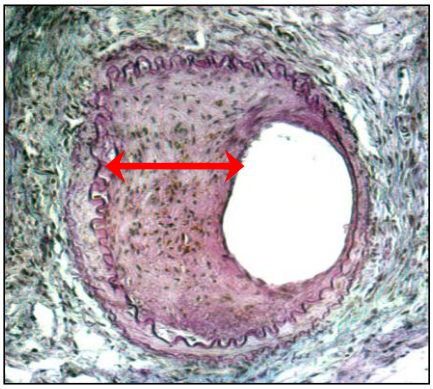
Eplerenone reduced the macrophage content in both C57Bl/6J and 11β-HSD2$^{-/-}$ mice (Figure 5.6) but had no effect on collagen and smooth muscle cell content of neointimal lesions (Figure 5.7).

Figure 5.3: Effect of eplerenone administration and 11β-HSD2 deletion on body weight and blood pressure

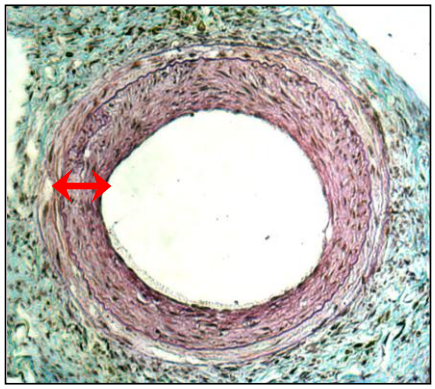


Body weights were similar in vehicle or eplerenone (200mg/kg/day, mixed with chow, 4 weeks) treated C57Bl/6J or 11β-HSD2$^{-/-}$ mice throughout the experiment, indicating no effect of eplerenone treatment (p=0.12) or 11-HSD2 deletion (p=0.4) (a). 11β-HSD2$^{-/-}$ mice had substantially higher blood pressure (p<0.001) than C57Bl6 controls. Eplerenone treatment had no effect on blood pressure in either C57Bl/6J or 11β-HSD2$^{-/-}$ mice (p=0.6) (b).
Data are mean ± SEM for n=6/group and were analysed by two-way ANOVA.

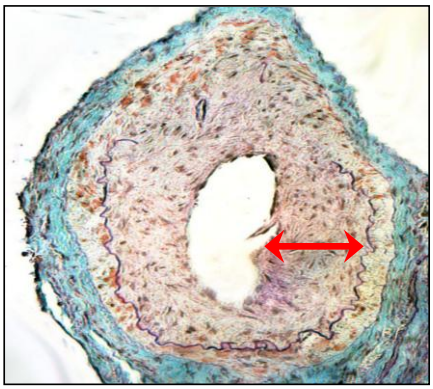
Figure 5.4: Wire-angioplasty produced fibro cellular neointimal proliferation



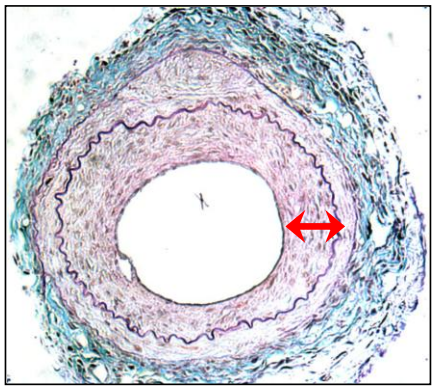
C57BL/6J – vehicle treated



C57BL/6J – Eplerenone treated



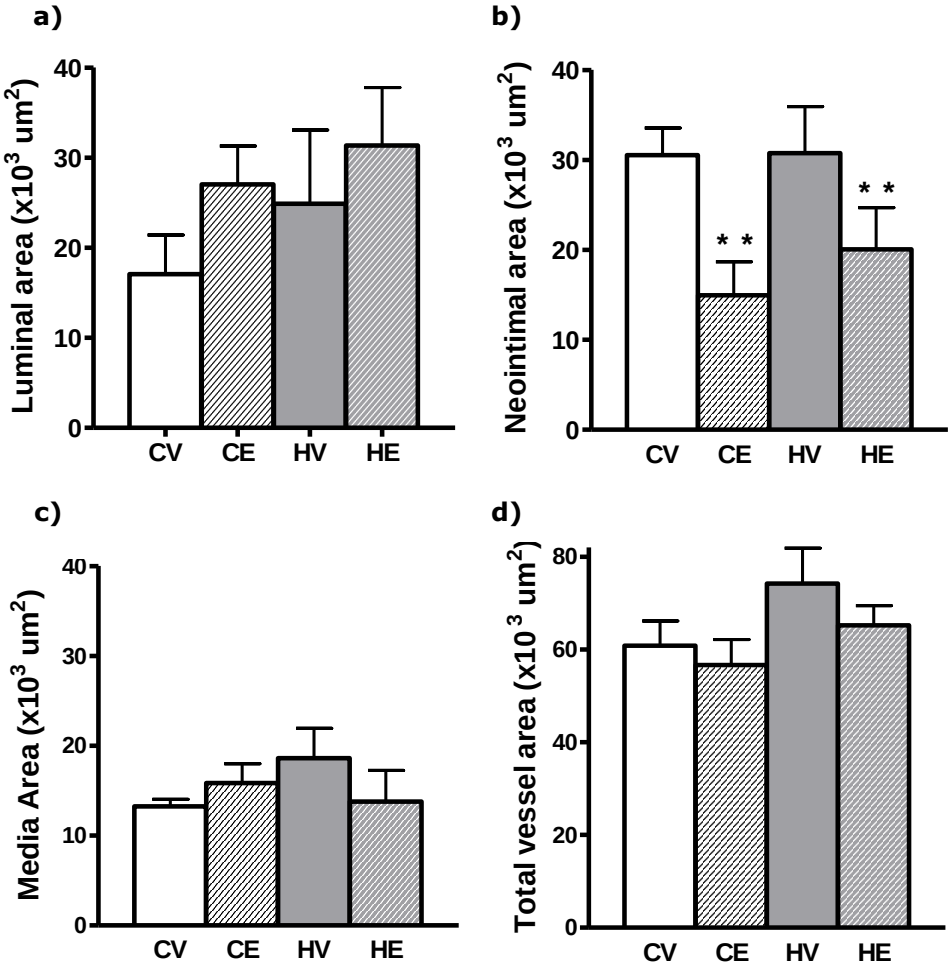
11β-HSD2$^{-/-}$ - vehicle treated



11β-HSD2$^{-/-}$ - eplerenone treated

All groups developed fibro-proliferative neointimal lesions (indicated by arrows) in response to wire-angioplasty (x 20 magnifications). However, it appears that size of lesions was smaller in eplerenone treated, as compared with vehicle treated, groups.

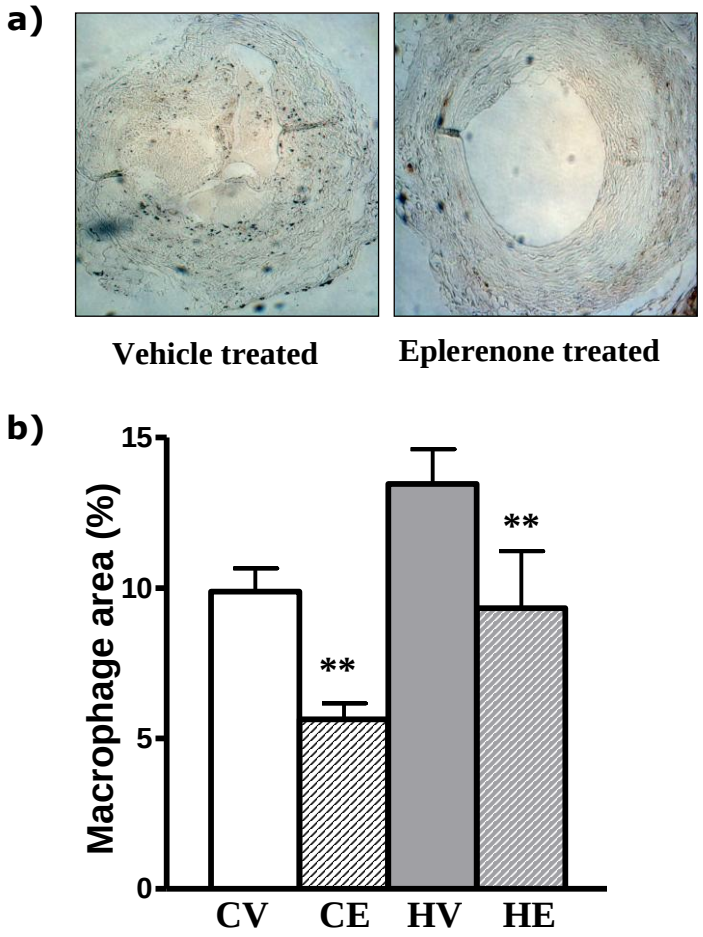
Figure 5.5: Eplerenone reduced neointimal proliferation in C57Bl/6J and 11β-HSD2$^{-/-}$ mice



C57Bl/6J and 11β-HSD2$^{-/-}$ mice received vehicle or eplerenone (200mg/kg/day) for four weeks, starting one week before wire angioplasty of femoral arteries. (a) Lumen size was not influenced by eplerenone treatment (p=0.2) or 11β-HSD2 deletion (p=0.3). (b) Eplerenone reduced neointimal area in both C57Bl/6J and 11β-HSD2$^{-/-}$ mice (p=0.005). (c & d) Eplerenone had no effect on medial or vessel area, whereas 11β-HSD2 deletion tended to increase vessel area (p=0.07). There was no genotype-eplerenone interaction. Data are mean ± SEM for n=6/group and were analysed by two-way ANOVA.

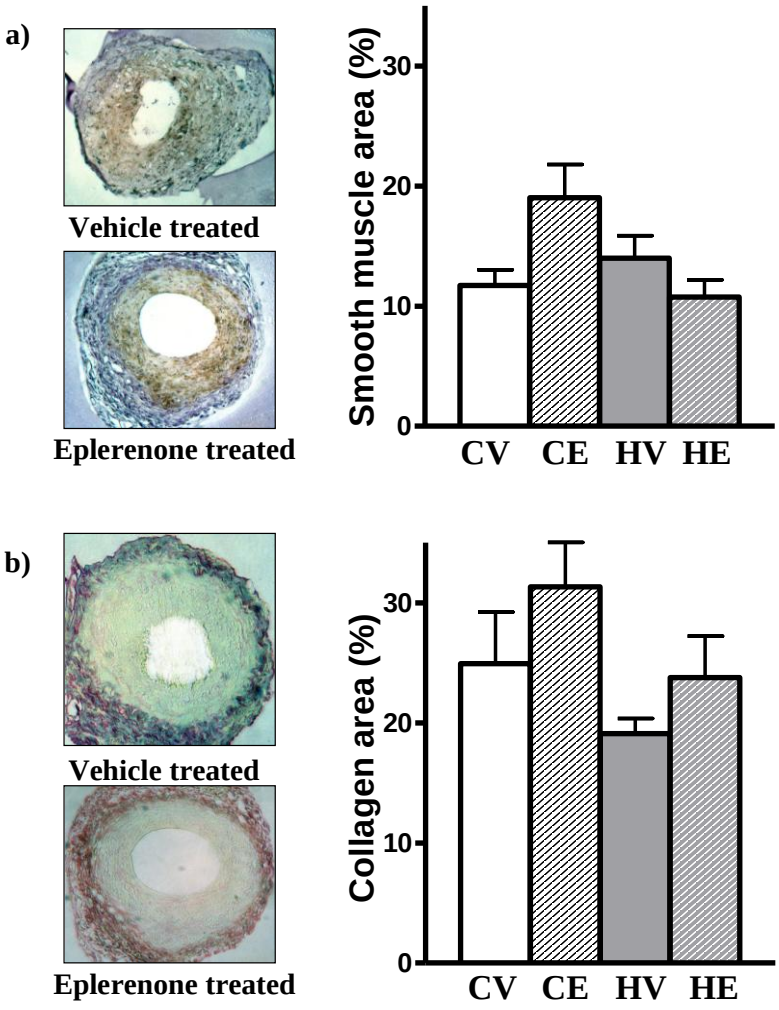
CV, C57Bl/6J mice receiving vehicle (open column); CE, C57Bl6 mice receiving eplerenone (hatched column); HV, 11β-HSD2$^{-/-}$ mice receiving vehicle (grey column); HE, 11β-HSD2$^{-/-}$ mice receiving eplerenone (grey hatched column).

Figure 5.6: Eplerenone decreases macrophage content of neointimal lesions



C57Bl/6J and 11β-HSD2$^{-/-}$ mice received vehicle or eplerenone (200mg/kg/day) for four weeks, starting one week before wire angioplasty. Macrophage content of neointimal lesions was assessed in transverse sections of femoral arteries with immunohistochemistry (representative pictures from two groups at x 20 magnification) (a). 11β-HSD2 deletion increased macrophage content of neointimal lesions (p=0.007). Conversely, eplerenone reduced macrophage content of lesions from both C57Bl/6J and 11β-HSD2$^{-/-}$ mice (p=0.002). There was no eplerenone and genotype interaction (p=0.9) (b). Data are mean ± SEM for n=6/group and were analysed by two-way ANOVA.
CV, C57Bl6 mice receiving vehicle (open column); CE, C57Bl/6J mice receiving eplerenone (hatched column); HV, 11β-HSD2$^{-/-}$ mice receiving vehicle (grey column); HE, 11β-HSD2$^{-/-}$ mice receiving eplerenone (grey hatched column).

Figure 5.7: Effect of eplerenone administration and 11β-HSD2 deletion on smooth muscle and collagen content of lesions



C57Bl/6J and 11β-HSD2$^{-/-}$ mice received vehicle or eplerenone (200mg/kg/day) for four weeks, starting one week before wire angioplasty. Collagen content of neointimal lesions was assessed by picrosirius red staining and smooth muscle content by immunohistochemistry (representative pictures from two groups at x 20 magnification). Neither eplerenone administration nor 11β-HSD2 deletion had a significant effect on smooth muscle (a) or collagen (b) content of lesions. Data are mean ± SEM for n=6/group and were analysed by two-way ANOVA.
CV, C57Bl/6J mice receiving vehicle (open column); CE, C57Bl/6J mice receiving eplerenone (hatched column); HV, 11β-HSD2$^{-/-}$ mice receiving vehicle (grey column); HE, 11β-HSD2$^{-/-}$ mice receiving eplerenone (grey hatched column).

5.4 Discussion

The data in this chapter highlight that MR, but not 11β-HSD2, play a significant role in vascular remodelling after wire angioplasty in mice. These results not only indicate a potential therapeutic role of selective MR antagonists in preventing neointimal lesion formation but also suggest that glucocorticoids act as MR ligands in murine vessel wall.

5.4.1 Effect of 11β-HSD2 on vascular remodelling

11β-HSD2$^{-/-}$ mice had evidence of outward remodelling in uninjured vessels. This is probably due to substantially higher systolic blood pressure in 11β-HSD2$^{-/-}$ mice (compared with C57Bl/6J mice) which is consistent with previous data from these knockout mice on C57Bl/6J and other genetic backgrounds [Kotelevtsev et al., 1999;Bailey et al., 2008].

Neointimal proliferation after wire-angioplasty was similar in C57Bl/6J and 11β-HSD2 knockout mice, suggesting a limited, if any, role of 11β-HSD2 in neointimal lesion formation. This contrasts with the role of 11β-HSD2 proposed in the development of atherosclerotic lesions, where *ApoE*/11β-HSD2 double knockout mice developed extensive plaques without need for dietary intervention (Deuchar et al., unpublished data). This could possibly be explained by mechanistic difference in lesion formation during atherogenesis and post-angioplasty neointimal proliferation (section1.2.1).

11β-HSD2$^{-/-}$ mice had more macrophage content of neointimal lesions compared with C57Bl/6J group. This is consistent with literature showing that pharmacological inhibition (with carbenoxolone) [Wilson et al., 2009] or transgenic deletion [Armour et al., 2009] of 11β-HSD2 can lead to increased monocyte/macrophage infiltration and consequent vascular inflammation. In the absence of convincing evidence for 11β-HSD2 expression in the resident or inflammatory macrophages [Schmidt et al., 2005;Lim et al., 2007;Gilmour et al., 2006], the difference in macrophage count in lesions in 11β-HSD2 knockout mice might represent a paracrine effect of neighbouring (e.g. endothelial) cells on macrophage recruitment, differentiation or survival and merits detailed mechanistic studies in future. There was no difference in the smooth muscle content of lesions

between the two groups. 11β-HSD2$^{-/-}$ mice have amplification of glucocorticoid activity in target cells leading to not only increased GR activation but these glucocorticoids can also mediate MR activation in the absence of 11β-HSD2. GR activation is known to inhibit proliferation of VSMCs [Longenecker et al., 1984] and MR activation, on the other hand, stimulates VSMCs proliferation [Ishizawa et al., 2005]. This could have resulted in no net effect on smooth muscle content of neointimal lesion in 11β-HSD2$^{-/-}$ mice. Furthermore, 11β-HSD2$^{-/-}$ mice showed a trend towards less collagen formation in the lesions (22.91±2.7 vs. 31.9±3.9, p=0.09). This is contrary to the expectation that inappropriate MR activation will result in more collagen accumulation [Wilson et al., 2009]. However, it is consistent with the observation that mice with transgenic over-expression of 11β-HSD2 have three fold increase in collagen deposition in their heart [Qin et al., 2003]. Furthermore, *ApoE*/11β-HSD2 double knockout mice have also been shown to have decreased collagen in the atherosclerotic plaques as compared with *ApoE* knockout mice [Armour et al., 2009].

5.4.2 Effect of Eplerenone on vascular remodelling

The data presented here confirm that eplerenone, a selective MR antagonist, reduces neointimal proliferation. These findings, novel in a mouse model, are consistent with data from other experimental models. Spironolactone, a mineralocorticoid antagonist, has been shown to inhibit neointimal proliferation, both in the iliac artery and in the aorta, after balloon angioplasty in rabbits [Van et al., 1995]. Eplerenone administration significantly increased luminal area (~60%) with modest reduction in neointimal proliferation after balloon angioplasty in pigs [Ward et al., 2001]. Wakabayashi et al. have shown that eplerenone treatment reduced the neointimal lesion size with consequent increase in lumen area after angioplasty and stent implantation in porcine coronary arteries [Wakabayashi et al., 2006].

Surprisingly, eplerenone did not have any effect on sBP in this study despite the fact that inappropriate MR activation is the underlying mechanism of hypertension in 11β-HSD2 knockout mice [Kotelevtsev et al., 1999]. This may be due to irreversible vascular or renal tubular remodelling during development in these mice [Bailey et al., 2008]. Furthermore, although eplerenone treatment has been shown to reduce blood pressure in

rodents [Bayorh et al., 2006], this effect is not invariable [Nehme et al., 2006;Kuster et al., 2005]. Eplerenone has also been shown to significantly reduce atherosclerosis [Suzuki et al., 2006] and cerebral ischemia/neurological deficit [Iwanami et al., 2007], without any effect on blood pressure. Eplerenone has been shown to reduce atherosclerotic lesions in DKO mice without affecting BP (Deuchar et al., Abstract at 90th Annual Meeting of The Endocrine Society, June 2008, San Francisco, USA). However, the substantial hypertension in 11β-HSD2$^{-/-}$ mice, though associated with vascular remodelling at baseline, did not increase the size of neointimal lesions. This is consistent with data from clinical studies in which hypertension, though strongly linked to the development of atherosclerosis [Yusuf et al., 2004], has not been shown to correlate with post-angioplasty restenosis [Bauters et al., 1998;Rensing et al., 1993]. Moreover, blood pressure reduction is not inextricably related to reduction in neointimal proliferation: hydralazine had no effect on neointimal proliferation despite lowering blood pressure in wild type and hypertensive mice [Kitada et al., 2009;Imai et al., 2002]. Finally, in the RALES and EPHESUS trials, the difference in sBP did not account for the observed beneficial effect of MR antagonism [Pitt et al., 1999;Pitt et al., 2001]. Thus the results presented here suggest that reduced neointimal proliferation in response to eplerenone treatment is not due to an effect on systolic blood pressure.

Restenosis after percutaneous revascularisation procedures develops as a result of arterial recoil with constrictive remodelling and neointimal proliferation [Bauters et al., 1996]. Collagen deposition in the vessel wall is suggested to mediate constrictive remodelling. It has been shown previously that eplerenone suppresses constrictive remodelling by reducing collagen content in the media [Ward et al., 2001]. The data shown in this chapter suggest no effect of eplerenone on collagen deposition in neointimal lesions in mice. The effect on collagen content, however, may be of limited clinical significance as restenosis in the era of stents mainly involves neointimal proliferation rather than the constrictive remodelling [Hoffmann et al., 1996]. Neointimal proliferation represents an inflammatory and proliferative response to iatrogenic vascular injury [Bennett, 2003]. MR activation is known to be pro-inflammatory in the vessel wall and MR blockade reduces vascular inflammation [Suzuki et al., 2006]. This study indicates that the beneficial effect of eplerenone in

reducing the size of neointimal lesions is associated with a reduction in macrophage, but not smooth muscle, content of lesions. Eplerenone has also been shown to reduce macrophage content of neointimal lesions in previous studies [Wakabayashi et al., 2006].

5.4.3 Glucocorticoids as ligand for vascular MR

Eplerenone reduced neointimal lesion size after wire-angioplasty in mice. If it is aldosterone that is displaced from the relevant MR by eplerenone, then local expression of 11β-HSD2 would have to protect these MR from corticosterone, so that this protection would fail in the absence of 11β-HSD2. Therefore, 11β-HSD2$^{-/-}$ mice would be predicted to have increased neointimal proliferation and, since corticosterone provides a higher level of occupancy of unprotected MR than does aldosterone [Edwards et al., 1988;Funder et al., 1988], a greater incremental response to eplerenone. The results show that deletion of 11β-HSD2 had no effect on neointimal proliferation and the effect of MR antagonism was not altered in 11β-HSD2$^{-/-}$ mice, so it can be concluded that 11β-HSD2 in the vessel wall does not protect the MR involved in modifying neointimal proliferation from glucocorticoids. Thus it is likely to be glucocorticoids, rather than mineralocorticoids, that occupy and activate the MR directly involved in post-angioplasty vascular remodelling. This concept is further supported by the data from different experimental models in which MR blockade (with spironolactone or eplerenone) significantly reduced neointimal proliferation whereas, in many cases, administration of aldosterone does not increase lesion size. For example, although aldosterone administration increased neointimal proliferation in rabbit iliac arteries [Van et al., 1995], it did not do so in rabbit aorta [Van et al., 1995] or pig coronary arteries [Ward et al., 2001]. This was despite MR blockade (with oral spironolactone or eplerenone) consistently reducing neointimal proliferation after balloon angioplasty in rabbits [Van et al., 1995] and pigs [Wakabayashi et al., 2006]. Finally, if MR is also not being protected by 11β-HSD2 in the human cardiovascular system, as others have also proposed [Funder, 2005], then it may explain the findings of the RALES and EPHESUS trials where patients with heart failure greatly benefited from MR antagonists despite having unremarkable aldosterone levels and salt status [Pitt et al., 1999;Pitt et al., 2001].

5.5 Conclusion

In conclusion, results in this chapter indicate that eplerenone substantially reduces the size of neointimal lesions associated with a reduction in macrophage, but not the smooth muscle or collagen, content of lesions and independent of any effect on systolic blood pressure. However, inhibition of 11β-HSD2 has no effect on the size of neointimal lesions. This suggests that MR blockade is a potential target to prevent post-angioplasty restenosis, but that it is glucocorticoids, rather than aldosterone, that activate vascular MR.

6. General discussion and future directions

Cardiovascular disease is the leading cause of death worldwide and, despite recent improvements in mortality, it is likely to remain number the one killer in the near future [Iqbal and Fox, 2010]. It is, therefore, important to understand the pathophysiology of (and to develop newer therapeutic targets for) atherosclerosis (the root cause of cardiovascular disease) and neointimal proliferation (cause of post angioplasty and in-stent restenosis). The main objective of the work described in this thesis was to examine the role of enzymes regulating intra-cellular levels of endogenous glucocorticoids in the development of atherosclerotic and neointimal lesions. The results suggest that 11β-HSD1 inhibition reduces development of vascular lesions whereas 11β-HSD2 does not play a significant role in modulation of neointimal proliferation. This chapter will discuss the overall results in the context of relevant literature, consider its implications and describe some pilot data suggesting further experiments to be performed in the future.

6.1 General discussion

6.1.1 Novel findings described in this thesis

The work described in the thesis has produced a number of novel observations, as summarised below:

1. Selective 11β-HSD1 inhibition or deletion lowers blood pressure in *ApoE*$^{-/-}$ mice

2. Selective 11β-HSD1 inhibition reduces the size of atherosclerotic lesions

3. Selective 11β-HSD1 inhibition improves markers of plaque stability

4. 11β-HSD1 inhibition does not alter neointimal proliferation in C57Bl/6J mice

5. 11β-HSD1 inhibition/deletion reduces neointimal proliferation in *ApoE*$^{-/-}$ mice

6. 11β-HSD1 inhibition reduces neointimal macrophage content in *ApoE*$^{-/-}$ mice

7. 11β-HSD2 deletion does not alter neointimal proliferation in C57Bl/6J mice

8. Eplerenone reduced neointimal proliferation in C57Bl/6J mice and 11β-HSD2 knockout mice without measurably altering blood pressure

6.1.2 Atheroprotective effect of 11β-HSD1 inhibition

Studies described in this thesis confirmed that selective 11β-HSD1 inhibition reduces the size of atherosclerotic lesions and improves markers of plaque stability. This is associated with reduced weight gain, lower blood pressure and a trend towards improved insulin sensitivity but without any change in circulating lipids.

The atheroprotective effect of selective 11β-HSD1 inhibition could possible be predicted from its ability to reduce cardiovascular risk factors [Hadoke et al., 2009]. But no previous study directly measured the effect of selective 11β-HSD1 inhibition on plaque size. The few relevant investigations available in the literature assessed lesion development indirectly by measuring intra-arterial lipids by aortic cholesterol extraction [Hermanowski-Vosatka et al., 2005], oil red O staining [Nuotio-Antar et al., 2007] or en face staining with Sudan IV [Lloyd et al., 2009]. These studies yielded variable results ranging from a dramatic reduction in aortic cholesterol with compound 544 (a triazole type selective 11β-HSD1 inhibitor) [Hermanowski-Vosatka et al., 2005], variable improvement in size of lesions with carbenoxolone (a non-selective inhibitor) [Nuotio-Antar et al., 2007] and no effect with compound 2922 (a thiazolone type selective 11β-HSD1 inhibitor) [Lloyd et al., 2009]. This study, for the first time, has histologically confirmed the atheroprotective and plaque stabilising effect of a selective 11β-HSD1 inhibitor. The precise mechanism for the atheroprotective effect, however, remains to be fully understood.

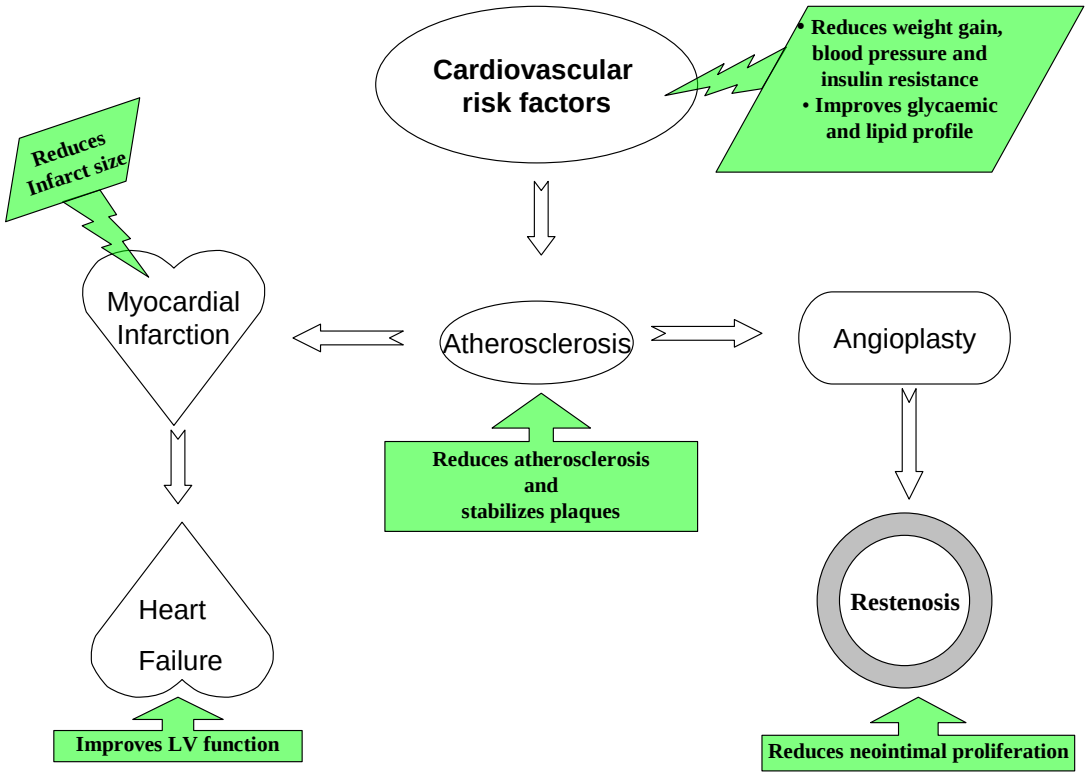
It is reassuring that the use of 11β-HSD1 inhibitors for the treatment of diabetes mellitus [Rosenstock et al., 2010] may have the additional benefit of reducing macro-vascular complications which are the main cause of morbidity and mortality in these patients [Wang et al., 2007]. This study also has implications for the use of selective 11β-HSD1 inhibitors as anti-atherosclerotic agents. However, it would not be appropriate to extrapolate results directly from the mouse model to humans and, therefore (and also due to issues of safety and economy), this atheroprotective effect should be further confirmed using other animal models (such as fat-fed pigs), before contemplating trial in humans.

6.1.3 Effect of 11β-HSD1 inhibition on neointimal proliferation

11β-HSD1 inhibition reduced neointimal proliferation in western diet fed *ApoE*$^{-/-}$ mice but had no effect on chow-fed C57Bl/6J mice. The effect of glucocorticoids (and glucocorticoid regulation by 11β-HSD1) on post-angioplasty neointimal proliferation is complex and determined by a number of variables including metabolic and inflammatory status [Liu et al., 2004]. Systemic or local glucocorticoid administration inhibited neointimal lesion formation in rats [Villa et al., 1994;Guzman et al., 1996], rabbits [Van Put et al., 1995;Poon et al., 2001;Ribichini et al., 2007] and dogs [Strecker et al., 1998]. However, not all studies in animals have yielded positive results, as dexamethasone treatment did not reduce neointimal hyperplasia after angioplasty in rabbits [Karim et al., 1997] or pigs [Lincoff et al., 1997]. In humans, the IMPRESS and STRIDE trials showed a reduction in restenosis with oral glucocorticoid administration [Versaci et al., 2002;Ferrero et al., 2007b;Liu et al., 2003]. On the other hand, oral methylprednisolone did not inhibit restenosis after coronary angioplasty [Pepine et al., 1990] or stenting [Reimers et al., 1998] and dexamethasone-drug eluting stents (D-DES) did not reduce the incidence of restenosis [Hoffmann et al., 2004;Ribichini et al., 2007]. These results probably indicate opposing local (beneficial) and systemic (adverse) effects of glucocorticoid administration. In current studies (using a mouse model of wire-angioplasty), 11β-HSD1 inhibition improved systemic risk factors without having any deleterious local effects and therefore, produced a reduction in neointimal proliferation.

The results presented here along with previously published data highlight the potential use of 11β-HSD1 inhibitors in the treatment of cardiovascular diseases (Figure 6.1). These data suggest that initiation or continuation of 11β-HSD1 inhibitors for the treatment of diabetes mellitus or metabolic syndrome is unlikely to have any detrimental effect on post-angioplasty restenosis and may indeed decrease neointimal lesion formation in high risk patients undergoing coronary angioplasty. The magnitude of reduction in neointimal proliferation is, however, small and its usefulness in clinical practice as an anti-restenotic target may be limited but needs to be explored in future experiments (section 6.2.2).

Figure 6.1: Potential role of 11β-HSD1 inhibitors in the treatment of cardiovascular diseases



There are robust data to suggest that 11β-HSD1 inhibition ameliorates cardiovascular risk factors. Studies described in this thesis have shown a reduction in the size and vulnerability of atherosclerotic plaques and a potential reduction in neointimal proliferation after angioplasty. A recent study (McSweeney *et al*., 2010) has also shown a reduction in infarct size and an improvement in heart function in a murine model of myocardial infarction. These potential therapeutic effects of 11β-HSD1 inhibition are highlighted in green (shaded) boxes.

6.1.4 Role of 11β-HSD2 and MR in neointimal proliferation

Studies described in chapter 5 have shown minimal, if any, role of 11β-HSD2 in neointimal lesion formation. Since the physiological role 11β-HSD2 is to protect MR from glucocorticoids, this could either mean that vascular MR is not playing a role in neointimal proliferation or 11β-HSD2 is not protecting vascular MR from inappropriate (glucocorticoid-mediated) activation. However, eplerenone treatment reduced neointimal proliferation to a similar extent in both 11β-HSD2$^{-/-}$ mice and C57Bl/6J controls and, hence, provided the evidence that MR play a key role in development of neointimal lesions but that it is the glucocorticoids rather than aldosterone that occupy MR in murine vessel wall. These findings promote a significant shift from the idea that aldosterone is the major culprit in cardiovascular pathologies and MR antagonists derive their benefit by blocking effects of aldosterone. This is consistent with data from various studies showing beneficial effect of MR blockade with spironolactone or eplerenone but minimal or no effect with aldosterone itself [Van et al., 1995;Wakabayashi et al., 2006].

The effect of eplerenone in reducing neointimal proliferation (chapter 5) in the mouse wire-angioplasty model is consistent with data from porcine angioplasty [Ward et al., 2001] and porcine stenting models [Wakabayashi et al., 2006]. The underlying mechanism seems to be a reduction in vascular inflammation leading to reduced neointimal proliferation [Keidar et al., 2003;Ward et al., 2001;Wakabayashi et al., 2006]. These results suggest a possible therapeutic application of eplerenone in preventing restenosis in human (discussed in 6.2.3).

6.1.5 Limitations of work

The studies described in this thesis have several potential limitations, some of these inherent to the nature of this work, and some specific to the experiments.

One potential limitation is that these studies used a single compound (Compound 544, a triazole) as a selective 11β-HSD1 inhibitor. However, most of the work presented here has been validated by using genetically modified mice to ensure that results indeed represent effects of enzyme inhibition, and not any off-target effects of this compound.

Moreover, studies were conducted using a single animal model (mouse model of atherosclerosis or wire-angioplasty), and therefore it would be unwise to directly extrapolate results to humans. However, it was not possible to conduct studies in other animal models at the same time due to limitation of time and resources.

Finally, the effect of systemic administration or whole body deletion of enzymes may be due to direct local effects or indirect systemic effects which could not be tested in current experiments. To overcome this limitation, experiments could be set-up giving locally administered inhibitors or using tissue specific knockout mice. It was not possible to carry out these further experiments during the time-scale of the current project; however, this and other un-answered questions can be addressed in future studies as outlined below.

6.2 Future directions

The work described in this thesis has not only helped to clarify the role of endogenous glucocorticoid metabolism by 11β-HSD enzymes in vascular lesion development but has also provided ideas for future experimental and translational investigations. A few pilot studies were undertaken with a view to future development of the field and some preliminary data is also presented here to illustrate the future directions.

6.2.1 Mechanism of vascular lesion reduction with 11β-HSD1 inhibition

Experiments in this thesis have confirmed the atheroprotective effect of 11β-HSD1 inhibition and suggested improvements in systemic risk factors, including a novel blood pressure lowering effect, as a possible mechanism for this effect. However, it has also been suggested that 11β-HSD1 inhibition can also directly influence vascular inflammation [Hermanowski-Vosatka et al., 2005] and VSMCs, but this remains to be established unequivocally. A better understanding of whether (and how) these enzymes interact with inflammatory or smooth muscle cells will provide insight into the cellular mechanism by which 11β-HSD1 inhibition/deletion reduces vascular lesions and may identify novel therapeutic targets. The data presented in chapter 4 suggest that 11β-HSD1 inhibition may be modulating inflammatory, but not the VSMC proliferative, responses. Therefore, experiment(s) to investigate the effects of 11β-HSD1 inhibition on macrophages and T-cells during vascular lesion development is an important next step. In current experiments, arteries were collected three weeks after angioplasty when lesions would have stabilised and acute inflammation settled, therefore, it may be appropriate to evaluate effect of 11β-HSD1 inhibition on adhesion and migration of inflammatory cells at earlier time points. This could be achieved by designing short term experiments where mice lacking 11β-HSD1 activity (pharmacologically or genetically) get wire angioplasty and are killed at day 1 and day 7 after the procedure (as opposed to 21 days in current studies). The role of macrophages in mediating effects of 11β-HSD1 inhibition on lesion formation can also be studied in macrophage-specific knock-outs of 11β-HSD1. Since such knockout models are not available at the moment, another useful but relatively complex experiment may be to perform bone marrow transfer from 11β-

HSD1$^{-/-}$ mice into wild-type animals (and vice versa) which will selectively attenuate 11β-HSD1 activity in circulating haematic and inflammatory cells (or in whole body except these cells). This may help to confirm whether the atheroprotective effect of 11β-HSD1 is mediated by inflammatory cells. Finally, investigating the effect of 11β-HSD1 inhibition on inflammatory chemokines/cytokines (e.g. MCP-1 or IL-1) during post-angioplasty vascular remodelling can provide insight into the pathways modulated by these enzymes.

The data presented in chapter 3 and chapter 4 suggests a novel blood pressure lowering effect of 11β-HSD1 inhibition/deletion. However, the precise underlying mechanism remains unknown. Systolic blood pressure was measured using the tail-cuff photo-plethysmography technique which is a relatively crude method and provides a measurement of stressed blood pressure. Since glucocorticoids are stress hormones, it is possible that the blood pressure lowering effect of blocking glucocorticoid activation may represent blockade of the stress response. This blood pressure effect should first be confirmed with invasive blood pressure monitoring (e.g. using indwelling telemetry catheter) to get accurate (and non-stressed) measurement of blood pressure. Once the blood pressure lowering effect is confirmed, further experiments to elucidate potential mechanism (such as central nervous/sympathetic, vascular reactivity or renovascular), can be carried out.

6.2.2 Systemic vs. local effect of 11β-HSD1 inhibition on neointimal proliferation

The studies described in this thesis explored the effect of abolishing whole-body 11β-HSD1 activity by systemic inhibition or transgenic deletion. Glucocorticoids may have opposing systemic and local effects on vascular lesion development (Macdonald et al., unpublished data) and it has been suggested that the atheroprotective effects of 11β-HSD1 inhibition may partially be mediated by direct effects on the vessel wall [Hermanowski-Vosatka et al., 2005]. Therefore, it would be a logical next step to dissect the effects of local versus systemic inhibition of 11β-HSDI activity in vessel wall. This could be achieved either by local administration of an inhibitor or by development of transgenic tissue-specific models of 11β-HSD1 knockout in vascular smooth muscle cells or macrophages. The tissue-specific knockout models of 11β-HSD1 are not

available at the moment and, therefore, local administration of inhibitor via a drug eluting stent, implanted pellet or a sub-cutaneous mini-pump may be a more practical option.

Furthermore, it would be important to determine whether the beneficial effect of 11β-HSDI inhibition on lesion formation (seen here using the wire-angioplasty model) can be reproduced in a stent model as the mechanism of post-angioplasty stenosis may differ in these models. This could be achieved using a recently-described mouse model of stenting [Chamberlain et al., 2010] or in a porcine coronary stenting model [Lowe et al., 2003]. Use of porcine angioplasty models may also help to establish whether the magnitude of reduction in neointimal lesions is likely to be of any clinical significance in humans.

6.2.3 Effect of eplerenone on in-stent restenosis in man

Eplerenone, a selective MR antagonist, reduces neointimal proliferation after wire-angioplasty. These findings, novel in the mouse model, are consistent with data from other experimental models [Ward et al., 2001;Wakabayashi et al., 2006]. Spironolactone, a mineralocorticoid receptor antagonist, inhibited neointimal proliferation after balloon-angioplasty in rabbits [Van et al., 1995e]. In humans, there is association of elevated baseline plasma aldosterone level with subsequent post angioplasty restenosis [Amano et al., 2006]. However, a single centre, placebo-controlled, double-blind, randomised study using spironolactone (50mg twice a day for six months) did not reduce the incidence of angiographic in-stent restenosis [Kursaklioglu et al., 2004]. It could be argued that eplerenone, where the 17α-thioacetyl group of spironolactone has been substituted with a carbomethoxy group, differs considerably in terms of selectivity, intensity and action. Certainly, the interest in mineralocorticoid blockade with eplerenone as a potential therapeutic target in restenosis has been revived with very promising experimental data from different animal models Eplerenone administration reduced neointimal proliferation after wire angioplasty in mice (current study), balloon angioplasty [Ward et al., 2001] and angioplasty with stent implantation in pigs [Wakabayashi et al., 2006], suggesting its potential role as an anti-restenotic agent.. Currently used drug eluting stents (Sirolimus and Paclitaxel) activate

anti-proliferative mechanisms by pathways that are independent of MR [Poon et al., 1996;Suzuki et al., 2001]. Eplerenone may have a complimentary role in preventing neointimal proliferation (and in-stent restenosis) and, hence, merits translational studies in man.

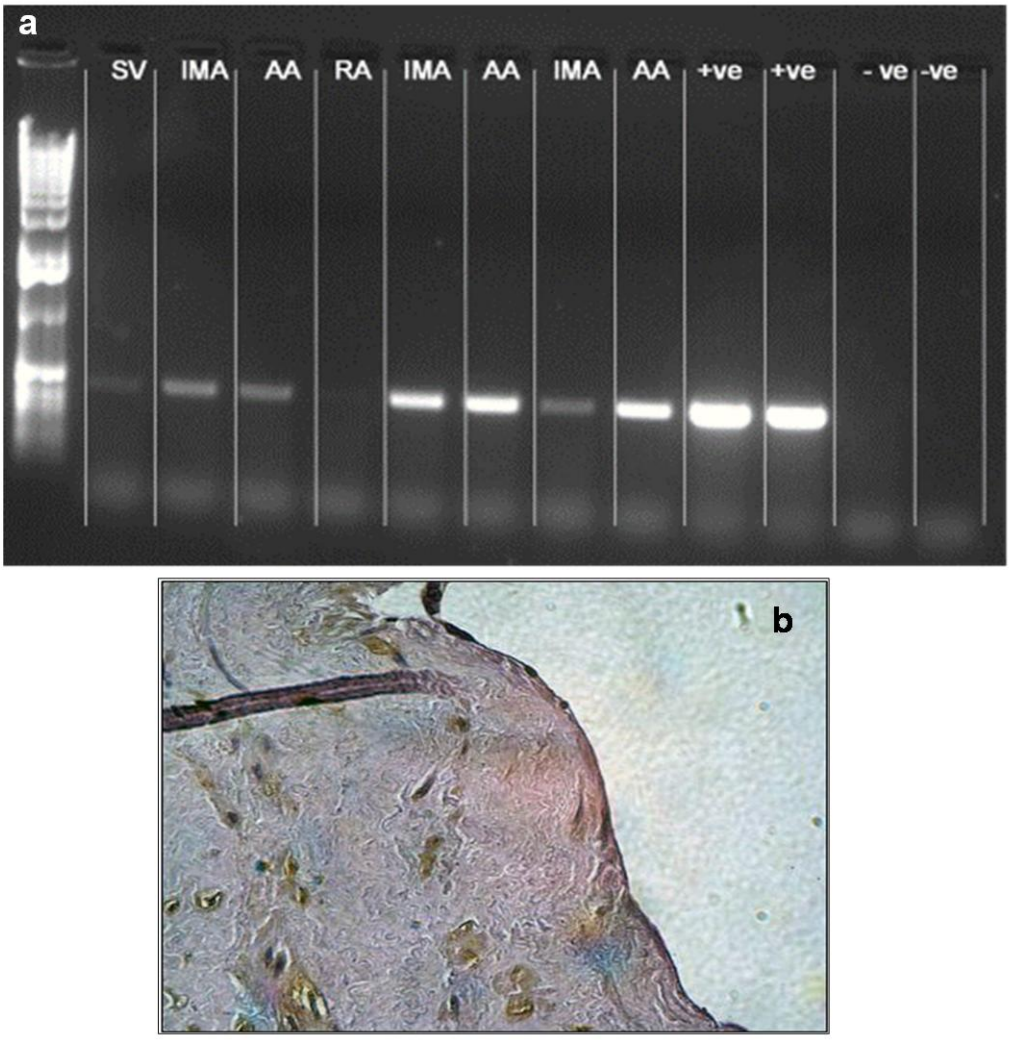
6.2.4 11β-HSD in the human cardiovascular system

The work presented in this thesis examined the role of 11β-HSD enzyme activity in regulation of the cardiovascular system, using rodent models. To translate these studies to humans, a basic first step is to establish the expression and activity of 11β-HSD in the human cardiovascular system.

Studies in rodents have suggested that the predominant 11β-HSD isozyme in the heart is 11β-HSD1, with trivial 11β-HSD2 activity (section 1.4). In humans, however, the pattern of expression may be different. Lombès et al., obtained myocardial biopsies during cardiac surgery or from endocavity catheterization and provided evidence for MR and 11β-HSD expression in the human heart [Lombes et al., 1995]. Cardiac 11β-HSD activity was detected and was dependent on the presence of the cofactor NAD, but not NADP, suggesting indirectly that there is predominant 11β-HSD type 2 activity [Lombes et al., 1995]. Other groups have also showed detectable 11β-HSD2 mRNA in human heart and also confirmed that 11β-HSD2 activity is distributed evenly between atria and ventricles [Slight et al., 1996;Kayes-Wandover and White, 2000].

Tissues from atrial appendage and vessels (including radial artery, internal mammary artery and saphenous vein) were obtained from adult patients undergoing elective cardiac surgery (at the Royal Infirmary of Edinburgh) and able to give informed consent. During heart surgery, a small sample from the right atrial appendage and trimmings from blood vessels were collected for the purpose of this study. Ethical approval was obtained from Lothian Research Ethics Committee. Provisional data, using polymerase chain reaction (PCR) technique (kindly performed by Ms Eileen Miller) confirmed expression of GR, MR, 11β-HSD1 and 11β-HSD2 in human cardiovascular tissues (Figure 6.2a). 11β-HSD1 expression was also shown in human vessels using immunohistochemistry (Figure 6.2b).

Figure 6.2: Expression of 11β-HSD1 in the human cardiovascular system



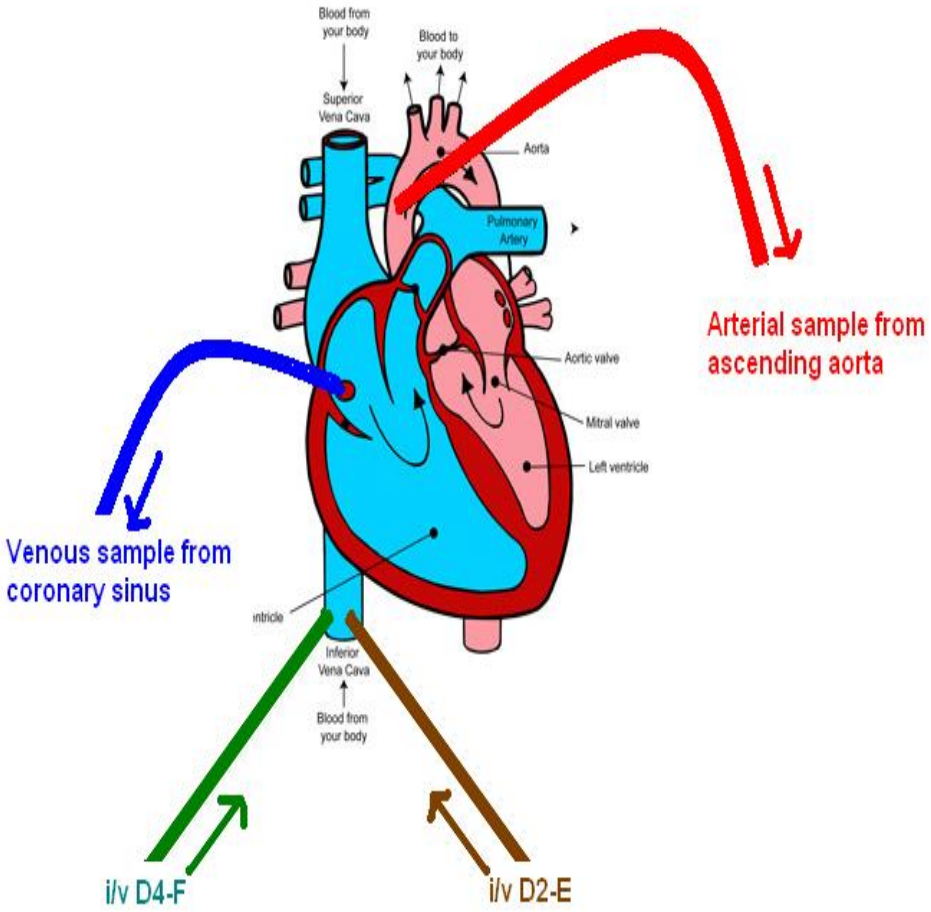
Human samples obtained during coronary artery bypass grafting (CABG) surgery were used to detect expression of 11β-HSD1 in saphenous vein (SV), internal mammary artery (IMA), atrial appendage (AA) and radial artery (RA) using polymerase chain reaction (PCR) with specific primers (forward, 5'-AAA GTG ATT ACW GGG GCC AGC AAA-3'; reverse, 5'-ATC CAR AGC AAA CTT GCT TGC-3') to detect the hsd11b1 allele. PCR conditions included an initial denaturing step (95ºC for 15min) followed by 34 cycles of annealing (90ºC for 1min, 56ºC for 1min and 72ºC for 2min) and final extension (72ºC for 5min). Gel electrophoresis showed expression in cardiovascular tissues at 440bp (a). Immunoreactivity for 11β-HSD1 antibody was then used to detect presence of 11β-HSD1 in arterial wall (brown staining, x 10 magnification, b).

However, so far there has been no study to measure the activity of 11β-HSD type 1 and type 2 in human heart *in-vivo*. This is obviously important for determining the effects of 11β-HSD1 inhibitors in the human cardiovascular system and to establish whether the human heart has sufficient 11β-HSD2 activity to exclude cortisol from MR binding sites.

It has been shown that stable isotope tracers can be used to quantify *in-vivo* 11β-HSD activity in man [Andrew et al., 2002]. Andrew *et al.* have successfully used D_4-cortisol tracers to measure the rate of regeneration of cortisol from cortisone by 11β-HSD1. More recently, using a D_2-cortisone tracer, it has been possible to measure the rate of conversion of cortisol to cortisone by 11β-HSD2 (Hughes et al., unpublished data). It is possible, using these tracers in combination with tissue-specific sampling, to elucidate 11β-HSD activities in human heart. A feasibility study was carried out where a patient requiring coronary angiography for clinical reasons, received these tracers (D_4-cortisol 3.5mg bolus and then 1.74mg/hr infusion; D_2-cortisone 150µg bolus and 210 µg/hr infusion) and once steady state was reached then blood samples were collected from atrial (ascending aorta) and venous (coronary sinus) sides of heart (Figure 6.3). Mass spectrometry analysis confirmed that the tracers and their metabolites can be reliably identified and quantified in both venous and arterial samples.

Therefore, future studies can be conducted in humans to determine the activity of the two isozymes of 11β-HSD in human heart *in-vivo* and explore whether human heart has sufficient 11β-HSD2 activity to prevent glucocorticoid-mediated MR activation. Once the activity of the two isozymes of 11β-HSD in the human cardiovascular system is established, it may be a useful next step to evaluate 11β-HSD1 and 11β-HSD2 activity in patients with confirmed coronary artery disease and heart failure against a matched population without these conditions. It will provide a useful insight into possible effects of manipulating these enzymes, since glucocorticoids (and therefore 11β-HSD enzymes) have been linked to coronary atherosclerosis, and heart muscle function [Small et al., 2005].

Figure 6.3: Schematic illustration of the methodology for evaluating *in-vivo* activity of 11β-HSD isozymes in the human heart



Stable isotope tracers, D_4-cortisol (D4-F) and D_2-cortisone (D2-E) are infused via peripheral veins. Once steady state is achieved, sampling from the arterial (ascending aorta or femoral artery) and venous (coronary venous sinus) sides of the myocardium can allow quantification of tracers and their metabolites (as a result of 11β-HSD1&2 activity) using mass spectrometry.

6.2.5 Ex-vivo assessment of in-stent restenosis using OPT

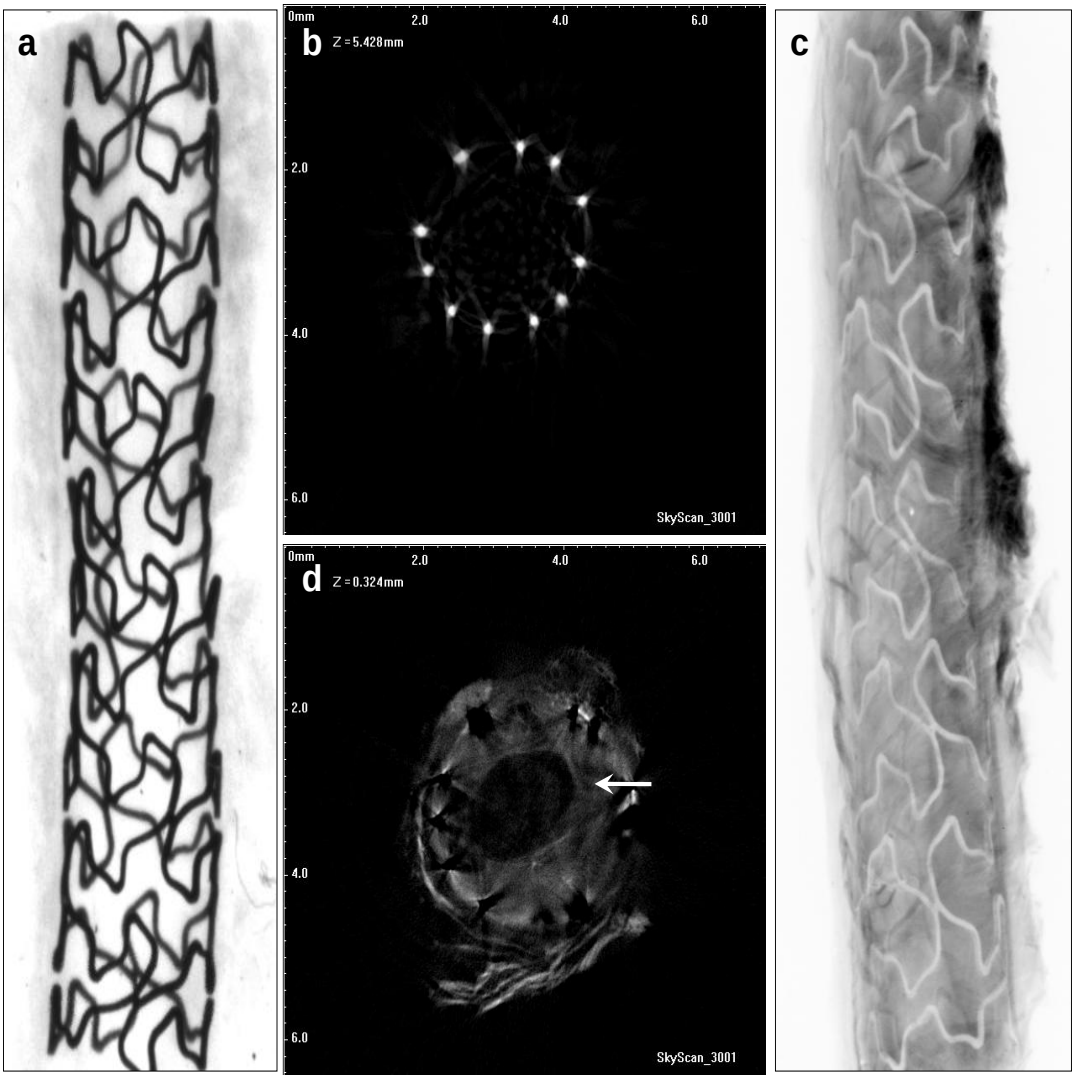
The studies presented in this thesis have identified a possible therapeutic role of 11β-HSD1 inhibition, using a wire angioplasty model (chapter 4) and it may be a desirable next step to evaluate the effect of this inhibition in a model of arterial stenting. OPT has emerged as a novel tool for assessment of vascular lesions but has not been used to study stented arteries. Therefore, it would be useful to establish if OPT can be used to quantify lesion area and volume in stented murine or porcine vessels.

A preliminary feasibility study using porcine coronary artery stenting model, a well established and clinically-relevant model of in-stent restenosis, was conducted. Circumflex and left anterior descending porcine coronary arteries, stented with bare metal stents (BMS, 3.5x15 mm) and excised at various time points, were kindly provided by Dr. Julian Gunn (Senior Lecturer, University of Sheffield).

These vessels were prepared for OPT analysis using the standard technique described in section 2.8. The scanning was done initially with white light and then with UV light.

Pilot data show that OPT can provide optimum images of both the metallic stent (white light imaging) and biological tissue (UV light imaging) which can be used to calculate 3-dimensional lesion volume (Figure 6.4). Comparison with standard histology to validate OPT measurements will help to establish this technique for routine use and thereby helping to improve analysis and reduce number of animals required to obtain statically valid results in this useful experimental model.

Figure 6.4: OPT appears to be a promising tool for *ex-vivo* assessment of in-stent restenosis



The left anterior descending (LAD) coronary artery from a juvenile Yorkshire white pig (weight~25kg) stented with a bare metal stents (BMS, 3.5x15 mm) was analysed with optical projection tomography (OPT). Scanning with white light (a) provided clear visualisation of metal stent (b), whereas scanning with UV light (c) enabled optimum visualisation of neointimal lesions (d) (indicated by arrow-head).

6.2.6 In-vivo assessment of vascular lesions in mice

In pre-clinical work, histological analysis of the cross-sections of vessels remains the gold standard for quantification of lesion size. However, histology has obvious limitations. It can only provide cross-sectional 2-dimensional assessment, is time consuming, requires culling of large number of animal at each time point and cannot be used for temporal assessment of lesion development in same animals. Optical Projection Tomography (OPT) can provide useful *ex-vivo* 3-dimensional analysis of these lesions, as demonstrated in chapter 4. However, it remains highly desirable to have a reliable and reproducible non-invasive imaging tool for *in-vivo* assessment of vascular lesions in longitudinal studies which can potentially increase the amount of information from each animal, increase statistical power and reduce the number of animal killed for each study. *In-vivo* imaging techniques used in pre-clinical work can also allow direct comparison with clinical data.

6.2.6.1 Magnetic Resonance Imaging

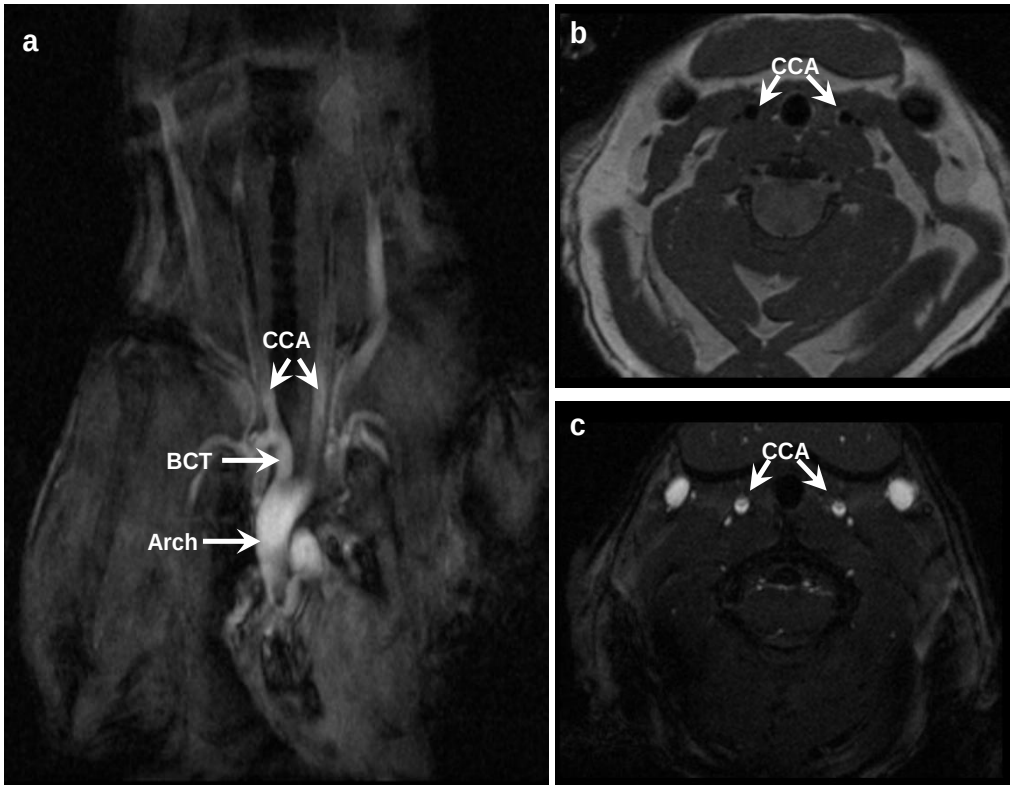
Magnetic resonance imaging (MRI) is a commonly used technique in clinical practice and increasingly employed in cardiovascular medicine for detailed structural and functional analyses [Rehwald et al., 2007]. It has the advantage of being free from ionising radiation; however, it is expensive and time consuming. It has been used in pre-clinical work to study atherosclerotic plaques in the murine aortic arch [Trogan et al., 2004;Weinreb et al., 2007]. However, there are limited data on feasibility of scanning the brachiocephalic trunk and carotid arteries (which are used more commonly for studying vascular lesions) particularly without use of contrast agents. A pilot study was, therefore, conducted to evaluate feasibility of MRI scan for the assessment of vascular lesions in mice using the currently available MRI scanner at the Biomedical Research Facility, University of Edinburgh.

MR imaging was performed with a Varian 7-Tesla scanner with 305mm bore system operating at a proton frequency of 300MHz. A gradient insert (internal diameter of 60mm) was capable of generating a maximum of 100 gauss/cm. Mice were anaesthetized with continuous inhaled isoflurane and were placed in a 39mm (inner

diameter) transmit/receive birdcage coil. Constant body temperature of 37ºC was maintained using a thermocouple/heater system. The aortic arch was identified in an approximately coronal section on a localizing sequence. Serial (0.5mm thick) transverse slices spanning from aortic arch to carotid bifurcation were acquired (117 x 117μm^2 in plane resolution) using a dark-blood (fat suppressed) fast spin-echo sequence. Repetition time (TR) and echo time (TE) for the proton density-weighted (PDW) images were 2000ms and 9ms, respectively. The total imaging time for this scan was ~20 minutes/mouse. The same area was then scanned using bright-blood gradient echo sequence with TR 30ms and TE 1.86ms. Sequential (0.5mm thick) images were acquired with field of view of 30 x 30mm, acquisition matrix 256 x 256 and flip angle 30°. The total imaging time for this gradient echo scan was ~30 minutes/mouse. On average, it took about 2hr to scan one mouse.

MRI provided images with a recognisable aortic arch, BCT, common carotid arteries and the carotid bifurcation (Figure 6.5a). However, resolution was not adequate to provide reliable measurements of vessel wall and lesions (Figure 6.5 b & c). It was concluded that MRI scanning using 7-Tesla scanner and the adopted protocol is suboptimal for imaging carotid vessels in mice. However, it may be possible to scan mice arteries using a scanner with higher magnetic flux density or different protocol with longer scanning time and providing higher resolution images.

Figure 6.5: MRI scan is of limited value for assessment of atherosclerotic lesions in mice



Magnetic resonance images were acquired from an adult male apolipoprotein-E deficient mouse fed a western diet for 8 weeks (using Varian 7-Tesla scanner). Heart, aortic arch and major neck vessels could be distinguished in coronal sections (a). However, resolution of scans did not allow reliable measurements of wall thickness or plaques in cross-sectional images using either dark-blood (b) or white-blood (c) techniques.

6.2.6.2 High frequency ultrasound

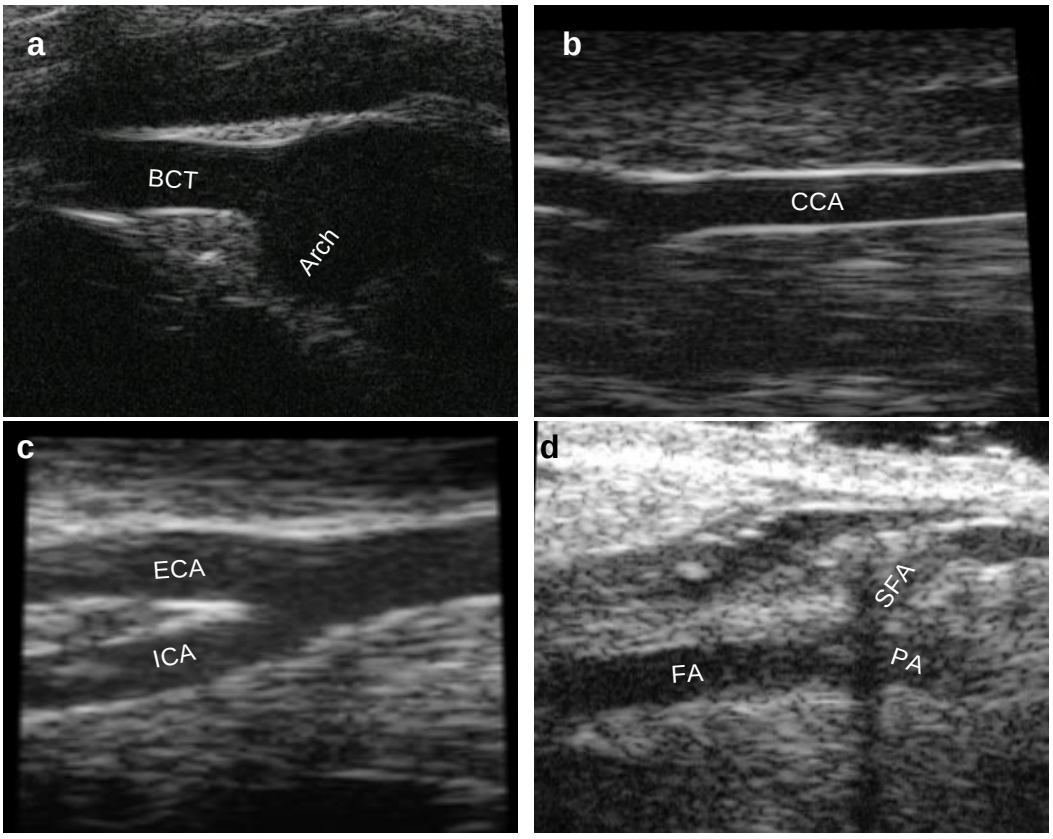
After rather disappointing results from MRI and due to the pressing need for having a technique for *in-vivo* assessment of vascular lesions, high resolution ultrasound using a Vevo-770 imaging system was tried. There are no published data to evaluate use of ultrasound for post-angioplasty lesion development in femoral arteries of mice, probably due to the concern that the lesions in these small calibre vessels may not be visualised with ultrasound. However, a small number of studies describe the use of ultrasound for evaluating advanced lesions in mouse aortic arch and common carotid arteries [Gan et al., 2007;Ni et al., 2008]. Thus, it remains to be established whether neointimal lesions in murine femoral arteries and mild to moderate atherosclerotic lesions in carotid arteries can be visualised with ultrasound.

A pilot study was conducted to explore the feasibility of high resolution ultrasound to image atherosclerotic and neointimal lesions. The Vevo-770 high resolution imaging system (VisualSonics, Canada) was used for ultrasound scanning. Mice were anaesthetised using inhaled isoflurane (4-5% for induction, 1-2% for maintenance). Hairs were chemically removed from the anterior thorax and neck, using a commercially-available depilatory cream (Veet, UK) and a generous amount of ultrasound transmission gel was applied.

For assessment of atherosclerotic lesions, a parasternal long-axis view to visualise the aortic arch and the origin of brachiocephalic trunk (BCT) and a right cervical longitudinal view to visualise the right common carotid artery, the carotid bifurcation, and the external and internal carotid arteries were obtained. For parasternal long-axis views, mice were in the left lateral position and the probe was positioned at anterior thorax with the notch pointing towards left shoulder of the animal. For right cervical longitudinal views, mice were lying supine and the probe was positioned with the notch pointing toward the chin of the animal. For assessment of neointimal lesions in femoral arteries, mice were lying flat with the left the hind limb pulled towards midline to align with vertebral column, and a soft cushion was placed under the left hip. 2D images of femoral arteries arising from iliac arteries and Doppler spectral display were obtained from the common femoral, superficial femoral and popliteal arteries.

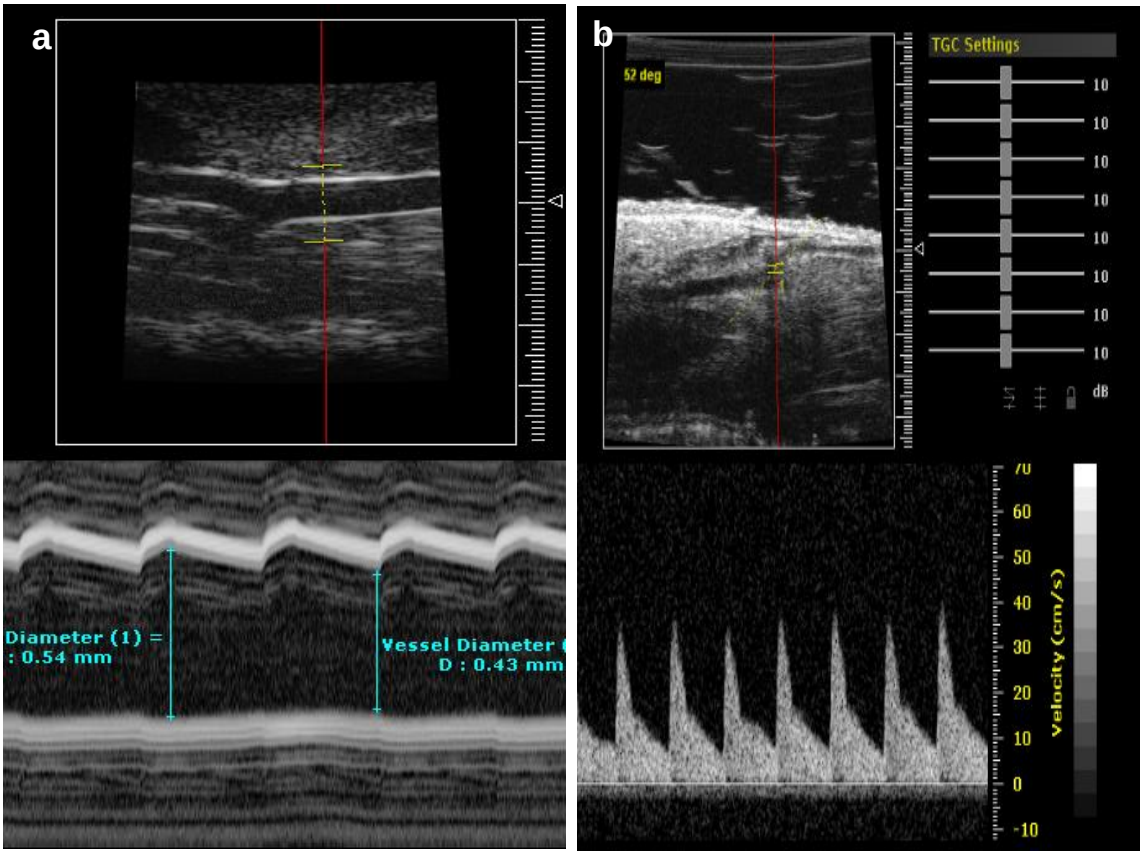
The pilot data suggest that susceptible arteries can be imaged (Figure 6.6) and ultrasound can provide reproducible measurements of vascular lumen, wall thickness and flow velocities (Figure 6.7). However, it remains to be seen how accurately the plaque size and associated changes in flow velocity can be determined. Furthermore, the Vevo-770 scanner is not equipped with colour Doppler, which could be very helpful to distinguish the lumen from the plaque in future studies. This scanner also lacks a linear array transducer which could have been useful to overcome the problem of getting a suitable angle of insonation for common carotid arteries (CCA) and BCT, as unsuitable angles of insonation can cause considerable measurement errors [Huo et al., 2008].

Figure 6.6: High frequency ultrasound can be used to image murine arteries *in vivo*



Ultrasound images of an adult apolipoprotein-E deficient mouse scanned using a Vevo-770 system shows that arteries of interest can be visualised. It was possible to image brachiocephalic trunk (BCT) originating from aortic arch (a), common carotid artery (CCA) with its bifurcation (b) and external/internal carotid arteries (ECA/ICA) (c), and femoral artery (FA) divining into popliteal artery (PA) and superficial femoral arteries (SFA) (d).

Figure 6.7: Ultrasound can be used to measure lumen size, wall thickness and flow velocities



Ultrasound images of an adult apolipoprotein-E deficient mouse scanned using a Vevo-770 system. Images illustrate that m-mode (a) can be used to assess lumen and intima-media thickness (IMT) and Doppler flow velocity (b) to detect hemodynamic significance of a vascular lesion.

6.3 Conclusion

The work described in this thesis aimed to investigate the role of intra-cellular glucocorticoid regulation (by 11β-HSD type 1 and 2) in vascular lesion (atherosclerosis and neointimal proliferation) development. The results presented in this thesis highlight the potential therapeutic application of selective 11β-HSD1 inhibition in reducing the size and vulnerability of atherosclerotic plaques. 11β-HSD1 inhibition also reduced neointimal proliferation in *ApoE*$^{-/-}$ mice, pointing towards a possible role in preventing post-angioplasty restenosis in high risk groups with "metabolic syndrome" phenotype. The data in this thesis also indicate that 11β-HSD2 has little, if any, role in modifying the development of neointimal lesions; however, eplerenone substantially reduced neointimal proliferation. This suggests that mineralocorticoid receptors are another potential target to prevent post-angioplasty restenosis, but that it is likely that glucocorticoids rather than aldosterone activate vascular mineralocorticoid receptors in this setting. These results improve our understanding of how intra-cellular glucocorticoid regulation influences vascular lesion development, identify potential therapeutic targets and pave the way for further mechanistic and translational studies.

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Ref Type: Abstract

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