Genetic and Pharmacological Manipulation of Urotensin II Ameliorate the Metabolic and Atherosclerosis Sequelae in Mice


Objective—Urotensin II (UII) is a potent vasoactive peptide that binds to the urotensin receptor—coupled receptor-14 (known as UT) and exerts a wide range of actions in humans and experimental animals. We tested the hypothesis that UII gene deletion or UT blockade ameliorate experimental atherosclerosis.

Methods and Results—We observed a significant reduction in weight gain, visceral fat, blood pressure, circulating plasma lipids, and proatherogenic cytokines and improvement of glucose tolerance in UII knockout mice compared with wild type (P<0.05). Deletion of UII after an apolipoprotein E knockout resulted in a significant reduction in serum cytokines, adipokines, and aortic atherosclerosis compared with apolipoprotein E knockout mice. Similarly, treatment of apolipoprotein E knockout mice fed on high-fat diet with the UT antagonist SB657510A reduced weight gain, visceral fat, and hyperlipidemia and improved glucose tolerance (P<0.05) and attenuated the initiation and progression of atherosclerosis. The UT antagonist also decreased aortic extracellular signal-regulated kinase 1/2 phosphorylation and oxidant formation and serum level of cytokines (P<0.05).

Conclusion—These findings demonstrate for the first time the role of UII gene deletion in atherosclerosis and suggest that the use of pharmaceutical agents aimed at blocking the UII pathway may provide a novel approach in the treatment of atherosclerosis and its associated precursors such as obesity, hyperlipidemia, diabetes mellitus, and hypertension. (Arterioscler Thromb Vasc Biol. 2012;32:1809-1816.)

Key Words: hyperlipidemia • hypertension • insulin • mice • obesity • tolerance

Urotensin II (UII) is a vasoactive peptide originally found in teleost fish with homologs later discovered in several other species including mice, rats, frogs, and humans.1-4 In the latter, UII is an 11 amino acid peptide with highly conserved C-terminal hexapeptide and disulfide bridge between 2 cysteine residues. Subsequent studies revealed that UII was the ligand for its own receptor, the rat orphan G-protein-coupled receptor-14/sensory epithelial neuropeptide–like receptor, which in humans is now known as the urotensin receptor (UT).5,6 Although it is considered the most potent vasoconstrictor peptide, studies of UII in various species have shown varied actions depending on the type of vessel, source of UII and the integrity of the endothelium, and the presence or absence of a disease state.6-11 In a study of 2 treatment groups, physiologically healthy individuals and patients with chronic heart failure, UII had vasodilator effects in healthy individuals, whereas patients with chronic heart failure showed vessel constriction.7 Plasma UII levels are elevated in patients with confirmed atherosclerosis.12,13 UII plasma levels predicted a 1.6-fold higher risk of carotid plaque formation when compared with currently recognized risk factors.13 UII and the UT are both upregulated in atherosclerotic lesions.14-17 We had demonstrated increased UII expression in areas of atherosclerotic plaques by immunocytochemistry and real-time polymerase chain reaction.14,16 UII is upregulated throughout the vessel wall (in endothelial, myointimal, and medial smooth muscle cell [SMC] layers) in human coronary atherosclerosis.18 In human abdominal aortic aneurysm and carotid atherosclerotic extracts, UT mRNA expression is increased.14 Among the inflammatory cell population, lymphocytes appear to be the largest producers of UII, whereas monocytes and macrophages appear to express the highest levels of UT.14 Inflammatory mediators such as lipopolysaccharide, tumor necrosis factor-α, and interferon-γ are known to upregulate UT mRNA expression.18 Indeed, UII-mediated chemotaxis has been demonstrated in monocytes to be ras homolog gene family, member A/rho-associated, coiled-coil containing protein kinase dependent.18 UII also upregulated acetyl-Coenzyme A acetyltransferase 1 activity resulting in increased production of foam cells in lesions.19 We have recently demonstrated that selective UT blockade significantly reduced the number of acetyl-Coenzyme
A acetyltransferase 1 producing cells in the atherosclerotic plaque of apolipoprotein E (apoE) knockout (KO) mice fed a high-fat diet (HFD). In addition, UII has direct effects on reactive oxygen species (ROS) levels by activating nicotinamide adenine dinucleotide phosphate oxidase subunits p22phox and nicotinamide adenine dinucleotide phosphate oxidase 4. ROS has also been shown to play a critical role in UII signal transduction. Thus, the effects of UII and ROS are intimately related along the progression of atherosclerosis.

Although a recent study has shown that chronic infusion of UII increases atherosclerosis in apoE KO mice, the exact role of UII in this disease process remains to be elucidated. Here, we used molecular and pharmacological approaches to determine the role UII plays in atherosclerosis. First, we generated UII KO mice and cross bred them with apoE KO mice. Second, we used a selective UT antagonist to determine the role of UII in the initiation and progression of atherosclerosis in apoE KO mice fed a HFD.

**Materials and Methods**

**Targeting the PreproUII Gene**

Gene targeting was performed in E14 embryonic stem cells using standard homologous recombination (Figure 1A). Generation of UII mutant mice is described in the online-only Data Supplement.

**Pharmacological Study**

ApoE KO mice (C57Bl6/J) were purchased from Jackson Laboratories (Bar Harbor, ME). Male and female mice were divided into 3 groups as follows.

**Prevention Groups**

apoE KO mice (6-week-old) were placed on the same HFD mentioned earlier containing either 50 mg/kg per day SB657510A (SB) or 1% methyl cellulose (V) for 10 weeks.

**Regression Group (I)**

apoE KO mice (6-week-old) were placed on the same HFD described above for 10 weeks, after which they were continued on a HFD containing either 50 mg/kg per day SB657510A (SB) or 1% methyl cellulose (V) for the ensuing 10 weeks.

**Regression Group (II)**

apoE KO mice (6-week-old) were placed on HFD for 10 weeks, after which they were placed on normal diet containing either 50 mg/kg per day SB657510A (SB) or 1% methyl cellulose (V) for the ensuing 10 weeks.

Food intake was monitored daily and did not significantly differ among all experimental groups. Body weight was measured at the beginning of the study and thereafter once weekly until the end of the study. Unless indicated, data shown here represent that of male mice.

**Blood Pressure Analysis**

First, blood pressure was monitored using the tail cuff technique. Second, before euthanizing, adult male mice were anesthetized with ketamine–xylazine (IM, 87 mg/kg:13 mg/kg), and a fluid-filled catheter was then inserted into the carotid artery for blood pressure monitoring.

**Statistical Analyses**

All values are presented as mean±SE. Multigroup comparisons were analyzed using ANOVA with the Tukey post hoc test. The 2 groups were compared using the Student t test. P <0.05 was considered statistically significant. All statistical analyses were carried out using SPSS version 11.5 (SPSS Inc, Chicago, IL).

**Results**

**UII KO Study**

Deletion of the UII gene was confirmed by reverse transcription polymerase chain reaction (RT-PCR) demonstrating successful preprourotensin II knockout (preproUII KO) generation. RNA was extracted from skeletal muscle of preproUII KO mice (+/+, +/-, and −/−) and preproUII gene expression was performed by RT-PCR. Tissue-dependent reduction of UII-like immunoreactivity in UII KO mice (male mice; n=13 per group). Levels of UII-like immunoreactivity were determined by radioimmunoassay (RIA). Proteins were extracted in acid–acetone followed by centrifugation. The RIA incubation mixture consisted of hUII or tissue extract, monoclonal antibody (mAb)UII-17, [125I]hUII, and standard buffer (50 mmol/L Na-phosphate buffer (pH 7.4) containing 50 mmol/L Na-EDTA and 0.1% BSA). After a 16-hour incubation at 4°C, bound and free ligands were separated using a secondary goat antimouse antibody (BioMagnetic Goat Antimouse IgG; Qiagen, Hilden, Germany). *P<0.001.
when the animals were placed on the HFD (Figure 2A; Table I in the online-only Data Supplement). Female mice on either diet did not exhibit a significant difference in weight gain. Food intake did not differ between UII KO (2.83±0.08 g/day) and wild-type (2.97±0.10 g/day) mice. UII KO mice also appeared more active and alert than their wild-type counterparts, and they exhibited significantly lower mean and systolic arterial blood pressure compared with wild-type mice (Table II in the online-only Data Supplement). There was no significant difference between wild-type and UII KO mice in terms of glucose or insulin tolerance when mice were fed normal diet. In contrast, when fed the HFD, the UII KO mice showed a significant improvement in glucose tolerance compared with wild type (Figure 2B). HFD-fed UII KO mice had <80% low-density lipoprotein (LDL)-cholesterol compared with wild type, and they both did not exhibit aortic atherosclerosis. Serum levels of free fatty acid were significantly lower in HFD-fed UII KO (0.63±0.06 mmol/L) compared with wild-type (1.00±0.059 mmol/L; P<0.05) mice. UII KO mice had greater serum lipoprotein particles containing apoA-I than wild type (Figure 3A). Similarly, the media of hepG2 cell culture incubated with serum from UII KO mice contained more lipoprotein particles containing apoA-I particles than those incubated with serum of wild-type mice (Figure 3B). Visceral adipocyte diameter of UII KO mice was significantly smaller than that of wild-type mice (Figure 4A and 4B; 696±31 versus 2019±54 pixels; P<0.01).

When UII KO mice were cross bred with apoE KO mice, the resulting apoE KO/UII KO (DKO) mice exhibited reduced mean arterial blood pressure (100.63±3.61 versus 121.31±5.71; P<0.05), serum very-low-density lipoprotein–cholesterol (23±2%), triglycerides (50±3%), and aortic atherosclerosis compared with apoE KO mice (Figure 4C and 4D), and increased cholesterol efflux of peritoneal macrophages. Serum levels of inflammatory cytokines, such as monocyte chemoattractant protein-1, monokine induced by γ-interferon (MIG), and keratinocyte chemoattractant, were reduced in UII KO compared with wild-type mice (Figure 5A). Similarly, DKO mice exhibited either a reduction or complete elimination of soluble intercellular adhesion molecule-1, monocyte chemoattractant protein-1 (Japanese encephalitis) MIG, and tissue inhibitor of metalloproteinase-1 compared with apoE KO mice (Figure 5B). Adipokines, such as dipeptidyl peptidase-4, endocan (endothelial cell–specific molecule-1), and

Figure 2. A, Growth curve. Mice from wild and urotensin II knockout (UII KO; 23 male mice per group for high-fat diet [HFD] and 10 male mice per group for normal diet [ND]) were put on HFD and ND, respectively. The body weights were measured every week. *P<0.05; **P<0.01. B, Glucose tolerance test. Data represent means±SEM for 6 male mice of each genotype. *P<0.05; **P<0.01.

Figure 3. A, Serums from mice with urotensin II knockout (UII KO) vs wild type (WT) were separated by 2-dimensional polyacrylamide gradient gel electrophoresis (PAGGE), and apolipoprotein A-I (apoA-I) was detected by anti–apoA-I serum. B, Culture media from hepG2 cell line containing lipoprotein particles containing apoA-I (LpA-I) particles were concentrated with a size exclusion centrifugal filter (spiral ultra filtration cartridge; molecular weight cutoff [MWCO, 40 000]; Amicon, Billerica, MA). LpA-I were separated by 2-dimensional PAGGE and detected by anti–apoA-I serum. Molecular size markers are shown.
insulin-like growth factor–binding protein-2, -3, -5, and -6 were reduced in the serum of DKO mice compared with apoE KO mice (Figure 5C). Analysis of the histological and immunohistochemical staining of aortic root of DKO mice revealed the presence of less atherosclerotic lesion in the aortic root of DKO mice compared with apoE KO mice (Figure 6). The latter showed more cholesterol clefts, with less \( \alpha \)-SMC staining in the atherosclerotic cap (3.73% of lesion), whereas the DKO mice exhibited mainly atheroma with foam cells and abundant presence of \( \alpha \)-SMC staining of the surface of the atherosclerotic cap (5.85% of lesion; \( P < 0.02 \); Figure 6). Western blotting and immunohistochemistry revealed a significant reduction in nitrotyrosine, 4-hydroxy 2-nonenal and soluble epoxide hydrolase protein expression, and extracellular signal-regulated kinase 1/2 (ERK1/2), and P44/42 phosphorylation in aortas of DKO compared with apoE KO mice (Figures I and II in the online-only Data Supplement).

**Pharmacological Study**

Selective UT blockade with SB657510A in male apoE KO mice fed a HFD significantly reduced body weight gain and visceral fat content (Table III in the online-only Data Supplement). There was no significant effect on body weight gain and visceral fat content in female mice fed the same HFD (Table III in the online-only Data Supplement). Systolic blood pressure was also reduced in SB657510A-treated mice (83±4.8 mm Hg) compared with vehicle-treated mice (91±1.7 mm Hg) without affecting diastolic blood pressure (60.6±2.2 versus 63±3.9 mm Hg) or heart rate. SB657510A significantly reduced serum lipids (Figure III in the online-only Data Supplement), insulin (0.127±0.035 versus 0.195±0.029 ng/mL; \( P < 0.05 \)), glucose (334±20 versus 410±15 mg/dL; \( P < 0.05 \)), and free fatty acid (1.05±0.26 versus 2.3±0.25 mmol/L; \( P < 0.05 \)) and improved glucose tolerance (Figure IV in the online-only Data Supplement). The receptor antagonist did not affect the extent of liver steatosis. SB657510A...
Atherosclerosis is the main cause of morbidity and mortality of cardiovascular diseases.\textsuperscript{25} Epidemiological studies have shown that atherosclerosis is associated, among other things, with the presence of serum hyperlipidemia and hyperglycemia, obesity, and hypertension.\textsuperscript{25} The vasoactive peptide UII modulates vascular tone, blood pressure, and insulin release.\textsuperscript{12} In the present study, we demonstrate that deletion of the UII gene in mice results in decreased body mass, visceral fat and blood pressure, and improved insulin and glucose tolerance. Furthermore, in the pathological setting of experimental atherosclerosis, UII deletion in apoE KO mice fed a HFD led to reduced aortic atherosclerosis and proatherogenic inflammatory cytokines and adipokines. Similarly, the use of the selective UT blocker SB657510A in apoE KO mice fed a HFD significantly reduced body weight gain, blood pressure, serum hyperlipidemia and hyperglycemia, circulating cytokines and aortic atherosclerosis, ERK1/2 and P44/42 mitogen-activated protein kinase phosphorylation and oxidant formation, and stabilized the lesion. Finally, patients with all parameters of the metabolic syndrome exhibited elevated levels of plasma UII. These findings demonstrate that UII is an important mediator of atherosclerosis and its associated sequelae.

A previous pharmacological study had shown that intracerebroventricular injection of UII produces anxiogenic effects and increases water and food intake in the mouse.\textsuperscript{36} Plasma UII level also correlates positively with body weight in the Hong Kong Chinese population.\textsuperscript{27} On the other hand, we have recently reported that UT KO mice exhibited a lean profile.\textsuperscript{28} In the present study, we monitored food intake and observed no significant difference between UII KO and wild-type mice. However, UII KO mice exhibited a significant reduction in body weight gain and visceral fat content compared with wild-type mice. In addition, apoE KO mice fed a HFD and treated with SB657510A exhibited similar reduction in weight gain to that of UII KO mice suggesting that UII plays an important role in the control of body mass and fat content in a manner independent of appetite suppression. Furthermore, the reduction in body weight gain appears to be related to gender and warrants further investigation.

Intravenous administration of UII led to increase in mean arterial blood pressure in cats,\textsuperscript{29} rats,\textsuperscript{30} and sheep.\textsuperscript{31} UII plasma levels and systolic blood pressure mirror one another.\textsuperscript{27} UII-like immunoreactivity was found in high concentrations in patients with essential hypertension or with hypertensive renal disease.\textsuperscript{32} The genetic variant, 3836C>T (S89N) in the UII gene is associated with essential hypertension in a Northwestern Chinese population.\textsuperscript{33} Moreover, elevated plasma level of UII and expression of UII and the UT have been reported in experimental animals of hypertension.\textsuperscript{24,35} A recent study has shown significant correlation between blood pressure and UII levels in patients with carotid atherosclerosis and vascular dementia.\textsuperscript{26} Here we demonstrated that UII KO mice tend to exhibit decreased mean arterial and, particularly, systolic blood pressure than wild type. Similarly, the use of UT antagonist SB657510A significantly reduced mean blood pressure and systolic pressure in apoE KO mice fed a HFD. A recent study has suggested that soluble epoxide hydrolase might play an important role in mediating UII vascular effects.\textsuperscript{37} Our data have shown that UII deletion is associated with a reduction in aortic soluble epoxide hydrolase protein expression. These findings point to an important role for UII in modulating blood pressure.

**Discussion**

Atherosclerosis is the main cause of morbidity and mortality of cardiovascular diseases.\textsuperscript{25} Epidemiological studies have shown that atherosclerosis is associated, among other things, with the presence of serum hyperlipidemia and hyperglycemia, obesity, and hypertension.\textsuperscript{25} The vasoactive peptide UII modulates vascular tone, blood pressure, and insulin release.\textsuperscript{12} In the present study, we demonstrate that deletion of the UII gene in mice results in decreased body mass, visceral fat and blood pressure, and improved insulin and glucose tolerance. Furthermore, in the pathological setting of experimental atherosclerosis, UII deletion in apoE KO mice fed a HFD led to reduced aortic atherosclerosis and proatherogenic inflammatory cytokines and adipokines. Similarly, the use of the selective UT blocker SB657510A in apoE KO mice fed a HFD significantly reduced body weight gain, blood pressure, serum hyperlipidemia and hyperglycemia, circulating cytokines and aortic atherosclerosis, ERK1/2 and P44/42 mitogen-activated protein kinase phosphorylation and oxidant formation, and stabilized the lesion. Finally, patients with all parameters of the metabolic syndrome exhibited elevated levels of plasma UII. These findings demonstrate that UII is an important mediator of atherosclerosis and its associated sequelae.

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Both patients with atherosclerosis and apoE KO mice are known to exhibit elevated serum levels of LDL and triglycerides. The extent of fat in the diet will influence the serum level of lipids, as well as the extent of atherosclerotic lesions. UII has been shown to influence lipase activity in the coho salmon, and UII plasma level is associated with LDL in atherosclerosis. However, since those initial studies, little has been done to assess the effects of UII on serum lipids. Here, we demonstrated for the first time that deletion of the UII gene alone or in the pathological setting of apoE KO in mice fed a HFD resulted in a decrease in serum LDL and triglycerides and an increase in serum pre-β high-density lipoprotein as evident in the increase of lipoprotein particles containing apoA-I. Furthermore, UT blockade with SB657510A again reduced serum lipid in apoE KO mice fed a HFD. The mechanism by which UII modulates serum lipid is under investigation in our laboratory. Indeed, microarray analysis of samples from SB657510A-treated mice have shown a downregulation of a number of adipokines known to play an important role in lipid metabolism (data not shown). These findings demonstrate an important role for UII in modulating serum lipid and open the possibility for the use of UT antagonists as therapeutic modality in reducing circulating LDL and triglycerides.

Atherosclerosis is associated with a buildup of cholesterol increasing vessel wall thickness and the induction of a chronic inflammatory response that includes the infiltration of monocytes and macrophages. We had previously shown that inflammatory cells express both UII and UT. Furthermore, inflammatory mediators, such as lipopolysaccharide, tumor necrosis factor-α, and interferon-γ have been shown to induce UT mRNA expression alluding to the chemotaxis and signaling roles that UII may play in the progression of atherosclerosis. Furthermore, chemotaxis effects of UII have been demonstrated in monocytes. UII is also known to induce expression of cellular adhesion molecules in coronary endothelial cells. Here, we demonstrated for the first time that UII deletion in combination with ApoE KO reduces protein expression of inflammatory cytokines, such as soluble intercellular adhesion molecule-1, monocyte chemotactic protein-1, MIG, and tissue inhibitor of metalloproteinase-1, known to play an important role in the pathogenesis of atherosclerosis. These data were further supported by the findings that the UT blocker SB657510A also reduced the serum protein level of C5a, tissue inhibitor of metalloproteinase-1, M-CSF, soluble intercellular adhesion molecule-1, and MIG in apoE KO mice fed on HFD.

Dyslipidemia associated with atherosclerosis leads to increases in LDL-cholesterol and mildly oxidized LDL, which subsequently cause development of atherosclerotic plaques through stimulation of ROS formation and vascular SMCs differentiation and proliferation. Indeed, studies have shown that UII increases the mitogenic effects of mildly oxidized LDL on vascular SMCs in a synergistic manner via both G-protein–dependent effects on protein kinase C and ERK. UII has also direct effects on ROS levels by activating nicotinamide adenine dinucleotide phosphate oxidase subunits p22phox and nicotinamide adenine dinucleotide phosphate oxidase 4. ROS has also been shown to play a critical role in UII signal transduction. Here, we demonstrated that deletion of the UII gene and the use of SB657510A significantly reduced the aortic expression of nitrotyrosine, a marker of protein oxidation by peroxynitrite, and 4-hydroxy 2-nonenal, a sensitive marker of lipid peroxidation and oxidative stress, both known to play an important role in the pathogenesis of atherosclerosis. These findings were also associated with a significant reduction in aortic ERK1/2 and P44/42 mitogen-activated protein kinase phosphorylation. Thus, the effects of UII and ROS appear intimately related along the progression of atherosclerosis, and the use of UT blocker significantly reduces aortic oxidative stress.

Hyperglycemia and insulin resistance are interrelated components of atherosclerosis, and both are related to UII. Both UII and the UT are expressed in the pancreatic islets. In salmon, UII increases glucose-6-phosphatase activity and reduces liver glycogen content. UII inhibited glucose- and arginine-induced insulin responses in the rat pancreas. In humans, single nucleotide polymorphism analysis of the Hong Kong and Han Chinese and Japanese populations confirmed that certain UT gene haplotypes are associated with insulin resistance and pancreatic β-cell function. A recent study has also demonstrated an association between UT gene and type II diabetes mellitus. Plasma UII levels were 2× as high in patients with type II diabetes mellitus without proteinuria when compared with healthy subjects (7.8 fmol/mL versus 4.4 fmol/mL). UII serum level and UII binding, as well as UT protein and mRNA expression, are all significantly enhanced in the skeletal muscle of 2DM mice. Here, we demonstrated that UII deletion significantly reduced serum insulin and glucose, and improved glucose and insulin tolerance. Furthermore, the use of SB657510A significantly improved glucose tolerance in apoE KO mice. More importantly, adipokines, such as dipeptidyl peptidase-4, endocan, and insulin-like growth factor–binding proteins known to play an important role in obesity and diabetes mellitus were all reduced in DKO mice compared with apoE KO mice. These findings again reinforce the role UII plays in insulin release and suggest a role for UT antagonists in the management of the metabolic syndrome.

Although we and others have previously examined the role of UII in atherosclerosis, the effects of UII deletion on the extent and type of atherosclerotic plaque and the mechanism involved had not been investigated. Here, we demonstrated that UII deletion and chronic receptor blockade did not only reduce the lesion but resulted in a more stable lesion by increasing the percentage of α-SMC and decreasing the percentage of carbohydrate-binding protein 35–producing cells in the atherosclerotic plaque. Furthermore, we show for the first time that SB657510A significantly reduced the extent of established atherosclerotic plaque in male apoE KO mice fed a HFD. We had previously shown that although UT deletion resulted in a lean profile, there was an increase in serum lipid and atherosclerosis. The discrepancy between UT and UII deletion could be attributed to the possibility of UT subtypes as evident from previous studies. For example, Coy et al demonstrated that although the somatostatin analog (Cpa-c[N-Cys-Pal-α-Trp-Lys-Val-Cys]-Cpa-amide) was able to inhibit UII–induced phasic contraction in the rat isolated aorta, the peptidic moiety was unable to block tonic contraction. In addition, Camarda et al demonstrated that [Orn]UII
acts as a full agonist at human embryonic kidney 293 cells expressing recombinant rat UT but is a competitive antagonist in rat isolated aorta. Furthermore and before these observations, Northern blotting analysis of multiple human tissues showed different UT transcript sizes. The other possible explanation to the opposing effect may stem from the effect of UT gene deletion on hepatocytes function. This is further supported by the fact that the receptor antagonist used in the present study had no effect on cholesterol metabolism or hepatic steatosis (data not shown).

In summary, we used molecular and pharmacological approaches to determine the role of UI in the pathogenesis of atherosclerosis and successfully demonstrated that UI plays an important role in atherosclerosis through modulating body weight mass, blood pressure, and serum lipid, sugar, cytokines, adipokines, and oxidant formation. Furthermore, UI contributed to plaque remodeling and stability. These findings point to a potentially important role for future therapeutic approaches aimed at blocking the UT in the management of atherosclerosis and its associated sequelae.

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None.

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Genetic and Pharmacological Manipulation of Urotensin II Ameliorate the Metabolic and Atherosclerosis Sequalae in Mice
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SUPPLEMENT MATERIALS

Supplement Methods

Generation of mutant mice.

The KO strategy was designed to delete all the coding region downstream of the PstI restriction site in exon2, resulting in deletion of half of exon 2 and all of exons 3 and 4, ensuring deletion of the whole coding region of the mature peptide. Chimeras were bred to C57Bl6/J to generate N1 heterozygotes. Skeletal muscle of preproU-II KO mice (+/+, +/-, and -/-) was extracted and preproU-II gene analysis was performed by RT-PCR. Levels of “U-II-like” immunoreactivity were determined by radioimmunoassay (RIA). Proteins were extracted in acid-acetone followed by centrifugation. The RIA incubation mixture consisted of hU-II or tissue extract, mAbUII-17, [125I]hU-II and standard buffer (50mM phosphate buffer [pH7.4] containing 10μM Na-EDTA and 0.1% BSA). Following 16 hour incubation at 4°C, bound and free ligands were separated using a secondary goat anti–mouse antibody (BioMagnetic Goat Antimouse IgG; Qiagen). The KO strategy resulted in the deletion of the whole UII open reading frame. The UII KO mice were backcrossed at least 10 times to the C57BL6/J background and then bred to homozygosity. They were then crossed with the ApoE KO to generate DKO mice. The ApoE KO mice were ordered from Jacksons Laboratories (B6.129P2-ApoE<sup>tm1Unc</sup>/J).

The study utilized 4 mouse strains including WT (C57BL6/J; ApoE<sup>+/+</sup>, UII<sup>+/+</sup>; n=39), UII KO (ApoE<sup>+/+</sup>, UII<sup>-/-</sup>; n=28), ApoE KO (ApoE<sup>-/-</sup>, UII<sup>+/+</sup>; n=26), and UII/ApoE double knockout mice (DKO, ApoE<sup>-/-</sup>, UII<sup>-/-</sup>; n=27). Mice were bred and allowed to mature to 6 weeks of age at which point mice were weighed and put on a high fat diet (HFD) for a period of 10 weeks. The HFD (TD-88137, Harlan Teklad, IN, USA) consisted of 42% calories from fat with 0.15% cholesterol content.
Glucose and Insulin Tolerance:

One week prior to the end of the study, 6-8 male mice/group were injected with 1.5 g/kg/mouse of dextrose intraperitoneally. Blood samples were obtained from the tail vein immediately before and 15, 30, 60, 90 and 120 min after injection and glucose values will be determined from whole venous blood using an automatic glucose analyzer (Onetouch Ultra2, Lifescan, BC, Canada). For insulin tolerance tests (ITT), animals were injected with 0.75U/kg/mouse of insulin (Lilly, IN, USA) intraperitoneally. Blood samples were obtained from the tail vein immediately before and 15, 30, 45 and 60 min after injection and glucose values were measured as mentioned above. At sacrifice, serum levels of glucose (Cayman Chemical Company) and insulin (Millipore) were measured using commercial kits.

Two-dimensional gel electrophoresis

Serum from wildtype and UII KO male mice, and medium of hepG2 cell cultures incubated with serum from wildtype and UII KO male mice (for 4 hours) were separated according to charge (agarose, horizontal axis) and size (polyacrylamide gradient, 2% to 36% gel, vertical axis) as previously described (1, 2). ApoA-I was detected using a goat anti-apoA-I antibody (Meridian Life science, Biodesign, 1/1000) followed by a peroxidase conjugated donkey anti-goat antiserum (SantaCruz).

Serum Lipids

Fasting blood samples were harvested at the time of sacrifice. Serum was then collected and stored at –20°C. Serum from individual animals (male mice n=4 per group) was separated into lipoprotein fractions using high performance liquid chromatography (HPLC) with a Superose 6 10/300 GL column (GE Healthcare) attached to a Beckman Coulter System Gold™ apparatus. Total cholesterol and triglyceride concentrations were subsequently analyzed using the Infinity™ Cholesterol and
Triglyceride Liquid Stable Reagents (Thermo Electron Corporation) following the manufacturer’s instructions. Serum free fatty acid wasp measured using a commercial kit from Biovision (CA, USA).

**Western Blotting**

Western blotting was performed as previously described [14,20], with goat polyclonal anti-4-hydroxynonenal (HNE) (1:1000 dilution, R&D Systems), nitotyrosine (1:1000, R&D Systems), soluble epoxide hydrolase (sEH), ERK1/2 and phosphorylated ERK1/2(1/1000 dilution, Cell Signalling), TGF-b-induced P44/42 and PP44/42 MAPK; and a secondary antibody conjugated to peroxidase (1:5000 dilution, Santa Cruz Biotech). Protein levels were normalized to the housekeeping gene Histone H1 or actin (1/1000 Santa Cruz Biotech). Protein bands were then quantified using arbitrary units (AU) with the image analysis program, *Image J*.

**Assessment of inflammatory cytokines and adipokines:**

Equal amount of serum from 6 male animals per group were pooled together and circulating level of inflammatory cytokines and adipokines were assessed using mouse cytokine and adipokine proteomic profiler kits by R&D Systems (MN, USA) according to the manufacturer’s instructions.

**Tissue Histology and Immunohistochemistry:**

Formalin fixed aortae were surgically cleaned of all adventitial fat and extraneous tissue. These aortae were then either fixed in formalin and stained with Sudan IV and pinned to a rubber surface for *en face* presentation, or snap frozen in liquid nitrogen for Western blotting. The heart, lungs, spleen, liver,
kidneys and visceral fat were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin & eosin and Sirius red for histologic analysis. The aortic root was sequentially sectioned and immunostained with Mac 2 (1/2500, Cedarlane), α-smooth muscle cell (αSMC1/500, Cedarlane) actin, von Willebrand factor and elastin (1/400) as described previously [14,16]. The percentage of Mac2 and αSMC staining in the atherosclerotic lesion was measured using Image J program. Extra set of paraffin sections cut at the aortic roots were immunostained with antisera to nitrotyrosine (1/350) or HNE (1/300).

**Supplement References:**


**Supplement Figure Legends:**

**Supplement Figure I.** Western blot of nitrotyrosine and 4- hydroxynonena (HNE) proteins in the aortas of ApoE KO and DKO mice. Male mice N=5 mice/group. *P<0.05.

**Supplement Figure II.** Western blot for soluble expoxide hydrolase (sHE; panel A) protein and p44/42 and phosphorylated p44/42 (panel B) proteins in aorta of ApoE KO and ApoE/UII DKO mice
fed HFD for 10 weeks. Proteins were normalized to that of actin and the bands were quantified by Image J. Male mice N=5 mice/group. *P<0.05.

**Supplement Figure III.** Fractioning of serum lipoproteins by HPLC for ApoE KO mice treated with the UT receptor antagonist SB657510A or vehicle in the prevention group. A: Cholesterol profile. B: TG profile. Male mice N=4 mice/group; W: Wildtype, V: Vehicle, SB: SB657510A.

**Supplement Figure IV.** Effect of UT receptor antagonist SB657510A on glucose tolerance test in high-fat diet (HFD) fed ApoE KO mice in the prevention group. Mean±SEM; Male mice N=5 mice/group. 1.5gGlu/mouse/kg; **P<0.01.

**Supplement Figure V.** Effect of SB657510A on aortic atherosclerosis in male ApoE KO mice from regression groups (I) and (II) assessed by Sudan IV staining. A: male mice Sudan IV staining. B: Quantification of lesion area. Male or female mice N=6 mice/group; *P<0.05.

**Supplement Figure VI.** Cytokines in the serum of ApoE KO (vehicle, V) and ApoE KO treated with SB657510A (SB). Equal amount of serum from 6 male mice of each group were pooled together. 1 ml of serum was used for the assay with R&D proteome profiler array. 1: complement component C5 (C5a); 2: soluble intercellular adhesion molecule-1 (SICAM-1); 3: Granulocyte–macrophage colony stimulating factor (M-CSF); 4: monokine induced by gamma-interferon (MIG); 5: Tissue inhibitor of
metalloproteinase-1 (TIMP-1). Two dots represent duplicates for each single protein. Only those with equal intensity of duplicates were considered different in comparing the two experimental groups.

**Supplement Figure VII A-B.** Western blot of 4-hydroxynonenal (HNE) protein in the aortas of ApoE KO male mice in the prevention group treated with SB657510A or vehicle. Lanes 1-4 were from SB657510A group; Lanes 5-11 were from Vehicle group.

**Supplement Figure VII C-D.** Western blot for nitrotyrosine in the aortas of ApoE KO male mice in the prevention group treated with SB657510A or vehicle. Lanes 1-4 were from SB657510A group; Lanes 5-11 were from Vehicle group.

**Supplement Figure VIII.** Immunohistochemical localization of nitrotyrosine (A and B) and 4-HNE (C and D) in aortas of ApoE KO male mice treated with vehicle (A and C) or SB657510A (B and D). Arrows indicate the immunoreactions in the atherosclerotic lesion.
Supplement Figure I.

ApoE KO  ApoE/UII DKO

Nitrotyrosine

HNE

β-Actin

A

Ratio of Nitrotyrosine/Actin

B

Ratio of HNE/Actin

*
Supplement Figure II A.

ApoE KO  ApoE/UII DKO

<table>
<thead>
<tr>
<th>sEH</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Image</strong></td>
<td><strong>Image</strong></td>
</tr>
</tbody>
</table>

![Image of Western Blot Analysis](image)

![Graph showing Ratio of sEH/Actin](image)

**Graph:**
- Y-axis: Ratio of sEH/Actin
- X-axis: ApoE KO, ApoE/UII DKO
- Bars for ApoE KO show higher sEH/Actin ratio compared to ApoE/UII DKO, with a significant difference indicated by the asterisk (*)
Supplement Figure II B.
Supplement Figure III.
Supplement Figure IV.

The graph shows the relationship between blood glucose (mmol/dl) and time (min) for two conditions: SB and V. The graph indicates that blood glucose levels rise initially and then decline over time. The data is represented with error bars, and significance levels are indicated with asterisks: **. The x-axis represents time in minutes, ranging from 0 to 120, while the y-axis represents blood glucose levels, ranging from 0 to 30.
Supplement Figure V.
Supplement Figure VI.

SB657510A

Vehicle
Supplement Figure VII.

A

HNE

B

Actin

C

Nitrotyrosine

D

Actin
Supplement Figure VIII.
Supplement Table I: Effect of urotensin II gene deletion on visceral fat, and heart and lung weight compared to wildtype mice fed the same high-fat diet.

<table>
<thead>
<tr>
<th></th>
<th>Visceral fat weight (g)</th>
<th>Heart weight (g)</th>
<th>Right lung weight (g)</th>
<th>Left lung weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UII KO</td>
<td>1.48±0.16*</td>
<td>0.15±0.004</td>
<td>0.114±0.004</td>
<td>0.049±0.001</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.55±0.13</td>
<td>0.15±0.005</td>
<td>0.119±0.008</td>
<td>0.048±0.001</td>
</tr>
</tbody>
</table>

Number of mice/group=13. * P<0.05
Supplement Table II: Blood pressure in mmHg of UII KO and wildtype mice fed HFD (n=13 mice/group). *P<0.05

<table>
<thead>
<tr>
<th></th>
<th>Mean arterial pressure</th>
<th>Systolic arterial pressure</th>
<th>Diastolic arterial pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>UII KO</td>
<td>77.35±1.97*</td>
<td>90.47±2.49*</td>
<td>63.57±2.12</td>
</tr>
<tr>
<td>Wildtype</td>
<td>89.5±1.1</td>
<td>112.35±2.25</td>
<td>71.45±0.96</td>
</tr>
</tbody>
</table>
Supplement Table III. Effect of the UT antagonist SB657510 on body weight gain and visceral fat in ApoE knockout mice fed high-fat diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gain of body weight (g)</th>
<th>Fat (g)</th>
<th>Heart (g)</th>
<th>Lung (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Prevention Group V</td>
<td>6.73±0.50</td>
<td>4.04±0.47</td>
<td>2.30±0.17</td>
<td>0.39±0.05</td>
</tr>
<tr>
<td>Regression group (I) V</td>
<td>2.86±1.39</td>
<td>0.048±0.97</td>
<td>2.99±0.21</td>
<td>0.31±0.053</td>
</tr>
<tr>
<td></td>
<td>-3.26±0.68*</td>
<td>-0.11±0.97</td>
<td>1.23±0.45*</td>
<td>0.48±0.065</td>
</tr>
<tr>
<td>Regression group (II) V</td>
<td>-4.2±1.5</td>
<td>-1.43±0.52</td>
<td>1.32±0.14</td>
<td>0.41±0.067</td>
</tr>
<tr>
<td></td>
<td>-5.56±1.39</td>
<td>-1.83±0.55</td>
<td>1.11±0.14</td>
<td>0.42±0.050</td>
</tr>
</tbody>
</table>

Prevention group: ApoE KO mice (6 weeks old) were placed on HFD containing either 50mg/kg/day SB657510A (SB) or 1% methyl cellulose (V) for 10 weeks. Regression group (I): after 10 weeks on HFD, ApoE KO mice were continued on HFD containing either 50mg/kg/day SB657510A (SB) or 1% methyl cellulose (V) for 10 weeks. Regression group (II): After 10 weeks on HFD, ApoE KO mice were placed on normal diet containing either 50mg/kg/day SB657510A (SB) or 1% methyl cellulose (V) for 10 weeks. SB657510A significantly reduced weight gain and visceral fat without affecting food intake. Means ± SEM; *P<0.05.