Urotensin II Receptor Knockout Mice on an ApoE Knockout Background Fed a High-Fat Diet Exhibit an Enhanced Hyperlipidemic and Atherosclerotic Phenotype


Rationale: Expression of the vasoactive peptide Urotensin II (UII) is elevated in a number of cardiovascular diseases. Here, we sought to determine the effect of UII receptor (UT) gene deletion in a mouse model of atherosclerosis.

Methods and Results: UT knockout (KO) mice were crossed with ApoE KO mice to generate UT/ApoE double knockout (DKO) mice. Mice were placed on a high-fat Western-type diet for 12 weeks. We evaluated the degree of atherosclerosis and hepatic steatosis by histology. In addition, serum glucose, insulin, and lipids were determined. DKO mice exhibited significantly increased atherosclerosis compared to ApoE KO mice (P<0.05). This was associated with a significant increase in serum insulin and lipids (P<0.001) but a decrease in hepatic steatosis (P<0.001). UT gene deletion led to a significant increase in systolic pressure and pulse pressure. RT-PCR and immunoblot analyses showed significant reductions in hepatic scavenger receptors, nuclear receptors, and acyl-CoA:cholesterol acyltransferase (ACAT) expression in DKO mice. UII induced a significant increase in intracellular cholesteryl ester formation in primary mouse hepatocytes, which was blocked by the MEK inhibitor, PD98059. Hepatocytes of UTKO mice showed a significant reduction in lipoprotein uptake compared to wild-type mice.

Conclusions: We propose that UT gene deletion in an ApoE-deficient background promotes downregulation of ACAT, which in turn attenuates hepatic lipoprotein receptor-mediated uptake and lipid transporter expression. As the liver is the main organ for uptake of lipoprotein-derived lipids, DKO leads to an increase in hyperlipidemia, with a concomitant decrease in hepatic steatosis, and consequently increased atherosclerotic lesion formation. Furthermore, the hypertension associated with UT gene deletion is likely to contribute to the increased atherosclerotic burden. (Circ Res. 2009;105:686-695.)

Key Words: urotensin II receptor | aorta | liver | lipid | hypercholesterolemia | ACAT
UII was found to be elevated in human atherosclerotic arteries. In fact, we were the first to show increased mRNA and protein levels of UII in atherosclerotic human aortae.7 Furthermore, we demonstrated that UT mRNA levels were also increased in diseased human aortae. Later on, we demonstrated that UII protein and mRNA levels were significantly increased in arteries of patients with coronary atherosclerosis compared to normal coronary arteries.8 These findings suggest a potential role for UII in the pathogenesis of coronary atherosclerosis.

Our findings are supported by Maguire et al who found prominent UII immunoreactivity in atherosclerotic plaques of coronary arteries as well as in the thickened intima of failed saphenous vein grafts of explanted human hearts.9 In addition to increased UII levels in atherosclerotic lesions, reports have demonstrated increased UII levels in plasma of patients with atherosclerotic disease.10,11 On the other hand, UT mRNA was shown to be significantly upregulated and UII radio-ligand binding was significantly elevated in the aortae of apolipoprotein E (ApoE) knockout (KO) mice, an established model for the study of atherosclerosis.12 Furthermore, recent studies have shown that UII induced foam cell formation through increased acetyl-LDL–induced cholesterol ester accumulation without affecting scavenger receptor A activity; and it increases monocyte chemotaxis.13,14 UT is expressed in monocytes, and its mRNA and protein expression is induced by inflammatory cytokines through stimulation of NF-κB.7,14 Thus, both UII and UT are upregulated in atherosclerotic arteries of humans and in aortae of ApoE KO mice. Also UII induces SMC proliferation alone and in a synergistic fashion with oxidized LDL, a major contributor to atherosclerosis. These data support a role for UII as a contributing factor in the pathophysiology of atherosclerosis. To determine the pathological significance of UII in the etiology of experimental atherosclerosis, we examined the effect of UT deletion in ApoE KO mice fed a high-fat Western diet.

Methods

Study Design

We previously generated UTKO mice as described in the online supplement. 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.15 At the conclusion of the study period, mice were fasted for 4 hours, weighed, and then euthanized by exsanguination under anesthesia. We then collected serum and organs such as heart, aorta, lungs, liver, kidneys, and spleen. Each organ was weighed and then fixed in 10% formalin or flash frozen in liquid nitrogen and stored at −80°C.

Details regarding strain development, tissue histology, serum lipids, serum insulin, serum glucose, real-time RT-PCR, Western blotting, immunofluorescence, lipid uptake, acyl-CoA:cholesterol acyltransferase (ACAT) activity assay, and statistics are in the supplemental data (available online at http://circres.ahajournals.org).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Hemodynamics

In light of the vasoactive properties of UII and the fact that hypertension is a prominent risk factor in the development of atherosclerosis,16 we assessed hemodynamic profiles of UTKO mice. The latter exhibited significantly elevated systolic blood pressure and pulse pressure compared to wild-type (WT) mice (Figure 1).

Gross Anatomy

We measured body weight at the beginning and end of the 12-week study period. Interestingly, we found that UT KO mice had a significant reduction in body weight gain compared to WT mice (P<0.001; Online Table I). Of note, double knockout (DKO) mice experienced the least weight gain of all the strains. Specifically, DKO mice had significantly lesser weight gain compared to WT mice and ApoE KO mice (P<0.05), but not compared to UT KO mice.

We also measured organ weights at time of sacrifice including heart, right and left lungs, liver, right and left kidneys, and spleen. Interestingly, UT KO mice exhibited significantly reduced liver mass compared to WT mice (P<0.01; Online Table I), however all other organs had comparable weights. Similar to UT KO mice, DKO mice also had decreased liver weights, which was significant when compared to both WT and ApoE KO mice (P<0.05). Of note, DKO mice had significantly increased heart, lungs, kidneys, and spleen weights compared to all other strains (P<0.05). This latter data are especially remarkable in light of the fact that DKO mice had the least body weight gain.

Lesion Area Fraction

WT C57BL/6 mice demonstrated negligible atherosclerosis after exposure to a high-fat diet for 12 weeks (Figure 2A–D). Interestingly, UT KO animals also demonstrated negligible lesion formation. Conversely, ApoE KO mice exhibited apparent atherosclerosis especially in the aortic arch with a
few small lesions in the thoracic and abdominal aorta. Remarkably, DKO mice exhibited a significant increase (54%) in lesion area fraction compared to ApoE KO mice (4.23±0.41% versus 6.59±0.59%, P<0.001; Figure 2E).

Of note, 2 of 11 aortae from the DKO mice developed abdominal aortic aneurysms, a phenomenon that was not observed in any other strains (Online Figure II). The first of these 2 cases exhibited such extensive atherosclerosis that >85% of the aorta was diseased. The latter 2 aortas were not included in the lesion area analysis because of their extreme deviation from the mean.

Lesion Histology
As expected from the en face aortic analysis, lesions were absent from WT and UT KO aortic root (Figure 2F, 2G, 2J, and 2K). On the other hand, ApoE KO and DKO animals both presented with lesions of varying sizes. Atherosclerotic plaques varied from Type 1 lesions exhibiting intimal thickening to type V lesions bearing large fatty cores with overlying fibrous caps (Figure 2H, 2I, 2L, and 2M). All lesions had pronounced inflammatory infiltrates. No major histological differences in lesions were noted between ApoE KO and DKO animals as evident by Mac 2, α-SMC actin, and elastin staining (Online Figure III), however the largest lesions were found in DKO animals.

Serum Glucose, Insulin and Lipids
Serum glucose was similar for all strains; however, DKO mice exhibited a marked increase in serum insulin levels (P<0.001, Figure 3A). UT KO mice had serum total cholesterol levels comparable to WT mice. As expected, ApoE KO mice demonstrated a very significant increase in serum total cholesterol compared to WT and UT KO animals (P<0.001; Figure 3B), which is in accordance with levels reported by others.17 Remarkably, DKO had even greater serum total cholesterol levels, which were significantly increased compared to all other strains including ApoE KO (P=0.001; Figure 3B).

Fractioning of serum lipoprotein by HPLC showed increased content of cholesterol in the very low-density lipoprotein (VLDL)-sized fraction in ApoE KO compared to WT and UT KO, and was greatest in DKO mice (Figure 3C). Cholesterol content of LDL was higher in UT KO, ApoE KO, and DKO compared to WT mice. Cholesterol content of high-density lipoprotein (HDL) was lower in ApoE KO and DKO mice compared to UT KO and WT mice. Interestingly, cholesterol content was elevated in both the LDL and HDL fractions in UT KO mice compared to WT mice.

Triglyceride content of the VLDL-sized fraction was higher in ApoE KO mice compared to WT or UT KO mice, and again it was highest in DKO mice (Figure 3D). In addition, DKO also had higher free triglycerides than both ApoEKO and WT mice, however the highest levels were seen in UTKO mice (Figure 3D). It is worth mentioning that the hyperlipidemic and atherosclerotic phenotype seen in DKO mice is seen in male and female mice only when fed a high-fat diet (data not shown).

Liver Histology
The significant reduction in liver size in both UT KO and DKO mice led us to analyze liver histology. Analysis of H&E-stained livers demonstrated that WT had apparent hepatic steatosis (Figure 4A). Of note, the hepatic steatosis was significantly decreased in UTKO mice compared to WT mice (P<0.05) (Figure 4B and 4E). Similarly, DKO mice had markedly reduced hepatic steatosis which was significant when compared to all other strains (P<0.01; Figure 4D and 4E).

Lipid Uptake
To assess whether the observed reduction in hepatic steatosis was related to decreased lipid uptake we performed a lipoprotein binding and uptake assay of LDL and VLDL remnants in cultured mouse primary hepatocytes from WT, ApoE KO, and UTKO mice. Interestingly, there was a marked and significant reduction in LDL binding (data not shown) and uptake in UTKO mouse hepatocytes compared to both WT and ApoE KO mice (Figure 5).
found a significant decrease in βVLDL binding and a small but not significant reduction in βVLDL uptake compared to WT cells (data not shown), supporting a role of ApoE in the uptake of βVLDL. The decreased LDL uptake in UTKO mice supports the hypothesis of reduced lipid uptake in DKO mice generating reduced hepatic steatosis (Figure 4) and increased hyperlipidemia (Figure 3).

Expression of Liver Lipid Transporters and Nuclear Receptors

The marked reduction in hepatic steatosis in DKO mice and the reduction in lipid uptake in UTKO hepatocytes suggested that UT deficiency may be associated with a reduction in lipid transporters or uptake receptors. Therefore, we analyzed mRNA levels from liver tissue of the 4 strains of mice. Specifically, we analyzed the mRNA levels of the LDL receptor (LDLR), the LDLR-related protein (LRP), the ATP binding cassette-A1 (ABCA1), ATP binding cassette-G5 (ABCG5), scavenger receptor-A1 (SR-A1), and SR-B1. The mRNA expression for these transporters and receptors were reduced in UTKO mice compared to WT mice but this reduction did not reach statistical significance. In contrast, LRP, ABCA1, SR-A1, and SR-B1 were all significantly elevated in ApoE KO mice (P<0.05) compared to WT and UT KO (Online Table III). Of note, the mRNA levels for ABCA1, SR-A1, and SR-B1 were all significantly reduced in DKO mice compared to ApoE KO mice (P<0.05) (Figure 6A through 6C and Online Table III). Protein levels of SR-A1 were also significantly reduced in UT KO and DKO mice compared to WT and ApoE KO, respectively (P<0.05;
Interestingly, LRP levels remained significantly elevated in DKO mice compared to WT and UT KO mice (Online Table III). The mRNAs for LDL receptor and ABCG5 did not differ significantly among the different experimental groups.

These lipid transporters and uptake receptors are transcriptionally regulated by a group of nuclear receptors including liver X receptor-α (LXR-α), as well as the peroxisome proliferator activated receptors (PPARs) α, δ, and γ. Therefore, mRNA analysis of the latter receptors was evaluated. The results indicated that mRNAs for all the nuclear receptors were reduced in UT KO mice compared to WT mice, but this reduction did not reach statistical significance. In contrast, both LXR-α and PPAR-δ mRNA expression levels were significantly increased in ApoE KO mice compared to WT and UT KO mice (P<0.01). Importantly, there was a significant reduction in the expression of LXR-α, PPAR-δ, and γ in DKO mice compared to ApoE KO mice (Figure 6D; P<0.01). Western blot analysis showed that although expression of LXR-α protein was not significantly different between WT and UT KO, it was significantly elevated in ApoE KO mice. In contrast, DKO mice had a significantly reduced levels of LXR-α protein compared to ApoE KO mice (P<0.05; Figure 7B). There was no significant difference in the protein levels of other receptors and nuclear proteins between ApoE KO and DKO mice.

Figure 7A). Interestingly, LRP levels remained significantly elevated in DKO mice compared to WT and UT KO mice (Online Table III). The mRNAs for LDL receptor and ABCG5 did not differ significantly among the different experimental groups.

UII Upregulates ACAT Expression and Activity
Because UII has been previously shown to induce the expression of ACAT in macrophages, we sought to determine whether the reverse occurred in our mouse model of UT gene deletion. Indeed, we found that ACAT protein expression was significantly reduced in DKO mice compared to their ApoE KO littermates (Figure 7C). Furthermore, we determined whether UII had any effects on ACAT activity in hepatocytes. To this end, we performed a cholesterol esterification assay in primary mouse hepatocytes in which the basal level of intracellular cholesterol esterification was compared to cells treated either with an ACAT inhibitor, UII alone, UII + ACAT inhibitor, UII + a mitogen activated protein kinase and extra-cellular-signal regulated kinase kinase (MEK) inhibitor (PD98059), UII + a p38 kinase inhibitor (SB203580), or UII + ACAT inhibitor + PD98059 (Figure 8). Interestingly, we found that UII significantly increased ACAT activity evident by significantly elevated cholesterol esterification in mouse primary hepatocytes. UII-induced ACAT activity was abolished by the addition of the MEK inhibitor PD98059, but not by the p38 kinase inhibitor, SB203580. Furthermore, the addition of both the MEK inhibitor and the ACAT inhibitor simultaneously in the presence of UII did not further reduce cholesterol esterification than by addition of the UII and ACAT inhibitor. These results suggest that cholesteryl ester formation is inducible with UII administration in hepatocytes as in macrophages.
and this activation is dependent on ACAT and MEK. Furthermore, immunohistochemical analysis showed that ACAT protein expression was reduced specifically in the DKO hepatocytes (Figure 7D through 7G), suggesting impaired UII-induced ACAT expression.

**Discussion**

Here we show that UT gene deletion leads to (1) elevated systolic and pulse pressure, (2) decreased LDL uptake in the liver, (3) hypertriglyceridemia, (4) reduced hepatic steatosis, and (5) reduced liver mass. In addition, UT gene deletion combined with ApoE gene deletion also leads to: (1) hyperlipidemia, (2) hyperinsulinemia, and (3) exaggerated atherosclerotic lesion formation in the aorta.

The elevated serum lipids in DKO animals is likely to be the main contributor to the significant increase in atherosclerotic burden compared to ApoE KO mice. However, the elevated systolic pressure and hyperinsulinemia, two well-established risk factors of atherosclerosis, may also potentially contribute to the enhanced atherosclerotic state in DKO animals. Indeed together, the hyperlipidemia, hyperinsulinemia, and hypertension provide strong mechanistic evidence for the observed atherosclerosis in DKO mice.

UII has been shown as a potent vasoconstrictor of main blood vessels, conversely there was a strong evidence for vasodilatory role for UII in peripheral vessels.1,18–20 These findings are further supported by the report that UII deletion in mice is associated with increased systolic pressure.21 Furthermore, the striking hyperinsulinemia in a euglycemic
context in DKO mice suggests that these mice have insulin resistance. The hyperinsulinemia may be a consequence of the exaggerated hyperlipidemia in DKO mice; however, it may also be a direct result of UT deficiency. Indeed, UII has previously been shown to inhibit insulin secretion in the perfused rat pancreas.22

The liver is an integral organ in the regulation of lipid metabolism. UII and UT are both expressed in the liver, and recent data have suggested an important role for UII in chronic liver disease and metabolic syndromes,1,23–25 Therefore, it was of great interest to find that UT gene deletion led to significantly reduced liver mass and hepatic steatosis, regard-

Figure 6. mRNA levels of lipid transporters, lipid uptake receptors, and nuclear receptors as measured by RT-PCR. *P<0.05 vs WT and UT KO; #P<0.05 vs ApoE KO. Error bars indicate SEM.
less of strain background. The decreased steatosis is likely attributable to either a reduction in liver uptake and storage of lipids, increased lipid export, or an increase in lipid oxidation.

To determine whether the decreased steatosis and associated hyperlipidemia was in fact a result of reduced lipid uptake, we performed a lipoprotein uptake assay using hepatocytes isolated from WT, ApoE KO, and UTKO mice. We found that UTKO exhibited a significant reduction in LDL binding and uptake compared to both WT and ApoE KO mice, supporting the hypothesis that the reduced hepatic steatosis in UTKO and DKO mice is the result of reduced lipid uptake.

The reduced lipid uptake suggests that there would be a reduction in the abundance or activity of lipoprotein uptake receptors. Therefore, we analyzed the mRNA and protein expression of lipid transporters including the LDL receptor, LRP, ABCA1, SR-A1, and SR-B1 in the livers from all 4 strains. Interestingly, we found significant reductions in the mRNA levels of ABCA1, SR-A1, and SR-B1 in DKO mice. The protein

Figure 7. Pictures of representative Western blots, as well as graphs demonstrating the quantification of the blots for SR-A1 (A), LXR-α (B), and ACAT (C), normalized to Actin, Histone H1, and Actin, respectively (measured with arbitrary units). Photomicrographs of ACAT immunoreactivity in WT (D), UTKO (E), ApoEKO (F), and DKO (G) mouse livers. #P<0.05 vs WT; *P<0.05 vs WT and UTKO; †P<0.05 vs ApoE KO. Error bars indicate SEM.
expression of SR-A1 was also significantly decreased in DKO mice. Therefore, the reduction in lipoprotein uptake in the liver of DKO animals may be a consequence of the reduction in this latter receptor. This is in accordance with several studies which show that scavenger receptor deficiency increases plasma cholesterol and aggravates atherosclerosis in hyperlipidemic mice.26–29 Furthermore, overexpression of SR-B1 led to a significant decrease in plasma HDL and non-HDL cholesterol levels.30 Therefore, these latter studies support the notion that the reduction in scavenger receptor as observed in DKO mice can lead to serum hyperlipidemia.

To investigate whether the effect of UT deficiency plays a role upstream of the lipid transport and uptake receptors we analyzed the expression of nuclear receptors known to regulate the transcriptional expression of the former. Of note, we found that the mRNA levels for LXR-α, PPAR-δ, and PPAR-γ were all significantly decreased in DKO mice compared to ApoE KO mice. Furthermore, we went on to demonstrate that LXR-α was also significantly decreased at the protein level in DKO mice compared to ApoE KO mice. Therefore, the reduced levels of these nuclear receptors involved in transcriptional regulation of lipid transport and uptake receptors leads to the reduced expression of the latter, which in turn would account for reduced liver uptake of lipids and hence reduced hepatic steatosis. This is supported by a study which demonstrated that liver specific disruption of PPAR-γ attenuated hepatic steatosis.31 Consequently, the significant reduction of lipoprotein uptake by the liver resulted in significant increases in plasma cholesterol and triglycerides, which in turn likely caused increased atherosclerotic burden in DKO mice.

Previously published reports have shown that UII induces ACAT expression in macrophages.13 In addition, we have previously shown a reduction in ACAT expression with UII blockade using SB-657510, a potent UII antagonist in vivo.32 Therefore, we were interested in identifying whether there was any effect on ACAT expression and activity in our UT deletion mouse model. Interestingly, we found that there was a significant reduction in ACAT protein expression in DKO mice compared to ApoE KO mice. Next, we examined whether UII also mediates alterations in ACAT activity.

Indeed, UII significantly induced cholesterol esterification in primary mouse hepatocytes, and this effect was completely abolished with the addition of PD98059, a MEK inhibitor. Therefore, this supports the notion that UII signaling blockade by UT gene deletion attenuates ACAT activity, thus reducing cholesterol esterification and storage in the liver. Indeed, others have shown decreased hepatic cholesterol with low ACAT activity.33–36 In addition, Sahlin et al previously demonstrated increased hepatic steatosis was associated with increased ACAT activity.37 Furthermore, inhibition of UII-induced ACAT activity with a MEK inhibitor is in complete agreement with a previous study by Watanabe et al, who demonstrated MEK-sensitive UII-induced ACAT expression in macrophages.13

Here we provide strong evidence for the mechanism of hyperlipidemia in DKO mice. When we include this latter data with the fact that DKO mice are hyperinsulinemic and that UT gene deletion leads to a hypertensive phenotype with significantly elevated pulse pressure, there is a clear stimulus for the increased atherosclerosis found in these mice. Indeed hypertension, hyperinsulinemia, and hyperlipidemia are all major risk factors for endothelial dysfunction and atherosclerosis.16,38 Furthermore, increased systolic pressure and the severity of atherosclerosis may account for the increased heart weight seen in DKO mice.

To summarize, we show that UT gene deletion leads to increased blood pressure and hypertriglyceridemia, as well as decreased body weight gain, hepatic steatosis, and liver size in mice with a WT genetic background fed a high-fat diet. While UT deletion in an ApoE KO genetic background leads to significantly increased atherosclerotic lesion formation, serum lipids, as well as a significant decrease in body weight gain, liver mass, and hepatic steatosis. Here we show that the DKO phenotype of increased serum lipids and decreased hepatic lipid content is the result, at least in large part, of decreased lipoprotein uptake or decreased cholesterol esterification and storage resulting from attenuated ACAT expression and activity in our UT deletion mouse model. The combination of increased serum lipids, hyperinsulinemia, and hypertension in these mice clearly supports the increased atherosclerosis found in these mice.
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Disclosures
None.

References


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Methods

Animals

The study utilized 4 mouse strains including WT (C57BL6/J; ApoE^{+/+}, UT^{+/+}; n=54), UT KO (ApoE^{+/+}, UT^{--}; n=35), ApoE KO (ApoE^{--}, UT^{+/+}; n=11), and UT/ApoE double knockout mice (DKO, ApoE^{--}, UT^{--}; n=20). All mice utilized in this study were male. The generation of UT KO was previously described.  

Briefly, gene targeting was performed in murine E14.1 ES cells, replacing the single coding exon of the UT receptor locus with a positive selection cassette containing the neomycin phosphotransferase gene driven by the phosphoglycerate kinase I promoter. The KO strategy resulted in the deletion of the whole UT open reading frame. These animals were backcrossed at least 10 times to the C57BL6/J background and then bred to homozygosity. The UT KO mice were then crossed with the ApoE KO to generate DKO mice. The ApoE KO mice were ordered from Taconic (B6.129P2-ApoelmoI1Unc N11). Genotypes were verified by PCR and gel electrophoresis (Supplemental Figure 1).

Blood pressure analysis

Measurements were taken from adult male mice as previously described.  

Briefly, mice were firstly anesthetized with ketamine-xylazine (i.m. : 87 mg/kg-13 mg/kg) and the jugular vein and the carotid artery were thereafter cannulated for intravenous injection and blood pressure monitoring plus blood sampling, respectively.
**Tissue Histology**

Formalin fixed aortae were surgically cleaned of all adventitial fat and extraneous tissue. These aortae were then stained with Sudan IV and pinned to a rubber surface for *en face* presentation. Flash frozen aortae were embedded in OCT freezing media for cryostat sectioning (10 µm thickness). The heart, lungs, spleen, liver and kidneys were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin & eosin for histologic analysis. The aortic root was sequentially sectioned and immunostained with Mac 2, α-SMC actin and elastin as described previously. From the liver sections, 5 random photomicrographs were taken (100X magnification) from each animal. These photomicrographs were then downloaded into an image analysis program, *Image ProPlus*, which allowed for the quantification of hepatic steatosis in the livers.

**Serum Lipids**

Fasting blood samples were harvested at the time of sacrifice. Serum was then collected and stored at −20°C. These serum samples were then analyzed for serum total cholesterol using a cholesterol assay kit (BioVision, CA, USA) according to the manufacturer’s instructions. Similarly, serum total glycerol (a surrogate for triglyceride levels) was determined using a triglyceride analysis kit (Sigma, St-Louis; Missouri). Protocol was carried out as per manufacturer’s instructions.

In addition, serum from individual animals 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.

**Real Time RT-PCR analyses**

Liver samples were retrieved from storage at -80°C for RNA extraction using Trizol (Invitrogen, Ontario, Canada) and performed as per the manufacturer’s instructions. RNA integrity was then verified for all samples by evaluating the clarity of the 28S and 18S bands and verifying that the bands were 28S>18S by gel electrophoresis. Following this, 1 μg of each RNA sample was reverse transcribed to synthesize cDNA using the Omniscript reverse transcriptase kit from Qiagen (Ontario, Canada). From this, 1 μl of cDNA was used to amplify target genes using specific primers (Table 2 supplement). Genes were amplified individually in the LightCycler (Roche, Montreal, Canada) using Quantitect SYBR Green reagent (Qiagen, Ontario, Canada) with the following amplification conditions: DNA polymerase activation, 15 minutes at 95 °C followed by 40 cycles of denaturation, annealing and extension for 15 seconds at 94°C, 20 seconds at 50-60°C (depending on primer Tm) and 20-30 seconds at 72°C (depending on amplicon size), respectively. Primers were designed using PrimerQuest biotool [www.idtdna.com] (Supplemental Table 2). All values for mRNA expression determined by RT-PCR are expressed as the ratio of the copy number of the mRNA transcript of interest to the copy number of the mRNA transcript of the housekeeping gene GAPDH. The copy number of mRNA
transcript is determined by the threshold cycle of the PCR reaction for each sample. A homogeneous amplification of the products was rechecked by analyzing the melting curves of the amplified products.

**Western Blotting**

Western blotting was performed as previously described, with goat polyclonal anti-LXR-α antibody (1:300 dilution), SR-B1 (1:350), SR-A1 (1:500); ABCA1 (1:500) and PPARγ (1/300); and a secondary antibody conjugated to peroxidase (1:5000 dilution). Protein levels were normalized to the housekeeping gene Histone H1 or actin as previously described. Protein bands were then quantified using arbitrary units (AU) with the image analysis program, *Image J*.

**Isolation of LDL**

LDL was isolated from a peripheral blood sample by sequential density ultracentrifugation. The concentration of LDL was calculated using the Markwell Lowry protein assay. Acetylated LDL was prepared by sequential addition of acetic anhydride. βVLDL was isolated from the plasma of apoE deficient mice and then re-enriched by incubation with bacterially expressed apoE (kind gift of V. Narayanaswami).

**Isolation and assay of hepatocytes**

All mice were maintained on a normal chow diet in a 12-h light/12-h dark schedule and used between the ages of 4 and 6 months. All experiments
performed were in accordance with protocols approved by the McGill University Animal Care Committee. Primary mouse hepatocytes were isolated from C57BL/6, C57BL/6 Apolipoprotein E-/-, and C57BL/6 UT-/- mice. Primary hepatocytes were isolated from these mice by liver collagenase perfusion according to the established protocols. \(^9,10\) Briefly, the 12-well plates were precoated with fibronectin (25 µg/well) and then the hepatocytes were seeded at an initial density of 1 x 10^6 cells/well in Williams' Medium E with 10% fetal bovine serum (containing penicillin (100 units/ml), streptomycin sulfate (100 units/ml), Fungizone (250 ng/ml). Cells were incubated with 10% lipoprotein deficient serum for 24 h prior to the uptake and binding assays. For the binding assay, lipoproteins (LDL and \(\beta\)VLDL) were radiolabeled with \(^{125}\)I using iodobeads (Pierce; according to manufacturer’s instructions). The binding assay was performed at 4°C and increasing concentrations of labeled lipoproteins (10-1000 ng/well) were left on the cells for 1 h before extensive washing and determination of bound radioactivity (normalized to non-specific binding and total cell protein) \(^{12}\). For the uptake assay, [3H]cholesteryl oleate (15 µCi/well) was incorporated into lipoproteins according to Vassiliou et al. \(^{12}\) Radiolabeled lipoproteins (25 µg) were added to the cells (hepatocytes and macrophages) for 30 minutes, 1 h or 4 h, and after extensive washing, the amount of radiolabel taken up by the cells was normalized for non-specific uptake and total cell protein. For the ACAT assay, hepatocytes were incubated with \([^3\text{H}]\)-cholesterol incorporated into FBS for 48 h to uniformly label the cells. Then the cells were switched to media containing cold 10% FBS without and with ACAT inhibitor (58035; 1µg/mL), UII
(500nM), or PD98059 (a MEK inhibitor; 20μM). After 24h, cells were washed 3 times in PBS, and radioactive lipids were extracted with hexane. Lipids were separated by thin layer chromatography using hexane/diethyl ether/acetic acid (105:45:1.5, v/v/v) as a solvent system. Cholesterol and cholesteryl ester (CE) spots were scraped from the plate and radioactivity determined by liquid scintillation. Results are expressed as percent CE of the total radioactive cholesterol (free and esterified) ±SD.

**Mouse Peritoneal Macrophages**

Peritoneal macrophages from wild-type and ApoE knockout mice were collected 3 days after an i.p injection of thioglycollate and seeded on 24-well plates in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS. Macrophages were transfected with either UT small interfering RNA (siRNA) or control scRNA (Santa Cruz, CA) for 24 hours and then treated with 50 µg/mL acetyl-LDL in DMEM media containing 0.2% BSA. Cells were treated for 24 hours before cholesterol efflux studies.

**Cholesterol Mass Analysis**

Cholesterol efflux was performed in DMEM containing 0.2% BSA in presence of with and without 25 µg/mL human apoAI (both from Merck, Darmstadt, Germany)and HDL (20 µg/mL). After 8 hours incubation, medium was aspirated; cells were lysed, and counted for ³H-activity by gas-liquid chromatography. Cholesterol efflux was calculated as the percentage of ³H-activity in the medium from the total ³H-activity in medium and lysate.
**Statistical analyses**

All values are presented as mean ± standard error. Multi-group comparisons were analyzed using ANOVA with the Tukey post-hoc test. Direct two group comparisons were carried out using the student’s t-Test. A P value <0.05 was considered statistically significant. All statistical analyses were carried out using SPSS version 11.5.
Results

**Effect of UT inhibition on cholesterol efflux in macrophages**

In order to establish the contribution of UT in the regulation of cholesterol efflux, an siRNA approach was used to reduce UT gene expression. The efficacy of the siRNA incubation for reducing UT protein expression was monitored by Western blotting. Reduction in target protein expression after siRNA incubations averaged 40%, whereas control scRNA did not influence UT expression (5%). Reduction of UT expression did not significantly influence basal cholesterol efflux to apoA-I in both WT and ApoE KO-derived cells. In the WT macrophages, cholesterol efflux in control, scrRNA and siRNA treated cells was 9.6±1.0%, 10.2±0.5%, 11.1±1.0%; respectively. In the ApoE KO macrophages, cholesterol efflux in control, scrRNA and siRNA-treated cells was 11.3±0.8%, 10.8±0.3%, 10.3±0.3%; respectively. Similarly, Western blot analyses revealed no significant difference in the protein expression of lipid transporters or nuclear proteins in the above mentioned groups (data not shown).

**Lung Histology**

Histological sections from lungs of DKO mice revealed the presence of pulmonary edema and a slight thickening of pulmonary arteries compared to other groups (Figure 3 A-B supplement). The kidneys of DKO mice exhibited mild glomerular sclerosis as evident by increased deposition of collagen (data not shown).


**Supplementary table 1**: Change in body weight over the course of the study, and organ weight in the four experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=54)</th>
<th>UT KO (n=35)</th>
<th>ApoE KO (n=11)</th>
<th>DKO (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW week 0</td>
<td>21.7 ± .24</td>
<td>22.2 ± .44</td>
<td>21.9 ± .47</td>
<td>22.8 ± .61</td>
</tr>
<tr>
<td>BW week 12</td>
<td>45.9 ± .39</td>
<td>43.2 ± .49</td>
<td>44.6 ± .81</td>
<td>41.7 ± 1.06</td>
</tr>
<tr>
<td>BW Change</td>
<td>24.2 ± 0.46</td>
<td>**20.9 ± 0.56 ***</td>
<td>22.8 ± 0.58</td>
<td><strong>18.9 ± 1.05 * †</strong></td>
</tr>
<tr>
<td>HW/BW</td>
<td>0.0030 ± 0.00004</td>
<td>0.0031 ± 0.00006</td>
<td>0.0033 ± 0.00011</td>
<td><strong>0.0044 ± 0.00014 * †</strong></td>
</tr>
<tr>
<td>RLV/BW</td>
<td>0.0025 ± 0.00003</td>
<td>0.0024 ± 0.00004</td>
<td>0.0026 ± 0.00008</td>
<td><strong>0.0030 ± 0.00010 * †</strong></td>
</tr>
<tr>
<td>LLW/BW</td>
<td>0.0011 ± 0.00002</td>
<td>0.0012 ± 0.00002</td>
<td>0.0013 ± 0.00005</td>
<td><strong>0.0015 ± 0.00005 * †</strong></td>
</tr>
<tr>
<td>LW/BW</td>
<td>0.089 ± 0.0018</td>
<td>**0.078 ± 0.0031 ***</td>
<td>0.078 ± 0.0040</td>
<td><strong>0.062 ± 0.0031 * †</strong></td>
</tr>
<tr>
<td>RKW/BW</td>
<td>0.0038 ± 0.00005</td>
<td>0.0040 ± 0.00008</td>
<td>0.0042 ± 0.00011</td>
<td><strong>0.0051 ± 0.00020 * †</strong></td>
</tr>
<tr>
<td>LKW/BW</td>
<td>0.0037 ± 0.00004</td>
<td>0.0039 ± 0.00007</td>
<td>0.0040 ± 0.00008</td>
<td><strong>0.0049 ± 0.00021 * †</strong></td>
</tr>
<tr>
<td>SW/BW</td>
<td>0.0028 ± 0.00007</td>
<td>0.0028 ± 0.00009</td>
<td>0.0034 ± 0.00014</td>
<td><strong>0.0047 ± 0.00040 * †</strong></td>
</tr>
</tbody>
</table>

BW, body weight; HW, heart weight; RLV, right lung weight; LLW, left lung weight; LW, liver weight; RKW, right kidney weight; LKW, left kidney weight; SW, spleen weight. *P<0.05 vs wild type; † P<0.05 vs ApoE KO.
**Supplementary table 2.** Primers used for genotyping and liver mRNA analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-F (WT)</td>
<td>GCCTAGCCGAGGGAGAGCCG</td>
</tr>
<tr>
<td>ApoE-R (WT)</td>
<td>TGTGACTTGGAGCTCTGACGC</td>
</tr>
<tr>
<td>ApoE-F (KO)</td>
<td>TTGGTGACCGCATCCGAGG</td>
</tr>
<tr>
<td>ApoE-R (KO)</td>
<td>GCCTCGTCTGCAGTTCATTCA</td>
</tr>
<tr>
<td>NEO-F</td>
<td>CCATTCGACCACAAAGCGAAACA</td>
</tr>
<tr>
<td>NEO-R</td>
<td>CCATGATATTCGGCAAGCAGGCA</td>
</tr>
<tr>
<td>UT-F</td>
<td>CTCTTCTGGGCATGCTTTTCTGC</td>
</tr>
<tr>
<td>UT-R</td>
<td>GATGCGAGCTGTGGCGTAAGTG</td>
</tr>
<tr>
<td>LDLR-F</td>
<td>AAT GAG GAG CAG CCA CAT GGT A</td>
</tr>
<tr>
<td>LDLR-R</td>
<td>TGT TGA TGT TCT TCA GCC GCC A</td>
</tr>
<tr>
<td>LRP-F</td>
<td>AGG AGC AGG TTG TTA GTC AGC A</td>
</tr>
<tr>
<td>LRP-R</td>
<td>ACT CGC CGC TTA TAC CAG AAC A</td>
</tr>
<tr>
<td>ABC-A1-F</td>
<td>TGA AGC CTG TCC AGG AGT TC</td>
</tr>
<tr>
<td>ABC-A1-R</td>
<td>ATG ACA AGG AGG ATG GAA GC</td>
</tr>
<tr>
<td>SR-A1-F</td>
<td>AAG AAC AGG CCG AGC TGG AA</td>
</tr>
<tr>
<td>SR-A1-R</td>
<td>ACC AGT TTG TCC AGT AAG C</td>
</tr>
<tr>
<td>SR-B1-F</td>
<td>TGC AGC TGA GGC TCT ACA TCA A</td>
</tr>
<tr>
<td>SR-B1-R</td>
<td>AAC CAC AGC AAC GGC AGA ACT A</td>
</tr>
<tr>
<td>LXR-F</td>
<td>TGA CTT TGC CAA ACA GCT C</td>
</tr>
<tr>
<td>LXR-R</td>
<td>AGC ATG ACT CGA TTG CAG AG</td>
</tr>
<tr>
<td>PPAR-α-F</td>
<td>AAT TTG CTG TGG AGA TCG G</td>
</tr>
<tr>
<td>PPAR-α-R</td>
<td>AGG TGT CAT CTG GAT GGT TGC</td>
</tr>
<tr>
<td>PPAR-δ-F</td>
<td>AGC CCA AGT TCG AGT TTG CTG</td>
</tr>
<tr>
<td>PPAR-δ-R</td>
<td>ACA GAA TGA TGG CCG CGA TGA A</td>
</tr>
<tr>
<td>PPAR-γ-F</td>
<td>AGG ACA TCC AAG ACA ACC TGC</td>
</tr>
<tr>
<td>PPAR-γ-R</td>
<td>TCT GCC TGA GGT CTG TCA TC</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>AAG AAG GTG GTG AAG CAG GCA</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>AGT TGC TGT TGA AGT CGC AGG A</td>
</tr>
</tbody>
</table>
**Supplementary table 3:** Liver mRNA analysis by RT-PCR

mRNA levels relative to GAPDH mRNA and normalized to the WT group. LXR: Liver X Receptor; PPAR: Peroxisome Proliferator Activated Receptor; LDLR: Low Density Lipoprotein Receptor; LRP: Oxidized LDL (lecithin-like) Related Peptide; ABC: ATP Binding Cassette transporter; SR: Scavenger Receptor. * indicates P<0.05 vs. WT & UT KO, † indicates P< 0.05 vs. ApoE KO.

<table>
<thead>
<tr>
<th>Genes</th>
<th>WT (n=8)</th>
<th>UT KO (n=8)</th>
<th>ApoE KO (n=7)</th>
<th>DKO (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>1.0 ± 0.18</td>
<td>0.95 0.28</td>
<td>2.3 ± 0.14*</td>
<td>1.5 ± 0.25†</td>
</tr>
<tr>
<td>SR-A1</td>
<td>1.0 ± 0.21</td>
<td>0.79 0.26</td>
<td>2.5 ± 0.17*</td>
<td>1.3 ± 0.27†</td>
</tr>
<tr>
<td>SR-B1</td>
<td>1.0 ± 0.11</td>
<td>0.97 0.16</td>
<td>1.6 ± 0.11*</td>
<td>1.4 ± 0.14†</td>
</tr>
<tr>
<td>LXR-α</td>
<td>1.0 ± 0.18</td>
<td>1.0 ± 0.14</td>
<td>1.8 ± 0.14*</td>
<td>1.4 ± 0.13†</td>
</tr>
<tr>
<td>PPARα</td>
<td>1.0 ± 0.08</td>
<td>0.96 ± 0.078</td>
<td>1.2 ± 0.09</td>
<td>1.1 ± 0.10</td>
</tr>
<tr>
<td>PPAR-δ</td>
<td>1.0 ± 0.12</td>
<td>0.96 ± 0.14</td>
<td>2.0 ± 0.12*</td>
<td>1.7 ± 0.16†</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.0 ± 0.16</td>
<td>0.88 ± 0.10</td>
<td>0.69 ± 0.23*</td>
<td>0.36 ± 0.14†</td>
</tr>
<tr>
<td>LDLR</td>
<td>1.0 ± 0.16</td>
<td>0.91 ± 0.25</td>
<td>0.86 ± 0.21</td>
<td>0.82 ± 0.25</td>
</tr>
<tr>
<td>LRP</td>
<td>1.0 ± 0.071</td>
<td>1.2 ± 0.11</td>
<td>1.99 ± 0.078*</td>
<td>1.99 ± 0.12*</td>
</tr>
</tbody>
</table>
**Figure 1 supplement. Genotyping analysis.**

DNA was isolated from WT mice (lanes 1-5), UT KO mice (lanes 6-10), ApoE KO mice (lanes 11-15), and DKO mice (lanes 16-20). This DNA was subjected to polymerase chain reaction (PCR) to amplify specific DNA segments with appropriate primers (Table 1). Amplicons were gel electrophoresed to verify appropriate amplicon sizes. Lanes 1,6,11,16: Ladder: bands demonstrating amplicons of 500, 400, and 300 BP from the top. Lanes 2,7,12,17: Presence of band (155bp) indicates an ApoE (+/+) genotype. Lanes 3, 8, 13, 18: Presence of band (250bp) indicates ApoE (−/−), since forward primer recognizes sequence in ApoE gene while reverse primer recognizes sequence in NEO gene. Lanes 4, 9, 14, 19: Presence of band (199bp) indicates NEO (+/+), which is gene used to replace both knockout genes (i.e. ApoE and UT). Lanes 5, 10, 15, 20: Presence of band (138bp) indicates UT (+/+).

**Figure 2 supplement: Aortic aneurysms.** Photographs of two aortae from DKO mice exhibiting aortic aneurysms (red arrow).

**Figure 3 supplement:** Immunohistochemical localization of α-SMC actin (A and C) and Mac2 (B and D) in aortic roots of ApoE KO (A and B) and DKO mice (C and D). Magnifications A and B X100, C and D X50.
Figure 4 supplement: Histological lung sections of DKO (A) and ApoE KO (B) mice. Note increased inflammatory infiltrate, pulmonary edema and thickening of pulmonary artery in the lungs of DKO mice. Magnifications X100.
**Figure 1**

|----|-----------|------------|------------|--------|-------|