Augmented Aortic Atherosclerosis in ApoE Deficient Mice with Targeted Overexpression of Urotensin-II Receptor

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Abstract

Urotensin-II (U-II) and its receptor UT are upregulated in the pathological setting of various cardiovascular diseases including atherosclerosis. However, their exact role in atherosclerosis remains to be determined. In the present study, we hypothesized that selective overexpression of UT in an SMC-specific fashion would increase atherosclerotic lesion formation in a hypercholesterolemic mouse model. The objectives were to demonstrate the role of UT in this mouse model of atherosclerosis, and to elucidate some of the mechanism involved in the process. We used four strains of mice; wildtype (WT), UT^+ (a transgenic strain expressing human UT driven by the α-SM22 promoter), ApoE knockout (ko), and UT^+/ApoE ko. All animals were fed a high-fat diet for 12 weeks. Western blot analysis revealed a significant increase in UT expression in UT^+ and ApoE ko mice ($P<0.05$). Serum cholesterol and triglyceride levels were significantly increased in ApoE ko and in UT^+/ApoE ko but not in UT^+ mice when compared to wild type mice ($P<0.0001$). Analysis of aortas showed a significant increase in atherosclerotic lesion in the UT^+, ApoE ko and UT^+/ApoE ko compared to WT mice ($P<0.05$). Oral administration of the UT receptor antagonist SB-657510A for 10 weeks in a group of ApoE ko mice fed a high fat diet resulted in a significant reduction of lesion ($P<0.001$). Immunohistochemistry revealed the presence of strong expression of UT and U-II proteins in the atheroma of UT^+, ApoE ko and UT^+/ApoE ko mice, particularly in foam cells. SB-657510A also significantly reduced ACAT-1 protein expression in the atherosclerotic lesion of ApoE ko mice ($P<0.05$). The present findings suggest that the use of UT receptor antagonists may reduce lesion formation through reduced foam cell

formation and lipid uptake, demonstrating an important role for UT in the pathogenesis of atherosclerosis.

Résumé

L'expression de l'urotensine-II (U-II) et celle de son récepteur (UT) sont régulées positivement dans diverses pathologies cardiovasculaires dont l'athérosclérose. Cependant, le rôle exact de ces protéines dans l'athérosclérose reste à déterminer. Dans la présente étude, nous avons présumé que la surexpression sélective du récepteur UT d'une mode SMC-spécifique augmenterait la formation athérosclérotique de lésion dans un modèle hypercholestérolémique de souris. Les objectifs étaient de démontrer le rôle de ce récepteur dans ce modèle de souris de l'athérosclérose, et d'élucider une partie du mécanisme impliquée dans le processus. Nous avons utilisé quatre souches de souris : sauvage (WT), transgénique exprimant l'UT humain sous le contrôle du promoteur α-SM22 (UT^+), knock-out pour le gène ApoE (ApoE ko), et knock-out pour les gènes UT et ApoE (UT^+/ApoE ko) Tous les animaux ont été soumis à un régime à haute teneur en graisses pendant 12 semaines. L'analyse par western blot a montré une augmentation significative de l'expression d'UT chez les souris UT^+ et ApoE ko ($P< 0.05$). Les niveaux de cholestérol et de triglycéride dans le sérum ont été sensiblement augmentés chez les souris ApoE ko et UT^+/ApoE ko comparativement aux souris WT alors qu'ils restent inchangés dans les souris UT^+ ($P< 0.0001$). L'analyse des aortes a montré une augmentation significative des lésions athérosclérotiques chez les souris UT^+, ApoE ko et UT^+/ApoE ko comparé aux souris WT ($P< 0.05$). L'administration par voie orale de l'antagoniste du récepteur UT, le SB-657510A, pendant 10 semaines dans un groupe de souris ApoE ko ayant suivi un régime à haute teneur en graisse, a eu comme conséquence une réduction significative de la lésion ($P< 0.001$). L'analyse en immunohistochimie a

révélé une forte expression des protéines UT et U-II dans l'athérome des souris UT^+, ApoE ko et UT^+/ApoE ko, en particulier dans les cellules spumeuses. Le SB-657510A a également réduit de manière significative l'expression de la protéine ACAT-1 dans la lésion athérosclérotique des souris ApoE ko ($P< 0.05$). Les résultats actuels démontrent que l'utilisation d'antagonistes du récepteur UT peut réduire les lésions athérosclérotiques en diminuant la formation des cellules spumeuses et par la prise de lipides. Ceci soutient que l'UT joue un rôle important dans la pathogénie de l'athérosclérose.

INTRODUCTION AND LITTERATURE REVIEW

Introduction

The morbidity and mortality rate of cardiovascular diseases (CVD) in the Western population is steadily increasing and has a considerable financial burden. Indeed, ischemic heart disease and cerebrovascular disease remain important causes of cardiovascular death. At present, about one-third of the total deaths worldwide are instigated by a cardiovascular cause of which, 43% result from coronary heart disease and 32% from stroke (World Health Organization 2003). In total, the cardiovascular deaths reach up to 16.6 million and the number of persons with these afflictions is expected to increase over the subsequent years (World Health Organization 2003).

While cardiovascular disease refers to many different types of heart or blood vessel problems, it's used most often to describe damages caused by atherosclerosis. Atherosclerosis, the primary cause of ischemic cardiomyopathy and ultimately the major contributing factor to death in the Western world today is obviously an enormous burden on society.

Numerous vasoactive factors have been implicated in the pathogenesis of atherosclerosis. The latest factor is an 11 amino acid cyclic peptide that binds specifically and irreversibly to the orphan receptor GPR14, now known as UT receptor (Ames et al., 1999). Both, U-II and UT are expressed in endothelial and vascular smooth muscle cells (Ames et al., 1999; Douglas et al., 2000). Pharmacological studies have shown a potential role for the urotensin system in modulating vascular function and morphology in normal and diseased conditions (Thanassoulis et al., 2004; Tolle et al., 2007). For example, a

vasoconstrictive and vasodilatory role has been attributed to U-II depending on the anatomic location and species studied (Thanassoulis et al., 2004; Tolle et al., 2007). U-II has also been shown to induce vascular smooth muscle cell growth alone and in concert with oxidized LDL (Watanabe 2001, Watanabe 2006, Sauzeau 2001). In addition, U-II has been shown to increase foam cell formation and enhances lipase activity (Watanabe 2005, Sheridan 1987). More recently, U-II has been shown to act as a chemotaxic factor for monocytes (Segain et al., 2007). U-II also induces ROS formation by inducing NADPH activity and plasminogen activator inhibitor-1 (PAI-1) expression (Djordjevic et al., 2005).

Bousette et al. 2003 have previously demonstrated increased expression of U-II and UT in atherosclerotic human carotid arteries and aortas, and showed that while U-II was primarily expressed in lymphocytes, UT was mainly present in macrophages. Increased expression of U-II and UT was also shown in human coronary atherosclerosis (Hassan 2005, Maguire 2004). A recent report has shown that UT mRNA expression and U-II binding are elevated in aortas of ApoE knockout mice (Wang et al., 2006). Despite all the above-mentioned studies, the exact role of the urotensin system in atherosclerosis remains to be elucidated.

Therefore, designs of therapeutic interventions have become a research priority for the past decades in an effort to stop or possibly delay the occurrence of CVD. The following review will summarize evidence pertaining to the role of U-II in vascular

remodeling, and the underlying cellular and molecular mechanisms suggested to date will be discussed.

Literature review

1. Development of Atherosclerosis

Atherosclerosis is a vascular disease that affects medium-sized and large conducting arteries. It is the most important and most common type of arteriosclerosis, a general term for several diseases in which the wall of an artery becomes thicker and less elastic.

A complex interaction exists between the significant cellular elements, including the endothelial cells, the smooth muscle cells, the platelets, as well as the leucocytes, which contribute to atherogenesis (Boudi et al. 2006). There are also complex and interrelated biological processes such as cellular inflammation, smooth muscle cell migration and proliferation, vasomotor function, the thrombogenicity of the blood vessel wall, the state of activation of the coagulation cascade and finally, the fibrinolytic system which have potentially serious pathologic implications in the atherosclerotic lesion formation (Boudi et al. 2006).

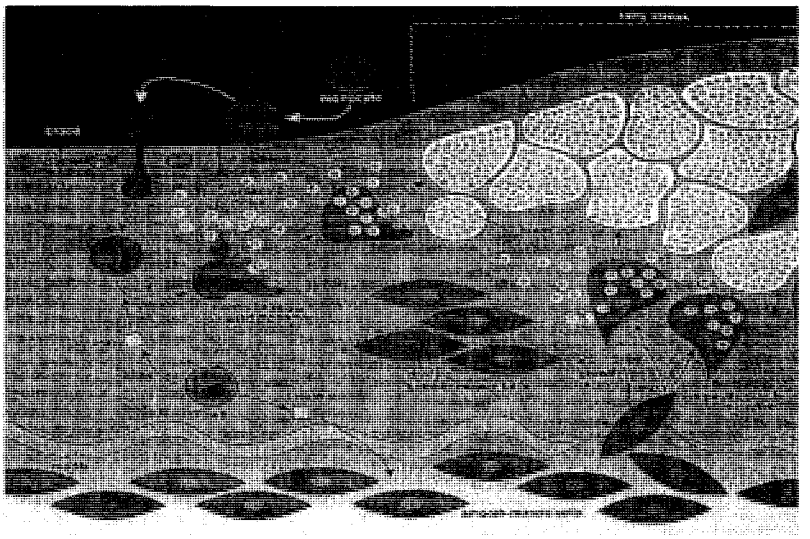
The lesions of atherosclerosis do not occur in a random fashion. Atheromas may be spread throughout medium-sized and large arteries, but they normally form at sites of curvature, such as the aortic arch, and branch points as in the carotid artery – presumably because the constant turbulent blood flow at these areas injures the artery's wall, making these areas more susceptible to atheroma formation (Stary et al., 1992). Coronary arteries are also frequently afflicted with the development of atherosclerotic lesions. Lesions typically begin in early adolescence, and often progress as people age, yet remain

clinically asymptomatic and undetectable. It is only when the lesion significantly accumulates that it eventually leads to plaque ruptures and stenosis (narrowing) of the artery, formation of a thrombus, disturbed blood flow and, therefore, an insufficient blood supply to the organ. Ultimately, death of the tissue fed by the artery or an infarction results

Atherosclerosis begins when monocytes are activated and move out of the bloodstream into the wall of an artery with endothelial injury; upon entry into the intima, these now lipoproteins are believed to bind to negatively charged proteoglycans (Camejo et al., 1993). Probable causes of endothelial injury include oxidized low-density lipoprotein (LDL) cholesterol; infectious agents; toxins, including the byproducts of cigarette smoking; hyperglycemia; and hyperhomocystinemia. These infiltrated macrophages act as scavenger cells – because of their specific lipoprotein receptors, including the LDL receptor and the LDL like receptor protein (LRP) which are not down-regulated by lipids (Hurt et al., 1990) – taking up LDL cholesterol and other fatty materials and hence, with time, becoming lipid filled foam cells. In fact, dyslipidemia or elevated serum levels of LDL cholesterol overwhelm the antioxidant properties of the healthy endothelium consequently inducing a dysfunctional endothelial metabolism. Activated macrophages produce numerous factors that induce the secretion of chemokines, which attract monocytes (Okada et al., 1998). With time, these foam cells accumulate and form patchy deposits – or atheromas – in the lining of the artery's wall, causing a thickening of the intimal wall, histologically described as fatty streaks.

Foam cell death and release of intracellular lipids ultimately occur with consistent accumulation of intimal lipids. Coalescence of small pools of the now extracellular lipids takes place as fibrosis of the overlying intima begins. This response persists until a fibrous cap, called the fibroatheroma, covers a necrotic fatty core. (Figure 1)

Figure 1. Schematic of atherosclerotic plaque development characteristically associated with complex interretaled biological processes, including inflammation, smooth muscle cell migration and proliferation (Boudi et al 2006).



2. Urotensin-II

2.1 U-II Synthesis and Structure

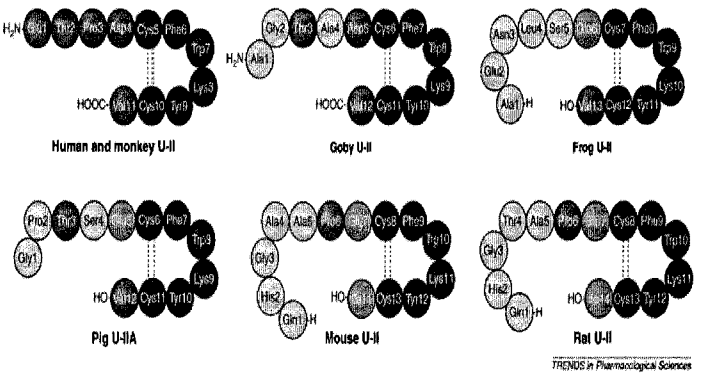
Urotensin-II (U-II), a cyclic undecapeptide (H-Glu-Thr-Pro-Asp-c{Cys-Phe-Trp-Lys Tyr-Cys}-Val-OH) with an intramolecular ring structure connected by two cysteine residues, originally isolated from the caudal neurosecretory system of the fish has been identified as a vasoactive peptide in mammals (Pearson et al., 1980). The U-II gene is located on human chromosome 1p36–p32 (Suzuki et al., 2004).

In humans, U-II is derived from pre-pro U-II of both 124 and 139 amino acid residues which differ in their amino acid terminal (Russel et al. 2004). These two alternative splicing variants are cleaved at a dibasic site to form an 11 amino acid mature peptide (Coulouarn et al., 1998). Several studies though indicate that there are many mono/polybasic cleavage sites suggesting U-II as a peptide of varying lengths (11, 16 and/or 19 amino acids long), although only an 11 amino acid peptide has been observed (Chartrel et al., 2004). In mice, U-II is synthesized from a gene with four exons and three introns that spans 4799 nucleotides, from which a 371 nucleotide mRNA transcript is spliced, which encodes a 123 amino acid prepropeptide (Elshourbagy et al., 2002). The latter is ultimately cleaved to form a mature 14 amino acid peptide in mice.

The amino terminal region of the proprotein greatly differs among species, yet all species have a conserved cyclic hexapeptide at the C-terminus of the gene – Cys-Phe-

Trp-Lys Tyr-Cys – where disulphide bridge connects the two cysteine resides. (Figure 2) The enzyme responsible for the maturation of human U-II from the prepropeptide has yet to be elucidated although it has been proven that both calcium-dependent furin and aprotonin-sensitive trypsin have urotensin converting enzyme activity in cell based and plasma based assays, respectively (Russell et al., 2004).

Figure 2. Schematic of mature U-II protein from several species with the conserved cyclic hexapeptide in black (adapted from Douglas et al. 2004)



This U-II endogenous ligand binds to a 389 amino acid seven transmembrane G protein coupled receptor, termed UT (Ames et al., 1999).

2.2 Physiological U-II expression

In mice and rats, U-II is mostly expressed in the central nervous system (CNS), particularly in the cerebellum, spinal cord and medulla oblongata (Coulouarn et al. 1998). Coulouarn et al. 1998 also found this peptide in the rat epididimis and mouse thymus. Alternatively, the mouse prepro-U-II mRNA is highly expressed in the heart, thoracic aorta, skeletal muscle, kidney, spleen, and testis (Elshourbagy et al., 2002).

In humans, U-II mRNA expression is strongest in spinal cord but is also apparent in other tissues such as the kidneys, small intestine, thymus, pituitary gland, adrenal gland, the stomach and liver (Coulouarn et al., 1998). In contrast, another study revealed that human prepro-U-II mRNA is more abundant in human kidney than in spinal cord and medulla oblongata (Nothacker et al., 1999). Immunocytochemistry indicated the localization of U-II-like immunoreactivity in human vascular and endocardial endothelium as well as in the renal epithelium (Maguire et al., 2004; Shenouda et al., 2002).

U-II is also found circulating in the plasma and may thus physiologically function through autocrine and paracrine mechanisms (Charles et al., 2005; Loirand et al., 2007; Matsushita et al., 2003; Richards et al., 2004; Yoshimoto et al., 2004). Interestingly, in normal anesthetized sheep, there were arteriovenous gradients in U-II levels present in organs such as the heart, the liver and the kidneys, proving that these organs are sources of circulating U-II (Charles et al., 2005). In addition, Matsushita et al. noticed, while

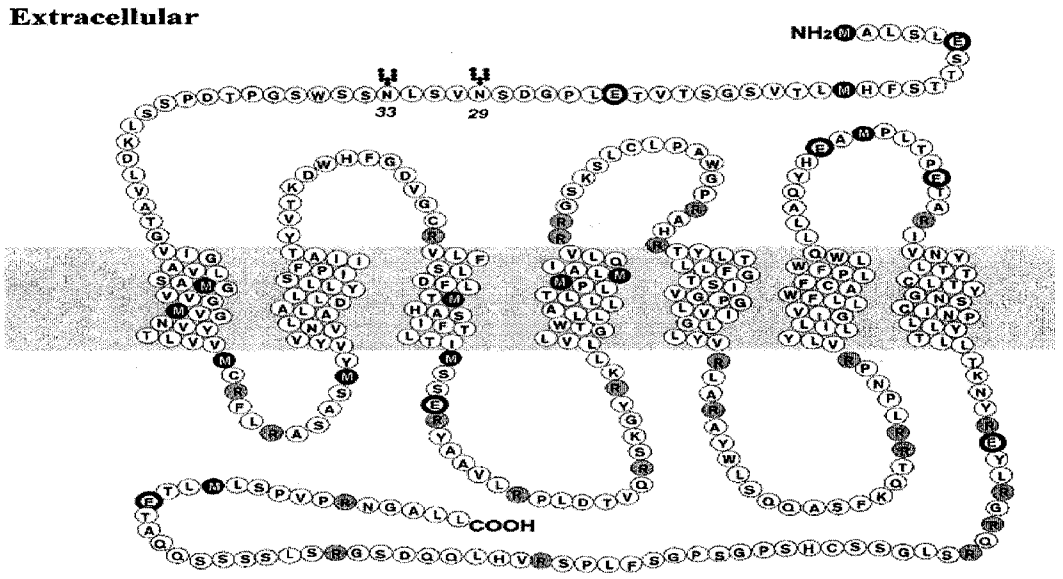
studying the effects of U-II on DNA synthesis in a porcine renal epithelial cell line (LLCPK1), that neutralization of endogenous UII by anti-U-II antibody, but not nonimmune serum, significantly suppressed DNA synthesis via activation of both protein kinase C and ERK1/2 pathways as well as Ca^{2+} influx via voltage-dependent Ca^{2+} channels.

3. Urotensin-II receptor (UT)

UT protein is synthesized from an intronless gene with an open reading frame of either 1158 or 1167 nucleotides (Elshourbagy et al. 1999). UT is made up of 386 and 389 amino acids in mouse and man, respectively (Figure 3) (Elshourbagy et al., 1999). The slight difference in the structure of the UT receptor in mouse and human appears to give rise to a significant pattern of expression and pharmacologic profile in these two species as will be elucidated in the subsequent section of the background. In addition to its predicted approximate 42 KDa molecular weight, the total molecular weight of UT adds up to 60 KDa due to its numerous potential glycosylation sites (Boucard et al., 2003).

UT is known to be the cognate receptor for U-II (Nothacker et al., 1999; Mori et al., 1999; Liu et al., 1999). In mice, UT prepro-mRNA presents predominant abundance of expression in the heart followed by a less significant expression in the thoracic aorta, bladder, and pancreas (Elsourbagy et al., 2002).

Figure 3. Schematic of UT receptor



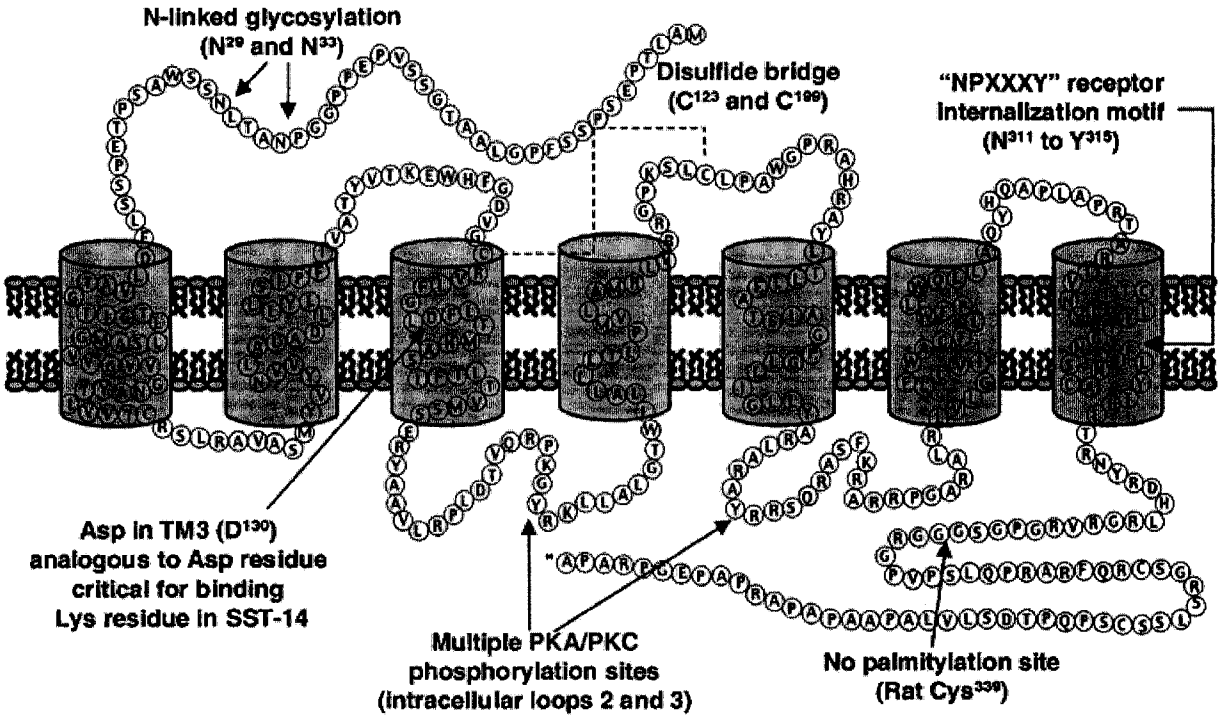
Human urotensin receptor (UT receptor) displays its greatest expression in the peripheral vasculature, heart, and kidney (Matsushita et al., 2001; Ames et al., 1999) although it is also found in other tissues, notably the central nervous system (Jegou et al., 2006). Using RT-PCR, Ames et al. 1999 revealed expression of the receptor in human cardiac and aortic tissue, as well as in endothelial cells and smooth muscle cells from coronary arteries. While some argue that UT is predominantly found in the heart and vasculature, others such as Matsushita et al., 2001 describe the UT mRNA expression that is equally expressed in both human cardiovascular and renal tissues.

4. U-II/UT signaling

Interestingly, biological activity found in the cyclic hexapeptide is maintained in all U-II isopeptides originating from all species (Elshourbagy et al., 2002; Itoh et al., 1988). In fact, the side chains of the residues Trp-7, Lys-8, and Tyr-9 of U-II are most important for receptor binding and activation (Flohr et al., 2002).

U-II binds to a seven transmembrane G-protein coupled receptor, which is part of the $G_{\alpha q}$ class of G-proteins. It binds by means of definite interactions with methionine residues at positions 184 and 185 of the fourth transmembrane domain (Boucard et al., 2003). Once activated, it activates in turn phospholipase C, which catalyzes the production of diacylglycerol, inducing certain isoforms of PKC, and inositol triphosphate, which releases intracellular calcium from the endoplasmic reticulum (Nothacker et al., 1999; Liu et al., 1999). In addition, several studies have provided evidence that U-II induces phosphoinositide hydrolysis (Saetrum-Opgaard et al., 2000), the activation of ERK ½ and protein kinase C (PKC) (Birker-Robaczewska et al., 2003; Tasaki et al., 2004; Shi et al., 2005; Chen et al., 2004 and Watanabe et al., 2001). U-II signaling is thought to also be mediated by phospholipase A_2 dependent arachidonic acid production (Mori et al., 1999). Ligand release, receptor desensitization, and/or receptor internalization including clathrin-coated pit endocytosis are mechanims by which termination of the ligand/receptor mediated signaling can occur (Camarda et al. 2002; Giebing et al. 2005).

Figure 4. Schematic representation of the UT receptor that selectively binds human urotensin-II. (Douglas et al. 2000)



5. Vasoactivity

Several recent reports have revealed the vasoconstrictive effect of U-II, which testifies to its potential significance in cardiovascular physiology and contribution to cardiovascular diseases. Its vasoactivity differs among the species and the anatomical location of its vasculature. It is also endothelial cell dependent.

It was demonstrated in the rat using an aortic bio-assay that doses as low as 0.1-0.5nM of U-II induce potent vasorelaxation dependent on endothelial function in coronary arteries while doses as high as 1-10nM induce vasoconstriction (Bottrill et al., 2000; Gibson et al., 1987). Since then, numerous studies predominantly done on rats have been pursued in describing U-II vasoactivity. This activity is mediated by the formation of endogenous nitric oxide (NO), prostacyclin (PGI2), prostaglandin E2, or endothelium-derived hyperpolarizing factor (EDHF) (Gray et al., 2001; Johns et al. 2004; Maguire et al., 2002). Smooth muscle contraction though is suggested to occur in the absence of endothelial cells and upon the inhibition of the release of the dilator factors mentioned above. Indeed, when administering nitro-L-arginine methyl ester, an inhibitor of endothelial nitric oxide synthase in addition to U-II, there was persistent vasoconstriction in the coronary vasculature (Gray et al., 2001). Thus, the biphasic vasoactivity is likely due to the endothelial effects. Interestingly, Lim et al. found that administration of U-II to patients with cardiac heart failure (CHF) and normal controls caused microcirculatory vasoconstriction and vasodilation, respectively (Lim et al., 2004).

The signaling pathway by which vasoconstriction is thought to be mediated is the Ca^{2+}/calmodulin/myosin light chain kinase system (Tasaki et al. 2004). It was also established that U-II-induced aortic contraction was attenuated by inhibitors of PKC, p38MAPK, and ERK½. Complementary to these findings, Rossowski et al. 2002 also revealed inhibition of the U-II-induced aortic ring contractions by calcium channel blockers, as well as inhibitors of phospholipase C, PKC, Rho kinase, and tyrosine kinase. The RhoA and Rho-kinase system was also linked to U-II-induced contraction although it was only tested in cultured SMCs (Sauzeau et al., 2001).

Studies done on humans in regards to this peptide's vasoactive functionality illustrate a conflicting image. U-II exhibited potent vasodilatory activity on endothelium intact small pulmonary and abdominal arteries, although potent, yet low efficacious vasoconstriction in the human umbilical artery and vein (Stirrat et al., 2001; Camarda et al., 2002). Likewise, in isolated human coronary, mammary, and radial arteries in addition to saphenous and umbilical veins, U-II demonstrated more potent yet less efficacious vasoconstriction compared to endothelin-1 (Maguire et al., 2000).

Human in vivo studies also reveal inconsistency, where for instance, some evidence suggest brachial artery vasoconstriction (Bohm et al., 2002), yet others do not report any effect (Affolter et al., 2002; Wilkinson et al., 2002). Additionally, studies have shown that U-II induces vasodilation in the microcirculation of normal volunteers while it induces vasoconstriction in patients with either congestive heart failure or essential hypertension (Lim et al., 2004; Sondermeijer et al., 2005).

6. Urotensin-II and vascular remodeling

6.1 U-II and UT receptor expression

Heringlake et al. 2004 found that patients with CAD had significantly higher U-II plasma levels than normal patients and that the severity of the disease increased proportionally to the U-II plasma levels. Also, Hassan et al. 2005 showed that U-II protein and mRNA levels were abundantly expressed in arteries of patients with coronary atherosclerosis in comparison to healthy arteries. In parallel, immunohistochemistry analysis against U-II revealed similar results. In fact, there was immunoreactivity against macrophages and endothelial cells where the strongest expression was seen in endothelial cells of atherosclerotic arteries. Even though Maguire et al. 2004 showed significant expression of U-II in macrophages, U-II mRNA expression analysis proved that lymphocytes are the largest producers of U-II among the inflammatory cells (Bousette et al. 2004).

U-II and UT receptor expressions are regulated by inflammatory mediators and neurohumoral peptides. More specifically, tumor necrosis factor (TNF-α), interleukin-6 and 1β, and interferon-γ produced by activated T lymphocytes in the plaque significantly increase U-II and UT levels in monocytes (Birker-Robaczewska et al., 2003; Segain JP et al. 2007). Furthermore, in endothelial cells, U-II alters collagen synthesis and expression of matrix metalloproteinase (MMP)-1 through ERK½ dependent pathway (Wang et al., 2004).

As mentioned above, U-II expression is observed in endothelial cells, foam cells, and myointimal and medial vSMCs of atherosclerotic human coronary arteries (Douglas et al., 2002; Hassan et al., 2005). It was also reported that monocytes and macrophages are the major cell types expressing UT receptors with relatively little expression in foam cells, platelets and lymphocytes (Bousette et al., 2006).

In addition, UT receptors have been shown to be expressed in smooth muscle cells in human coronary arteries (Maguire et al., 2000). Katugampola et al. 2005 revealed though that there are no density differences of UT receptors in the vSMC between healthy and atherosclerotic coronary arteries. On the contrary, Bousette et al. 2004 found that UT receptor mRNA expression is significantly enhanced in abdominal aortic aneurysm and carotid endarterectomy tissue samples compared to normal vessels. Interestingly, Wang et al. 2005 also evaluated the UT mRNA expression levels in the aorta of ApoE knockout mice and found similar results. (Wang et al. 2005).

6.2 U-II and monocytes

Studies have validated the importance of chemokine production by endothelial and vSMC, labelling it as fundamental in recruiting monocytes or macrophages from the bloodstream into vessel walls (Bjorkbacka et al., Kunjathoor et al., 2004; Boring L et al.,1998; Okada et al., 1998; Ross et al., 1999). Indeed, U-II induces a phenomenon called monocyte chemotaxis provoked by the pre-treatment with IL-1β (Loirand et al. 2007). The signaling pathway considered related to U-II-mediated chemotaxis is the

RhoA/Rho kinase-signaling cascade where the UT receptor and actin cytoskeleton are thought to be involved (Segain et al., 2007; Loirand et al., 2006; Sauzeau et al., 2001; Watanabe et al., 2006). These observations thus clearly demonstrate the potential inflammatory role that the U-II system exerts in the progression of atherosclerosis.

6.3 U-II and its mitogenic effects

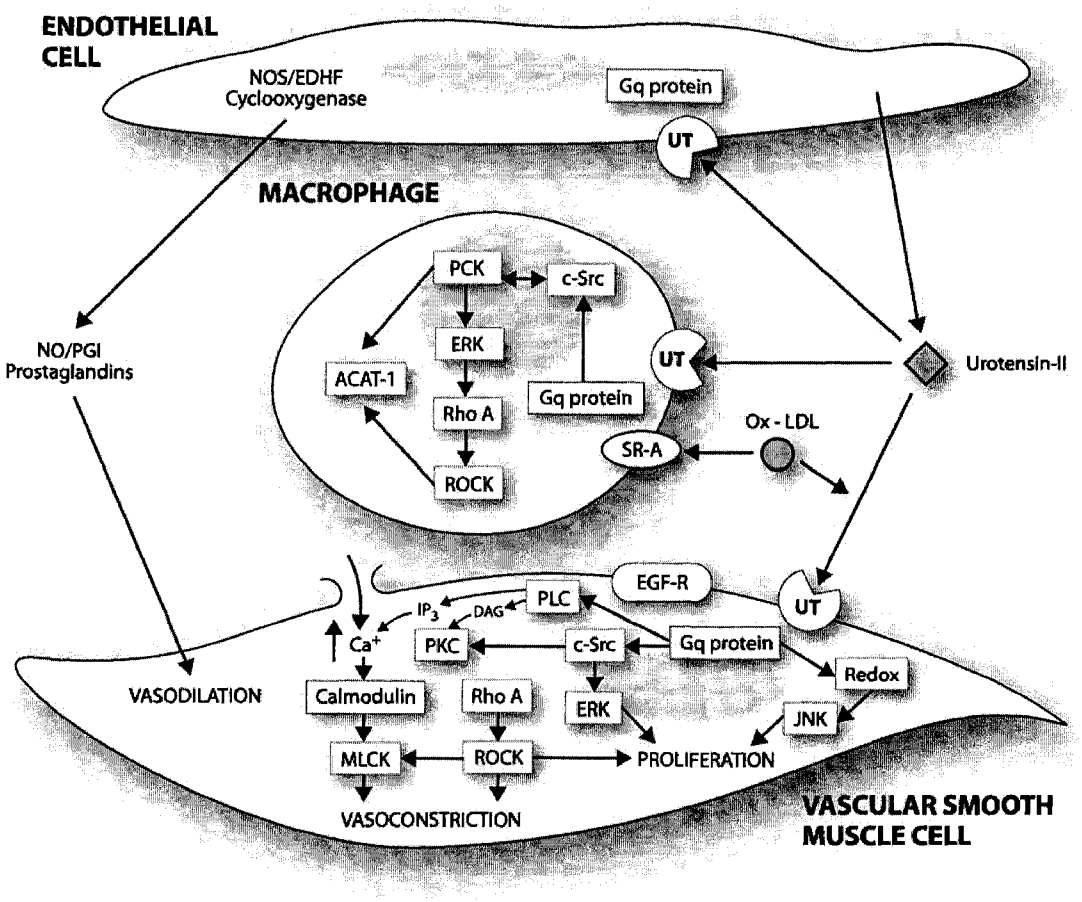
Another atherosclerotic remodeling process, which occurs in the early stages of plaque formation, is intimal thickening, one of the earliest stages of the atherosclerotic process. Indeed, it is these sites of increased intimal thickening that have the greatest predilection for atherosclerotic plaque formation. As such, vSMC proliferation is integral to the pathophysiology of atherosclerosis. Proliferation of vSMCs and endothelial cells, in addition to fibroblast-mediated collagen deposition are well established as integral to its development. U-II is said to suppress cellular apoptosis via the ERK pathway (Figure 4) (Shi et al., 2006). Watanabe et al. 2006 demonstrated that U-II acts in synergy with mildly oxidized LDL (mox-LDL) inducing vSMC proliferation (Watanabe et al., 2001). OxLDL is an important factor known to induce foam cell formation, inflammatory cell chemotaxis and smooth muscle proliferation (Witztum et al., 2001). The intracellular signaling mechanisms underlying this synergistic interaction are dependent on the activation of the UT receptor/Gq protein/protein tyrosine kinase/PKC/ERK and RhoA-ROCK-related pathways by U-II along with simultaneous activation of the JNK pathway by mox-LDL (Figure 4) (Peng et al., 2000; Sauzeau et al., 2001; Watanabe et al., 2006). Interestingly, Bousette et al. 2006 recently showed in a rat model of carotid artery

stenosis that the use of a UT antagonist reduces myointimal thickening and increases lumen size.

Another established factor known to induce a significant mitogenic effect of U-II in vSMC is reactive oxygen species (ROS). In fact, NADPH oxidases have been described to largely contribute to ROS production within the vasculature led by U-II induction and activation of ERK½, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and Akt (Djordjevic et al., 2005). In turn, activity of NADPH oxidase induced by U-II not only contributes to vSMC proliferation but it also triggers augmentation of plasminogen activator inhibitor-1 (PAI-1) levels (Djordjevic et al., 2005). Indeed, Kohler et al. 2000 demonstrated that PAI-1 mRNA levels are greater in atherosclerotic arteries compared to normal arteries.

As for the endothelial cells, which also proliferate with the induction of U-II, the same mitogenic pathways are in effect as those found in vSMCs. Indeed, DNA synthesis and suppression of apoptosis via the ERK½ dependent pathway are in play (Shi et al., 2006). Furthermore, in human endothelial cells, U-II has been shown to increase mRNA and protein expression of collagen-1 and decrease that of MMP-1 (Wang et al., 2004). Thus, several lines of evidence suggest that U-II has a functional and central role in aggravating the development of atherosclerosis.

Figure 5. The mechanism of U-II-induced actions in endothelial cells, macrophages and vascular smooth muscle cells of atherosclerotic vessels. UT, urotensin-II receptor; EGF-R, epidermal growth factor receptor; ACAT-1, acyl-coenzyme A:cholesterol acyltransferase-1; ox-LDL, oxidized low-density lipoprotein; SRA, scavenger receptor class A; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; PLC, phospholipase C; ROCK, Rho kinase; IP3, inositol-1,4,5- trisphosphate; DAG, diacylglycerol; MLCK, myosin light chain kinase; NO, nitric oxide; PGI2, prostacyclin. (Papadopoulos et al. 2007)



7. Rationale

U-II has been shown to exert vasoconstrictive and vasodilatory effects on blood vessels in vivo (Ames et al. 1999). Also, several studies have demonstrated a mitogenic effect for U-II on vSMC and endothelial cells (Djordjevic et al., 2005, Shi et al., 2006). Patients with coronary atherosclerosis have augmented U-II plasma levels (Heringlake et al. 2004). In addition, U-II and UT are found in abundance in atherosclerotic arteries of humans in aortas of hypercholesterolemic mice, a well-established atherosclerotic mouse model (Wang et al. 2006). In addition, U-II acts synergistically with oxidized low-density lipoprotein, a factor known to cause proliferation of vSMC (Watanabe et al. 2001). Furthermore, U-II increases foam cell formation and lipase activity (Watanabe et al. 2006). Therefore, there is significant evidence to support a role for U-II in the pathogenesis and progression of atherosclerosis.

8. Hypothesis and Objectives

Based on the well-known effects of U-II on the vasculature mentioned above, we hypothesized that selective overexpression of UT in a SMC-specific fashion would increase atherosclerotic lesion formation in a hypercholesterolemic mouse model.

Therefore, the objectives of the following study were to demonstrate the effects of a SMC-specific-UT overexpression mouse model of atherosclerosis and to elucidate a mechanistic role for this urotensin-II system using a selective U-II receptor antagonist.

METHODS AND RESULTS

Methods

1. Animals

All animal studies were performed in accordance to the guidelines described in the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. Throughout the study, 4 strains including WT (C57BL6/J; n=40), hUT^{+} (a transgenic strain expressing human UT driven by the α-SM22 promoter; n=32), ApoE ko (n=41), and hUT^{+}/ApoE ko (n=36) were used. The hUT^{+} cDNA bearing construct contains the SM22α promoter, which consists of an MscI restriction fragment containing 2086 of 5' flanking region, the 64bp of the first exon and 1253bp of the first intron of the SM22α gene. In addition, a lacZ ORF is linked to the hUT cDNA at the 3' end, by an IRES (internal ribosome entry site) element and capped with a SV40 poly adenylation element (17). This ensures co-expression of UT and β-galactosidase. The model was generated by injection of the construct into the pronuclei of fertilised eggs from mouse strain C57Bl6J. For the study offspring from WT and hUT^{+} crosses were utilized. Transgenics were always crossed with wild types to avoid doubling of the transgene in offspring.

hUT^{+}/ApoE $^{-/-}$ mice were generated by crossing hUT^{+} mice with ApoE $^{-/-}$, which initially produced hUT^{+}/ ApoE $^{-/+}$ and hUT^{-}/ApoE $^{-/+}$ mice. hUT^{+}/ ApoE $^{-/+}$ mice were then crossed again with ApoE $^{-/-}$ mice until ApoE knockout homozygosity was attained. hUT^{+}/ ApoE $^{-/-}$ were then crossed with ApoE $^{-/-}$ and offspring were utilized for the study.

Again male and female transgenic mice were never crossed so as to maintain single copies of the transgene in offspring. Genotypes were verified by PCR and gel electrophoresis. The ApoE ko mice were ordered from Taconic (B6.129P2-*Apoe*^{tm1Unc} N11).

2. Study design

Mice were bred and allowed to mature to 6 weeks of age at which point male mice were weighed and put on a high fat diet for a period of 12 weeks. The high fat diet (TD-88137, Harlan Teklad) consisted of 42% calories from fat with 0.15% cholesterol content (18).

A further group of male ApoE KO mice (n=26, 6 weeks old) was placed on the same high fat diet and divided into two subgroups in a randomized fashion to receive either a UT receptor antagonist, SB-657510A (30 mg/kg/day; n=13) or drug vehicle (0.1% methylcellulose; n=13) administered by gavage for 10 weeks. Food intake and body weights were measured daily.

SB-657510A is an arylsulfonamide UT antagonist (2,6-dichloro-N-(4-chloro-3-{[2-(dimethylamino)ethyl]oxy}phenyl)-4-(trifluoromethyl benzenesulfonamide). It was synthesized at GlaxoSmithKline, King of Prussia, PA (19). The Ki (binding affinity) is 39nM at rat UT and 14nM at human UT in recombinant systems. The pA2 for $Ca2^{+}$-

mobilization is 7.47 (34nM) in the rat and 7.49 (32nM) in the human UT recombinant systems. The pA2 for inhibition of aortic contraction is 7.25 (56nM) in the rat. A bolus I.V. dose of 10mg/kg bolus of SB-657510A prevented U-II-induced pressor response in the anesthetized monkey (unpublished data).

3. Tissue collection

At the end of the study period, mice were fasted for 4 hours, weighed, and then sacrificed by exsanguination under anesthesia. The heart, lungs and aorta were then harvested and either fixed in 10% formalin or snap frozen in liquid nitrogen. Blood serum was also collected and stored at -80 °C.

4. Measurement of serum cholesterol and triglyceride

Fasting blood samples were harvested at the time of sacrifice. Serum was then collected and stored at -80°C. The serum samples were then analyzed for serum total cholesterol and triglyceride using cholesterol (BioVision, CA, USA) and triglyceride (Sigma, Missouri, USA) assay kits according to the manufacturer's instructions, as described below.

4a. Serum total cholesterol

Standard curve preparations were done first. For the colorimetric assay, we first diluted the cholesterol standard to 0.5 µg/µl by adding 20 µl of the cholesterol standard to 180 µl of cholesterol reaction buffer, and then mixed them well. Next, we added 0, 4, 8, 12, 16, 20 µl into each well individually and adjusted the volume to 50 µl/well with cholesterol reaction buffer to generate 0, 2, 4, 6, 8, 10 µg/well of the cholesterol standard. For the fluorometric assay, we diluted the cholesterol standard to 50 ng/µl by adding 10 µl of the cholesterol standard to 990 µl of cholesterol reaction buffer, and then mixed well adding 0, 4, 8, 12, 16, 20 µl into each well individually. Next, the volume was adjusted to 50 µl/well with cholesterol reaction buffer in order to generate 0, 0.2, 0.4, 0.6, 0.8, 1.0 µg/well of the cholesterol standard.

Test samples were prepared in 50µl/well with the cholesterol reaction buffer in a 96-well plate and the serum (0.5-2 µl/assay) was directly diluted in the cholesterol reaction buffer. The extract was spun for 5-10 minutes at top speed in the microcentrifuge. The organic phase which was the lower phase was what was collected and then initially, air dried at 50°C to remove chloroform and then vacuum-dried for 30mins to remove trace chloroform. The dried lipids were then dissolved in 200 µl of cholesterol reaction buffer by vortexing extensively for 5 minutes. 1- 50 µl of the extracted sample is used per assay.

The 50 µl reaction mix preparation contained: 44 µl cholesterol reaction buffer, 2 µl cholesterol probe, 2 µl enzyme mix, 2 µl cholesterol esterase which hydrolyzes cholesteryl ester into cholesterol. This reaction mix was then added to each well containing the cholesterol standard and incubated for 60 minutes at 37°C. With a microplate reader, we then measured O.D. 570nm for colorimetric assay or fluorescence at Ex/Em = 535/590 nm. The background reading was subtracted from sample readings. Finally, the cholesterol concentrations of the test samples were calculated based on the standard curve generated.

4b. Serum total triglyceride

The free glycerol reagent and the triglyceride reagent are prepared according to the preparation instructions and warmed up to assay temperature. 0.8 ml of the free glycerol reagent was pipetted into each cuvet (blank, standard, and sample). Subsequently, 10 µl of water, glycerol standard, and sample were added to cuvets. They were incubated for 5 minutes at 37 °C. Initial absorbance (IA) of blank, standard, and sample at 540 nm were read off the spectrophotometer and recorded versus water as the reference. 0.2 ml of the reconstituted triglyceride reagent were added to each cuvet, mixed, and continued incubation at 37 °C for 5 more minutes.

Final absorbance (FA) of blank, standard, and sample at 540 nm were then read and recorded versus water as the reference.

5. Lesion assessment

The descending thoracic aorta and abdominal aorta (up to bifurcation of common iliac arteries) were removed, cleaned of all adventitial fat and extraneous tissue, split open longitudinally, and stained with Sudan IV to visualize the extent of atherosclerosis. Quantification of the percentage of aortic surface covered by atheroma was performed with computer-assisted planimetry of the Sudan IV-positive areas. Once these ratios were determined, the slides were decoded and the cases were grouped accordingly. The interobserver and intraobserver variabilities of these measurements were <1.5% and <0.5%, respectively.

6. Histological analysis

All hearts were sectioned between the atriums and ventricles. Serial paraffin 5-μm-thick sections (every fifth section from the middle of the ventricle until the appearance of the aortic valve and every second section from the appearance to the disappearance of the aortic valve leaflets) were collected for histological, morphological, and immunohistochemical analyses on poly-D-lysine-coated slides, and counterstained with hematoxylin and eosin.

7. Immunohistochemistry

Immunohistochemical staining for UII, UT, CD68, α-SMC-actin, β-galactosidase and acyl-coenzymeA:cholesterol acyltransferase-1 (ACAT-1) was done using the avidin-biotin peroxidase method as described below.

Paraffin sections were dewaxed in toluene for 20 min, rehydrated in alcohol (100%, 90%, 70%, and 50%) and washed in PBS for 5min. Following this, sections were incubated with Triton-X 100 (0.2%) for 30 min. After three washes with PBS, they were washed with H_2O_2 to block endogenous peroxidase activity and incubated with 30% normal goat serum for 30 min and then with the primary rabbit anti-sera for 18 h at 4 °C. Finally, sections were incubated with biotinylated IgG (1:200) for 45 min and after three washes with phosphate buffered saline (PBS) sections were stained with an immunoperoxidase technique according to the manufacturer's instructions (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) for 45 min at room temperature. The sections were developed using diaminobenzidine and hydrogen peroxide and then counterstained with hematoxylin, dehydrated, cleared and finally glass cover slips were placed on top. Negative control sections included immunoabsorption of the primary antisera with their respective antigens, and the use of non-immune serum in place of the primary antibody.

Sections were then incubated in 10% normal goat serum (NGS) for 30 minutes at room temperature after which they were incubated overnight at 4°C with the primary antibody. The sections were washed three times in PBS for five minutes following the cold storage, and incubated for 45 minutes with biotinylated goat-anti rabbit-IgG (1:200) at room temperature. They were then washed three times more in PBS for five minutes, and incubated with the avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, U.S.A.) for 45 minutes at room temperature. Sections were then washed three times in PBS solution for five minutes. Following this, sections were incubated in 3'3 diaminobenzidene solution (0.025%) for 2 minutes for chromogenesis. Sections were covered using permount (Fischer Scientific, New Jersey, USA) and glass coverslips and were allowed to dry. Negative control sections were incubated with the antisera:antigens mixture or a normal serum only. The antibodies against UT were raised in rabbit using epitopes from the 1st extracellular, 1st intracellular, and 2nd extracellular loops.

8. Western blotting

Western blotting was performed with specific antibodies against rabbit polyclonal anti-mouse ACAT-1 (1/400) developed at Santa-Cruz Biotechnology, California and rabbit polyclonal anti-rat/human UT antibodies (1/400). Protein samples were extracted from aortic tissue using an extraction buffer containing 50mM Tris/HCl, pH 7.2; 150mM NaCl; 1% (v/v) Triton X-100; 1mM sodium orthovanadate; 50 mM sodium pyrophosphate; 100 mM sodium fluoride; and a complete protease inhibitor cocktail

tablet (Roche, Montreal, Canada; 1 tablet /50 ml buffer). The extracted protein was then snap frozen in liquid nitrogen and stored at –80°C. Protein concentrations were determined using the Bio-Rad modified Bradford protein assay. 50µg of protein were then diluted 1:2 with Laemmli sample buffer (Bio-Rad, CA, USA) and electrophoresed on a 6% or 10% SDS-PAGE gel at 120 volts (V) for 1 hour. Following this, proteins were transferred to a PVDF membrane at 100 V for 90 minutes. The membrane was then blocked with 5% skim milk powder in Tris-buffered saline (TTBS; 0.05M Tris, 0.15M NaCl; pH 7.6) with 0.1% Tween-20 for 90 minutes. This was followed by an overnight incubation with the primary antibody. The following day, the membrane was washed with TTBS and incubated with secondary antibody-conjugated to horse radish peroxidase for 60 minutes. The membrane was then washed in TTBS and incubated with Lumi-Light chemiluminescent substrate (Roche, Montreal, Canada) for 5 minutes and exposed on radiographic film. Protein bands were quantified using arbitrary units (AU) with the image analysis program, *Image ProPlus* (Media Cybernetics, CA. USA).

9. Statistical analyses

Multi-group comparisons were analyzed using one-way ANOVA with the Tukey post-hoc test. Direct two group comparisons were carried out using the student's t-Test. All statistical analyses were carried out using SPSS version 11.5. A P value of <0.05 was considered statistically significant. Data are presented as mean $\pm$ standard error.

Results

Lesion measurement:

Sudan IV staining revealed the presence of little to no atherosclerostic plaques over the aortas of WT mice. In contrast, there was clear atherosclerotic plaques on the aortas of UT^+, ApoE ko and UT^+/ApoE ko groups located mainly at the aortic root and occasionally at the iliac bifurication (Figure 2). Measurement of atherosclerotic lesion area showed a significant increase in the latter three groups compared to the WT group ($P<0.05$) (Figure 3). There was significantly greater lesion area in the aortas of UT^+/ApoE ko compared to the UT^+ group ($P<0.01$). Hematoxylin and eosin staining of paraffin sections at the aortic root showed the presence of extensive atherosclerotic lesions in ApoE ko and UT^+/ApoE ko mice. UT^+ mice exhibited less severe lesions while WT mice had no lesion (Figure 4).

Measurement of serum cholesterol and triglyceride:

There was no significant difference in the levels of total serum cholesterol and triglycerides between UT^+ and WT mice. In contrast, there was significantly greater serum cholesterol and triglycerides in ApoE ko and UT^+/ApoE ko when compared to the WT and UT^+ mice ($P<0.05$) (Figure 5).

Immunohistochemistry and Western blotting:

Immunostaining for UII was apparent over the endothelium and smooth muscle cells of WT animals, however it was abundantly evident in the atheroma of UT^+, ApoE ko and UT^+/ApoE ko mice (Figure 6). Only weak immunoreactivity for UT was observed over the endothelium and vascular smooth muscle cells of the WT aortas. In contrast, UT immunostaining was more evident over the smooth muscle cell layer of UT^+ mice. Similarly, there was abundant UT immunoreactivity in the endothelium, smooth muscle cells and atheroma in ApoE ko and UT^+/ApoE ko animals (Figure 6).

Protein measurement of Western blotting showed increased protein expression of UT in the aortas of UT^+ (2.1 ± 0.29 folds) and ApoE ko (2.5 ± 0.13 folds) mice compared to WT mice ($P<0.05$). These findings were further supported by the presence of strong immunostaining for β-galactosidase over the smooth muscle cell layer of UT^+ and UT^+/ApoE ko groups (Figure 7).

There was abundant expression of ACAT-1 immunoreactivity of in the aortas of ApoE ko and UT^+/ApoE ko groups, which was apparent mainly in macrophages and foam cells. A lesser immunoreactivity was observed over the smooth muscle cells of the media (Figure 7). Analysis of ACAT-1 protein expression using Western blotting showed an increase in the aortas of ApoE ko (2.36 ± 0.71 folds) and UT^+/ApoE ko (1.99 ± 0.28 folds) mice compared to WT and UT^+ mice ($P<0.05$) (Figure 8a,b).

Effect of SB-657510A on the development of atherosclerosis:

Treatment of ApoE knockout mice fed on high fat diet with SB-657510A for 10 weeks resulted in a significant reduction in atherosclerotic lesions (4.9±0.6) when compared with vehicle treatment (13.7±0.9) ($P<0.001$) (Figure 9). There was no significant difference in body weight or food intake between the two experimental groups. ACAT-1 protein expression was significantly decreased in the SB-657510A-treated compared to the vehicle-treated mice ($P<0.05$) (Figure 7). The number of ACAT-1 cells in the atheroma also showed a significant decrease in the SB-657510A (122.22±17.99) compared to the vehicle-treated mice (186.46±16.87) ($P<0.05$) (Figure 10).

Figures:

Figure 1: Western blot analysis of UT protein expression in mice aorta

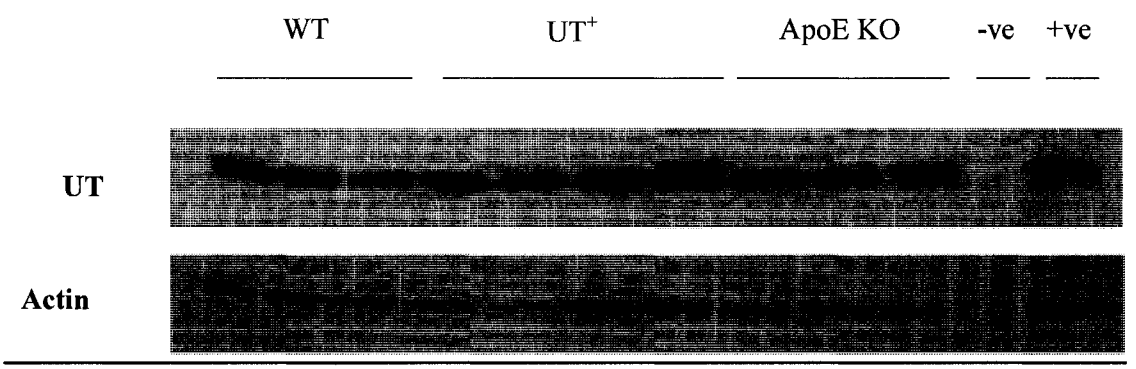


Figure 2. Sudan IV staining of aortas from WT (panel **A**), UT^+ (panel **B**), ApoE ko (panel **C**) and UT^+/ApoE ko (panel **D**) mice. Arrows indicate lesion area.

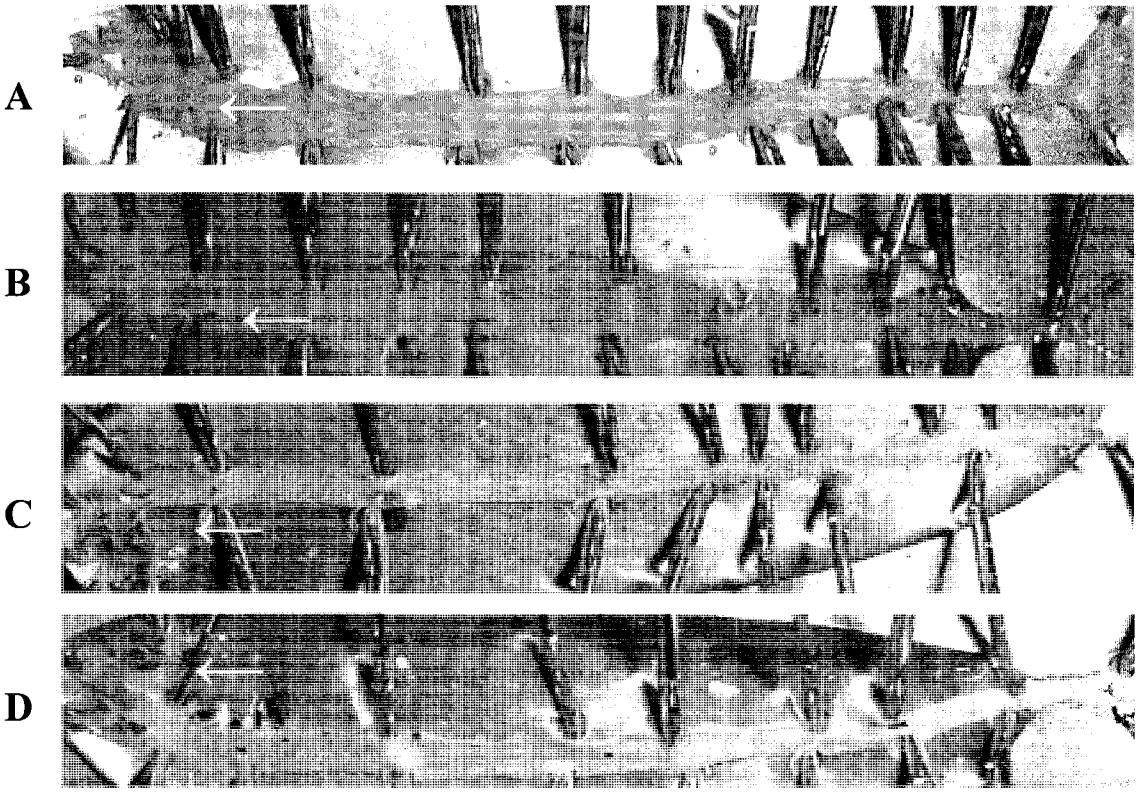


Figure 3. Graph representing atherosclerosis lesion ratio (percentage of aortic surface covered by atheroma) in WT ($N = 17$), UT+ ($N = 9$), ApoE- ($N = 7$) and UT+/ApoE- ($N = 10$) groups. * indicates P<0.001; all groups vs. WT; # indicates P<0.001; UT^+/ApoE ko vs. UT^+.

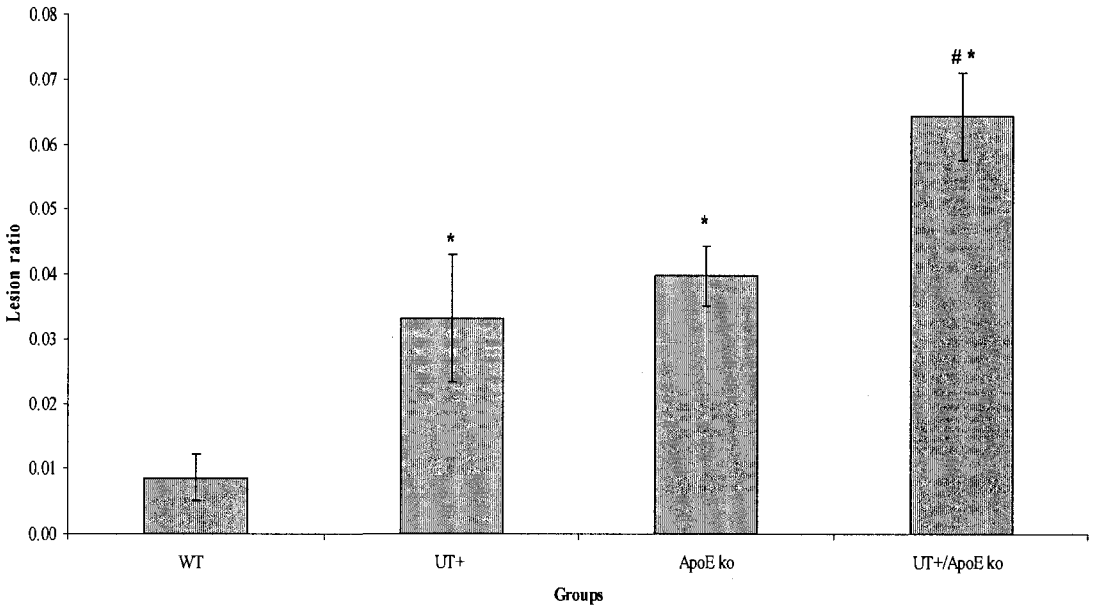


Figure 4. Hematoxylin and eosin stained paraffin sections from aortic roots of WT (**A**), UT^+ (**B**), ApoE ko (**C**), UT^+/ApoE ko (**D**) groups. Arrows indicate the area of atheroma. * indicates areas of sclerosis

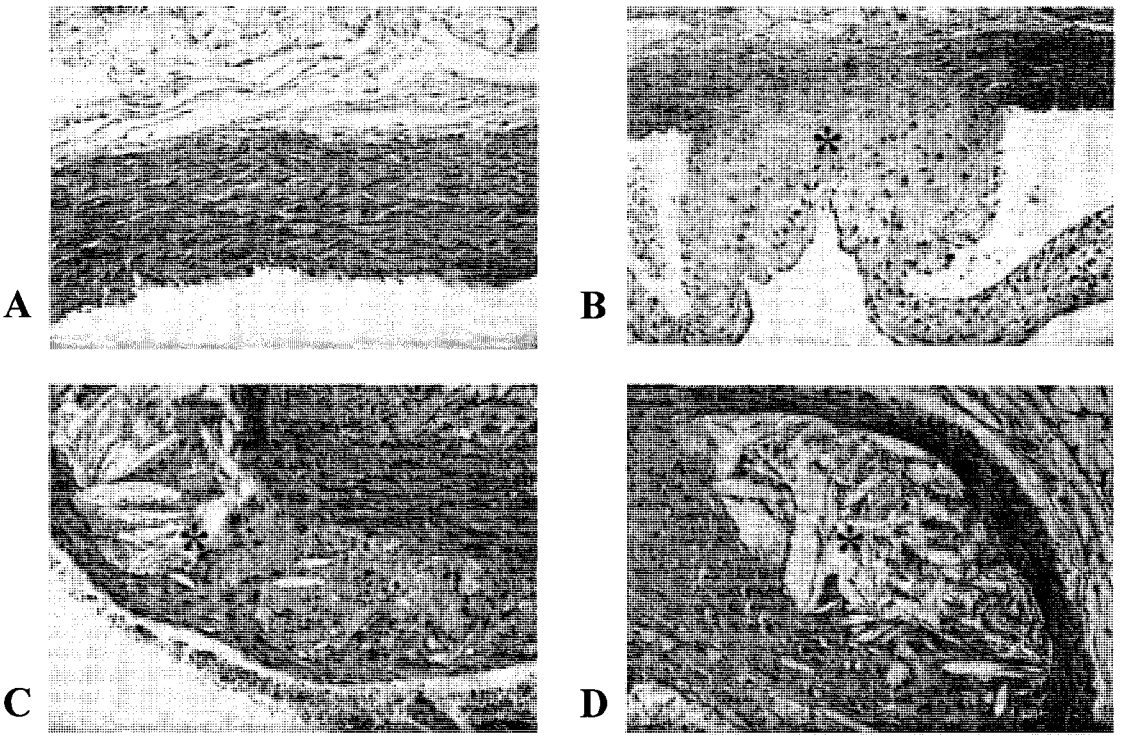


Figure 5. Serum total cholesterol in WT ($N = 9$), UT+($N = 8$), ApoE- ($N = 5$) and UT+/ApoE- ($N = 10$) groups and triglycerides in WT ($N = 12$), UT+($N = 7$), ApoE- ($N = 7$) and UT+/ApoE- ($N = 9$) groups. * indicates P<0.0001; ApoE ko and UT^+/ApoE ko vs. WT and UT^+.

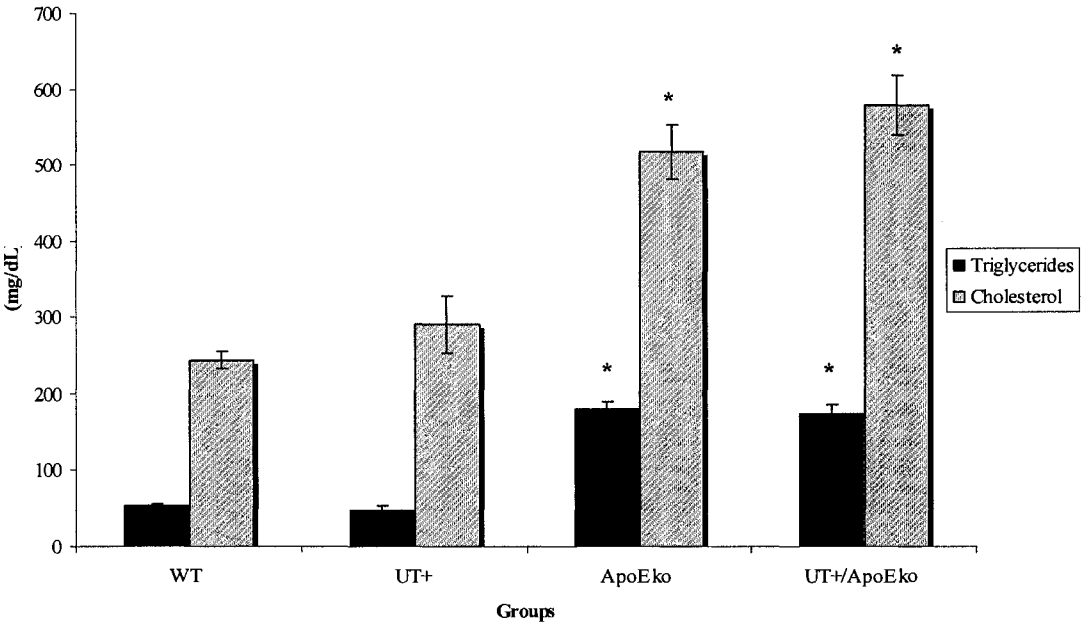


Figure 6. Immunohistochemical localization of UII and UT in the aortas of the WT (**A, E**), UT^{+} (**B, F**), ApoE ko (**C, G**), UT^{+}/ApoE ko (**D, H**) groups. Panels A-D show UII immunostaining; Panels E-H show UT immunostaining. * indicates areas of staining

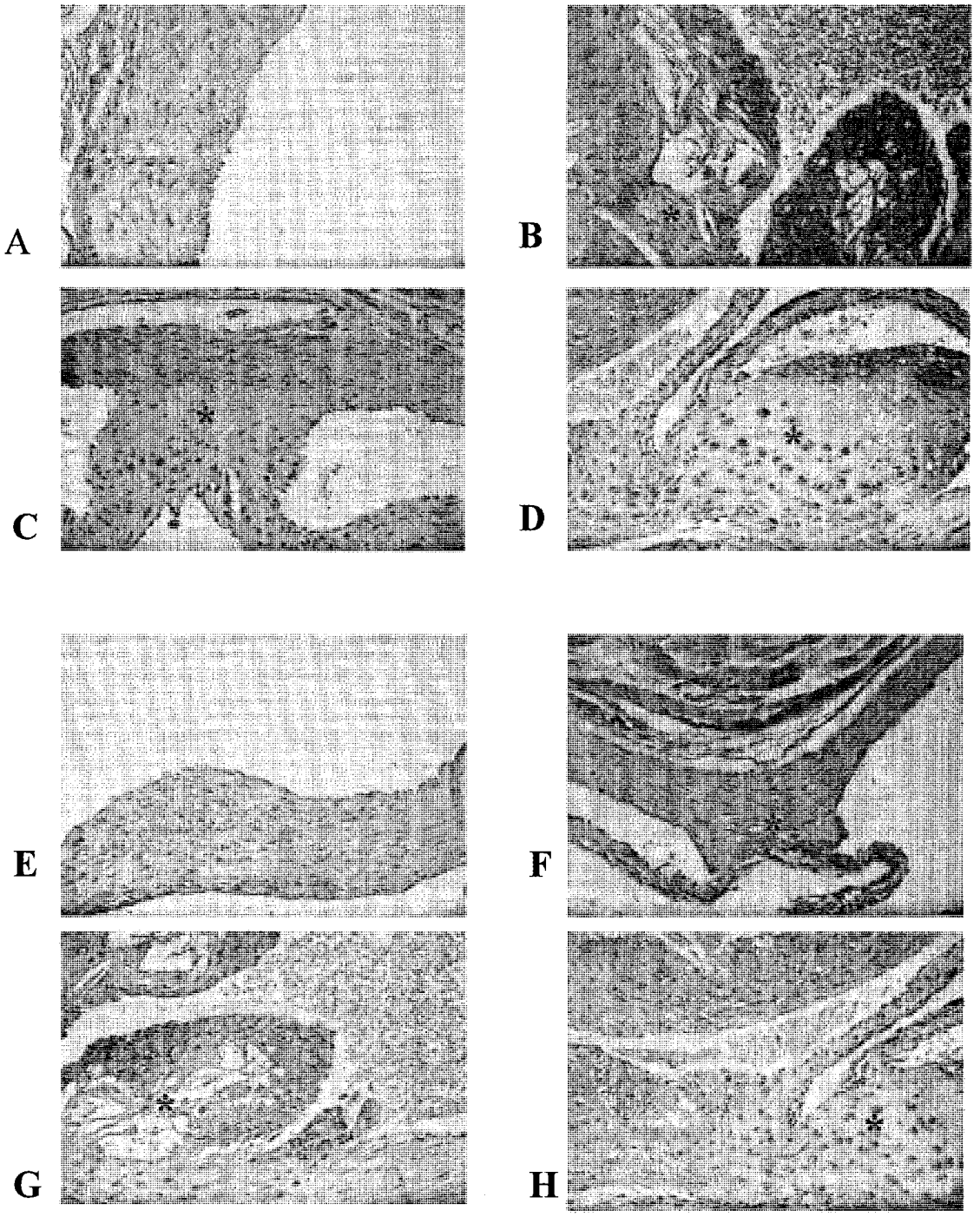


Figure 7. Immunohistochemical localization of ACAT-1 immunoreactivity in the aortic roots of vehicle-treated (**A**) and SB-657510A-treated (**B**) high fat diet-fed ApoE ko mice. Panel C represents a negative control section of none-immune serum from a vehicle treated group. Panel D demonstrated β-galactosidase immunostaining in the aortic smooth muscle cells of UT^{+} mice. * indicates areas of staining

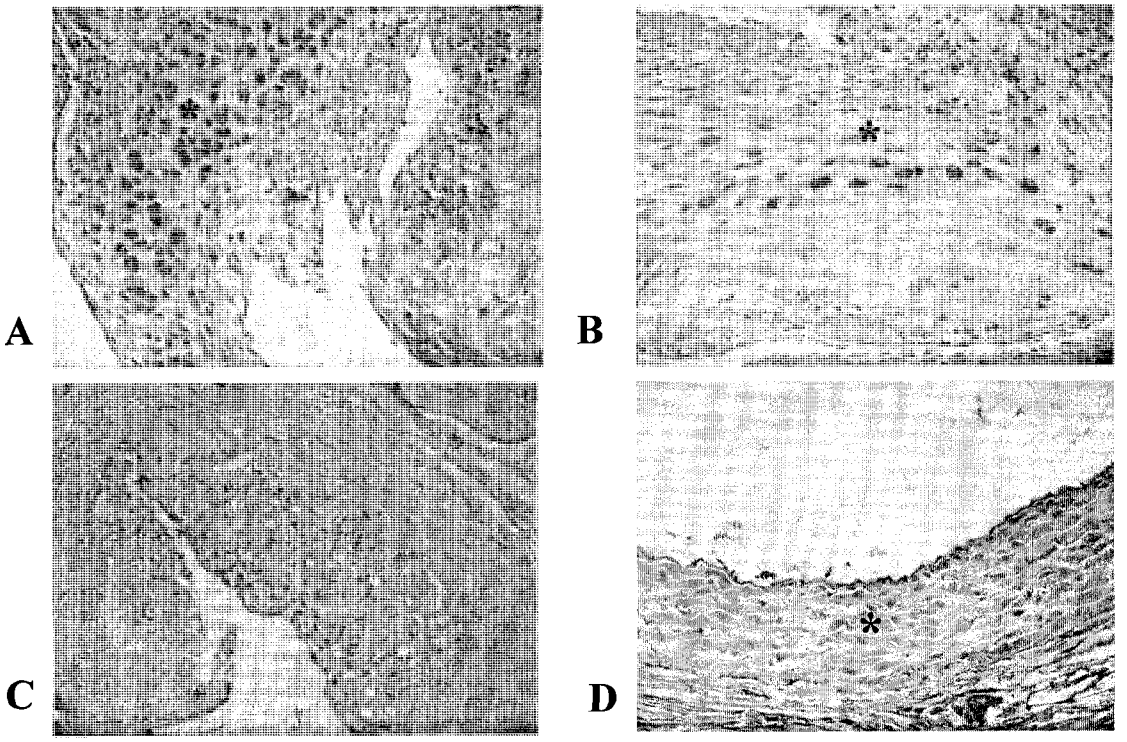


Figure 8a. Analysis of ACAT-1 protein expression using Western blotting in WT ($N = 6$), UT+($N = 5$), ApoE- ($N = 3$) and UT+/ApoE- ($N = 3$) groups.

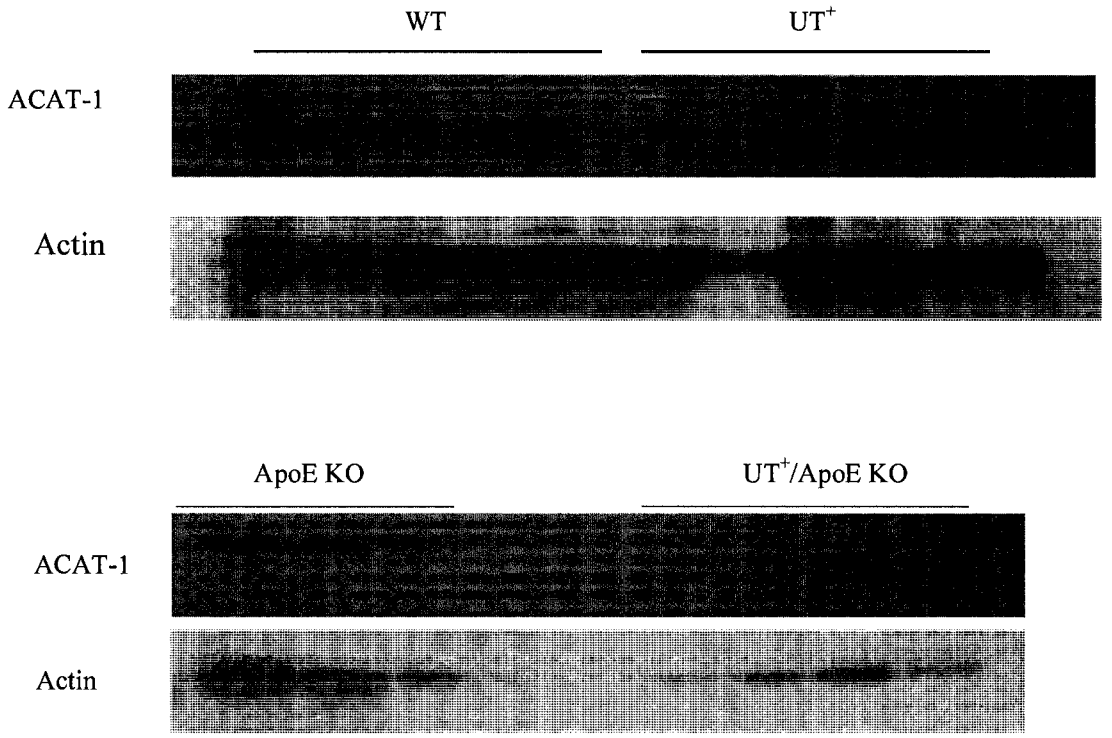


Figure 8b: Analysis of ACAT-1 protein expression using Western blotting showed an increase in the aortas of ApoE ko (2.36±0.71 folds) and UT^+/ApoE ko (1.99±0.28 folds) mice compared to WT and UT^+ mice ($P<0.05$)

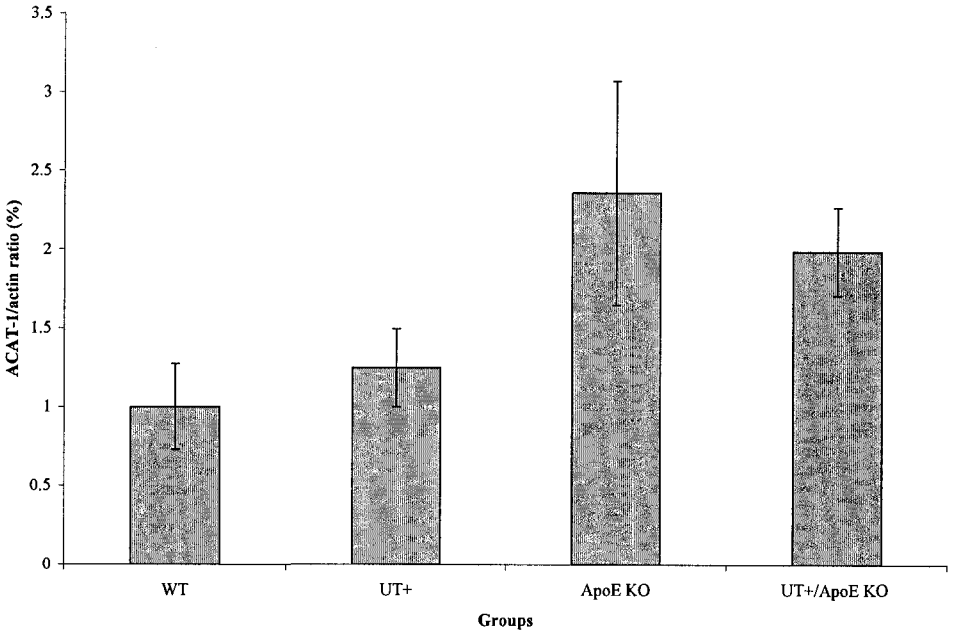


Figure 9. Sudan IV staining of aortas from vehicle-treated (**A**) and SB657510A-treated (**B**) ApoE ko mice fed on high-fat diet. Hematoxylin and eosin stained paraffin sections from the aortic roots of vehicle-treated (**C**) and SB657510A-treated (**D**) mice. * indicates areas of lesion

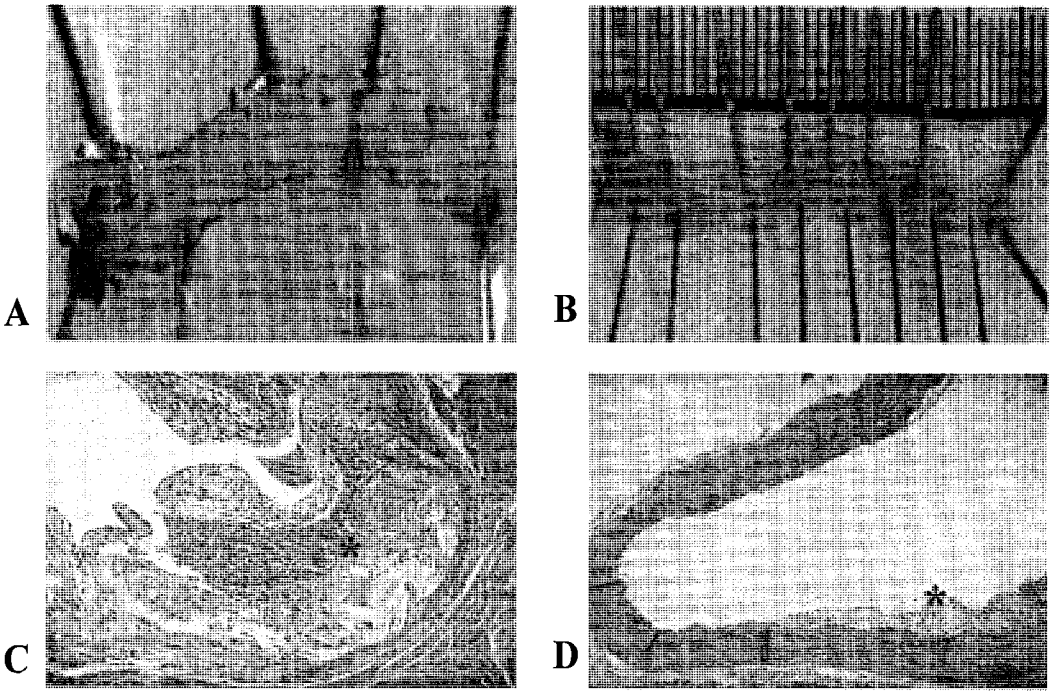
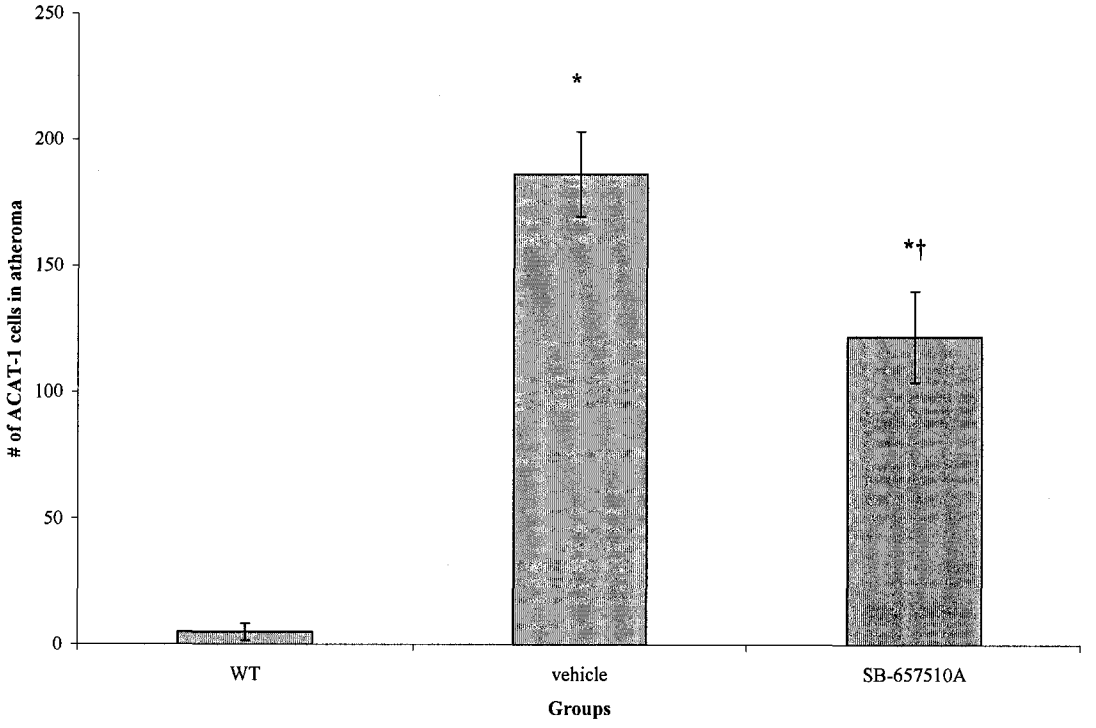


Figure 10. Analysis of the number of ACAT-1 cells in the atheroma. † indicates a significant decrease in the SB-657510A (122.22±17.99) compared to the vehicle-treated mice (186.46±16.87) ($P<0.05$) and * indicates a significant increase of ACAT-1 cells in the SB-657510A and vehicle-treated mice compared to the wild type (5.0±0.3) ($P<0.05$).



DISCUSSION AND CONCLUSION

Discussion

Although numerous studies have demonstrated an association between the urotensin system and atherosclerosis (Heringlake et al. 2004; Suguro et al. 2007), the exact role of this system in the pathogenesis of the disease remained to be determined. In the present study we used a binary system to express the UT gene in the smooth muscle cells, and examined its effects on the development of atherosclerosis using an established mouse model. We report here that overexpression of UT in smooth muscle cells, though it did not increase serum lipids, significantly increased lesion formation. The extent of lesion was exacerbated when the UT^{+} was back-crossed on an ApoE ko model. In addition, the use of the UT receptor blocker SB-657510A significantly reduced the atherosclerotic lesion in ApoE ko mice fed a high fat Western diet. These findings suggest that the urotensin system is one of the major players involved in the pathogenesis of atherosclerosis, and targeting the UT receptors may provide a new therapeutic tool in the management of this disease process.

In inflammatory cells, UT receptor mRNA is mainly expressed in monocytes and macrophages (Bousette et al. 2004). Analysis of cell surface UT receptor in human peripheral blood mononuclear cells by flow cytometry indicates that it is essentially expressed in monocytes and NK cells, but not in T lymphocytes (Segain et al. 2007). Macrophages are an important feature of atherosclerosis beginning with the early onset of the diseases and up to plaque rupture. Macrophage-derived foam cells constitute the core of the subendothelial atheroma lesion in diseased arteries. These are known to produce

numerous factors that contribute to arterial remodeling. These cells play an important role in lipid uptake. For example, free cholesterol is taken up by foam cells and converted into cholesterol ester by ACAT-1. Interestingly, recent studies have shown that U-II increases acetyl-LDL-induced cholesterol ester accumulation in human monocyte-derived macrophages through up-regulation of ACAT-1 protein expression (Watanabe et al. 2005). U-II-induced ACAT-1 expression occurs predominantly during differentiation of human monocytes into macrophages and involves receptor/trimeric G-protein/c-Src tyrosine kinase/MEK and Rho kinase pathways (Watanabe et al. 2005). As previously described in human atherosclerosis (Hassan et al. 2005), we have demonstrated in the present study an abundant expression of both UII and UT in foam cells of atheroma of UT^{+}, ApoE ko and UT^{+}/ApoE ko mice. We also showed that ACAT-1 expression is increased in ApoE ko and UT^{+}/ApoE ko mice, and that UT receptor blockade with SB-657510A significantly reduced the number of ACAT-1 producing foam cells in the atheromas of ApoE ko mice. Therefore, these findings suggest that UT receptor blockade may reduce lesion formation through a reduction in foam cell formation and lipid uptake in the arterial wall.

Interestingly, U-II has been shown to induce hyperlipidemia in fish. It increases plasma free fatty acid by enhancing triglyceride lipase activity and channeling glucose to free fatty acid synthesis (Sheridan et al. 1987). However, we did not see an increase in serum triglycerides or cholesterol in UT transgenic mice. Furthermore, the level of these factors in the serum of UT^{+}/ApoE ko was similar to that of ApoE ko mice. Therefore, it is

reasonable to conclude that over expression of the UT alone does not increase total serum lipid, though it appears to act locally in an autocrine/paracrine fashion to increase lipid uptake by macrophages since the use of UT receptor antagonist reduces ACAT-1 expression. Alternatively, increased expression of UT might induce lipid peroxidation especially since UII is known to induce ROS formation in macrophages (Djordjevic et al. 2005). However, this mechanism needs to be explored in future studies.

It is well known that UT receptors are expressed in smooth muscle cells throughout the human coronary artery tree, from large epicardial to small resistance arteries (Maguire et al. 2002). Though we have previously demonstrated that UT receptor expression is elevated in abdominal aortic aneurysm and carotid endarterectomy tissue samples (Bousette et al. 2004), there appears to be no differences in the UT receptor density in the medial smooth muscle cells between normal and atherosclerotic coronary arteries (Katugampola et al. 2002). Here, we demonstrate increased expression of UT in the aortas of UT^{+} and ApoE ko mice. In agreement with our findings, enhanced UT receptor mRNA expression and U-II binding sites are also described in aortic tissue of the ApoE knockout mice model of atherosclerosis (Wang et al. 2006). It was interesting to see that ApoE ko mice have an elevated expression of UT protein which was similar to that of the UT^{+} groups. Indeed, we have noted in the present study that deletion of ApoE seems to result in elevation of UT expression, and this is not related to type of diet or sex (data not shown). Therefore, in the antagonist study we proceeded with the use of ApoE ko instead of UT^{+} mice. Numerous peptidic and non-peptidic ligands for the UT receptor

have been reported in the literature (Lescot et al. 2007). Moreover, use of these UT receptor antagonists has been demonstrated in experimental animals and in humans in disease states such as hypertension, stroke, heart failure, diabetes nephropathy and metabolic syndrome (Dhanak et al. 2003; Sidharta et al. 2006; Ong et al. 2007). The antagonist we used in the present study is structurally-similar to the one we have previously used to demonstrate the role of UT in heart failure and restenosis (Bousette et al. 2006; Rakowski et al. 2005; Bousette et al. 2006). Administration of SB-657510A for 10 weeks resulted in significant reduction in the atherosclerotic lesion and the number of ACAT-1 producing foam cells. These findings indicated a role for this antagonist in preventing atherosclerosis in this animal model. The mechanism by which this occurs warrants further investigation.

Summary

In summary, we have demonstrated increased expression of UT in the aortas of mice overexpressing the UT gene using the α-SM22 promoter when fed a high fat diet. The size of lesions was further enhanced when the UT^{+} mice where cross-bred with mice lacking the ApoE gene and fed a high fat diet. We have also demonstrated that the increase in atherosclerosis seen in ApoE ko mice fed a high fat diet involves UT expression, and that use of UT receptor blockers significantly decreases the lesion. One of the mechanisms involved in the latter process was decreased ACAT-1 protein expression by foam cells. We therefore conclude that the use of UT receptor antagonist may prove to be a beneficial tool in treating atherosclerosis.

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APPENDIX

(Research compliance certificate)