Development of atherosclerotic lesions, one of the underlying causes of coronary heart disease (CHD), is a chronic pathological process with disorders of lipid metabolism and inflammation.1,2 Macrophages bind modified low-density lipoprotein (LDL) to eventually differentiate into lipid-laden foam cells, the prominent cells of advanced lesions in the intima-media. However, macrophages also express ATP-binding cassette transporter A1 (ABCA1) to efflux excess free cholesterol to extracellular acceptor, lipid-free apolipoprotein A1, which leads to generation of nascent high-density lipoprotein (HDL). This process enhances reverse cholesterol transport (RCT) to slow the progress of atherosclerosis.3 Both clinical and basic research studies have demonstrated the antiatherogenic properties of macrophage ABCA1.4,5 ABCA1 expression is regulated by liver X receptors (LXRs) α and β (LXR-α/β), the ligand-activated transcription factors.6 Synthetic LXR ligands inhibit atherosclerosis in animal models.7 Unfortunately, LXR activates fatty acid biosynthesis by activating the sterol-responsive element binding protein 1c pathway.8 Administration of LXR ligands in mice induces liver injury, such as enlarged liver, substantial hepatic lipid accumulation, increased serum alanine aminotransferase and aspartate aminotransferase, and hypertriglyceridemia.9,10 However, some divergence exists

**Objective**—Activation of liver X receptor (LXR) inhibits atherosclerosis but induces hypertriglyceridemia. In vitro, it has been shown that mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor synergizes LXR ligand–induced macrophage ABCA1 expression and cholesterol efflux. In this study, we determined whether MEK1/2 (U0126) and LXR ligand (T0901317) can have a synergistic effect on the reduction of atherosclerosis while eliminating LXR ligand–induced fatty livers and hypertriglyceridemia. We also set out to identify the cellular mechanisms of the actions.

**Approach and Results**—Wild-type mice were used to determine the effect of U0126 on a high-fat diet or high-fat diet plus T0901317-induced transient dyslipidemia and liver injury. ApoE deficient (apoE−/−) mice or mice with advanced lesions were used to determine the effect of the combination of T0901317 and U0126 on atherosclerosis and hypertriglyceridemia. We found that U0126 protected animals against T0901317-induced transient or long-term hepatic lipid accumulation, liver injury, and hypertriglyceridemia. Meanwhile, the combination of T0901317 and U0126 inhibited the development of atherosclerosis in a synergistic manner and reduced advanced lesions. Mechanistically, in addition to synergistic induction of macrophage ABCA1 expression, the combination of U0126 and T0901317 maintained arterial wall integrity, inhibited macrophage accumulation in aortas and formation of macrophages/foam cells, and activated reverse cholesterol transport. The inhibition of T0901317-induced lipid accumulation by the combined U0126 might be attributed to inactivation of lipogenesis and activation of lipolysis/fatty acid oxidation pathways.

**Conclusions**—Our study suggests that the combination of mitogen-activated protein kinase kinase 1/2 inhibitor and LXR ligand can function as a novel therapy to synergistically reduce atherosclerosis while eliminating LXR-induced deleterious effects. (Arterioscler Thromb Vasc Biol. 2015;35:948-959. DOI: 10.1161/ATVBAHA.114.305116.)

**Key Words:** atherosclerosis extracellular signal-regulated map kinases foam cells hypertriglyceridemia lipogenesis liver X receptor
between LXR-α and LXR-β. LXR-α is mainly expressed in fat, liver, and macrophages, whereas LXR-β is ubiquitously expressed in all tissues. LXR-α is a major subtype mediating the effects of LXR agonists on fatty acid synthesis, hepatic cholesterol excretion, and bile acid synthesis in the liver. Both LXR-α and LXR-β can regulate cholesterol metabolism and fatty acid synthesis in other tissues. Thus, the selective LXR-β modulators may inhibit atherosclerosis with acceptable undesired effects. However, the high identity in DNA and ligand binding domains between LXR-α and LXR-β isoforms limits the progress to identify the selective LXR-β modulators.

Extracellular signal–regulated kinases 1/2 (ERK1/2) belong to the mitogen-activated protein kinase family and function through the Ras-dependent Raf-MEK-MAPK cascade. Mitogen-activated protein kinase kinases 1/2 (MEK1/2) are the upstream dual specificity ERK1/2 kinases and are required for ERK phosphorylation. ERK1/2 activity is involved in different cellular processes, such as embryogenesis, cell proliferation/differentiation, and apoptosis, whereas the overexpression or constitutive activation of ERK1/2 can result in the progression of several cancers. Because of the high substrate specificity, inhibitors of MEK1/2 can consequently block ERK1/2 activity.

Accumulating evidence demonstrates an association between hypertriglyceridemia and atherosclerosis. Hypertriglyceridemia also seems central to the pathophysiology of dyslipoproteinemia in insulin resistance and type 2 diabetes mellitus. Although LXR can play important roles in different diseases, LXR-induced hypertriglyceridemia can have a variety of effects on atherosclerosis. We previously reported that MEK1/2 inhibitor and LXR ligand, both at low concentrations, can synergistically induce macrophage ABCA1 expression and cholesterol efflux. In this study, we have extended our previous work by hypothesizing that their combination can synergistically reduce atherosclerosis; moreover, this combination may eliminate the LXR ligand–induced undesired effects in blood vessels and other organs.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.
In aortic root, Figure 2B clearly shows that either T0901317 or U0126 alone inhibited sinus lesions. More importantly, greater inhibition on sinus lesions by the combination of T0901317 and U0126 than that of T0901317 or U0126 alone was observed. For instance, in female mice, the sinus lesions in the control group were (9.5±0.9)×10^5 μm^2, whereas the lesions were reduced to (5.9±0.7)×10^5, (6.50±0.5)×10^5, and (1.2±0.2)×10^5 μm^2 by either T0901317 or U0126 alone or their combination. We interpret these findings to suggest the synergistic inhibition occurs.

**Combination of T0901317 and U0126 Eliminates the Development of Fatty Liver and Hypertriglyceridemia in ApoE−/− Mice**

The effect of LXR ligand on hepatic lipid accumulation, fatty liver, and hypertriglyceridemia is proportional to the
doses used. After a long-term treatment with a reduced dose, T0901317 alone induced a moderate liver injury. Compared with the control group (HFD alone), T0901317 caused enlarged mouse liver by 15% to 20% (the ratio of liver weight to bodyweight) and reduced liver color (Figure 2C), indicating that it induces fatty liver. U0126 alone had little effect on both liver weight and color. It can also eliminate T0901317-induced liver color changes (Figure 2C) and T0901317-increased liver weight. Furthermore, Oil Red O staining demonstrates that T0901317 alone increased hepatic lipid accumulation (Figure 2D, second left column). In contrast, U0126 totally blocked T0901317-induced hepatic lipid accumulation (Figure 2D, right column). Similar results of liver/serum triglyceride levels and expression of microsomal triglyceride transfer protein (the molecule regulating triglyceride secretion from the liver) were observed using assays for triglyceride content and real-time reverse transcription polymerase chain reaction.
blocked T0901317-increased serum alanine aminotransferase and aspartate aminotransferase levels (Figure 2H and 2I). Thus, Figure 2 demonstrates that U0126 can eliminate T0901317-induced adverse effects and that the mice seem to tolerate the combination of these drugs reasonably well.

**Combination of T0901317 and U0126 Regresses Advanced Atherosclerotic Lesions**

To determine the therapeutic effects of the combination of T0901317 and U0126 on advanced lesions, apoE−/− mice were prefed HFD for 12 weeks and then divided into 5 groups (G1–G5) for the scheduled treatment (Figure 3A). In addition, the animals in G4 and G5 were switched from HFD to normal chow.

ApoE−/− mice developed lesions with time of HFD feeding (Figure 3B and 3C). Normal chow, containing no drugs, had little effect on lesions in both male and female mice (G4 versus G2), indicating a limited effect of reduced cholesterol uptake on advanced lesions. However, the combination of T0901317 and U0126 in normal chow reduced en face lesions (G5 versus G2, Figure 3B), with even fewer lesions than the baseline control (G5 versus G1, Figure 3B), indicating the regression of the advanced lesions. In aortic root, similar results of sinus lesions as en face aorta were obtained (Figure 3C). Thus, the combination of T0901317 and U0126 in normal chow reduced lesions (G5 versus G2, Figure 3B and 3C), suggesting a potential therapeutic effect.

Figure 3D shows that normal chow or plus T0901317 and U0126 can reverse HFD-induced hepatic lipid accumulation. This finding was confirmed by Oil Red O staining and liver photos (Figure 3E and 3F). Therefore, the combination of T0901317 and U0126 added to a healthy diet can lead to regression of the advanced lesions without the risk of fatty liver development.

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**Figure 3.** Regression of the advanced lesions by the combination of T0901317 (T0) and U0126 (U0) in normal chow. **A**, Experimental design: apoE−/− mice in 5 groups (15 per group) were scheduled for the indicated treatment; and the following assays were completed. **B** and **C**, Lesions in en face aortas and aortic root cross-sections were determined by Oil Red O staining. **D** and **E**, Hepatic lipid content was determined by triglyceride (TG) quantitative analysis and Oil Red O staining. **F**, Liver photos. *P<0.05; **P<0.01; ***P<0.001. HFD indicates high-fat diet; NC, normal chow; NS, not significantly different; TU, T0+U0 (1+3 milligram per kilogram of bodyweight); and w, week.
Mechanisms for Synergistic Reduction of Lesions

Serum lipid profiles were determined after treatment (Table II in the online-only Data Supplement). T0901317 alone increased total but not LDL- and HDL-cholesterol levels due to increased very LDL (VLDL) cholesterol, a triglyceride-rich lipoprotein in the serum. The increased serum triglyceride levels by T0901317 (Figure 2G) also confirms that the majority of increased total cholesterol is VLDL cholesterol. U0126 had little effect on serum LDL- or HDL-cholesterol levels, but it blocked T0901317-increased total and VLDL-cholesterol levels (Table II in the online-only Data Supplement) and serum triglyceride levels (Figure 2G). These results suggest that inhibition of atherosclerosis by T0 or U0 alone is not related to amelioration of serum LDL- or HDL-cholesterol levels. However, the synergistic inhibition of atherosclerosis by the combination of T0901317 and U0126 may be partially contributed by the decreased VLDL-cholesterol levels.

In the regression study (Figure 3), normal chow or plus the combination of T0901317 and U0126 improved cholesterol levels compared with the HFD with greater effects on female mice than male mice, in particular VLDL-cholesterol levels (Table III in the online-only Data Supplement). In female mice, the combined T0901317 and U0126 in HFD also decreased LDL-cholesterol levels, which could have contributed to the reduction of sinus lesions (Figure 3C).

To determine the synergistic induction of macrophage ABCA1 expression, the cells isolated from apoE−/− mice were treated with U0126 or PD98059 alone or plus T0901317. Similar to wild-type macrophages,16 MEK1/2 inhibitors alone increased ABCA1 expression (Figure 4A) in a dose-dependent manner, and they synergized T0901317-induced ABCA1 expression in apoE−/− macrophages (Figure 4B). In vivo, ABCA1 expression in peritoneal macrophages and in aortic root was also induced by the treatment with the greatest effect by the combination of T0901317 and U0126 (Figure I in the online-only Data Supplement).

To characterize the effect on RCT, apoE−/− mice receiving treatment were intraperitoneal injected with pre[3H]-cholesterol–labeled macrophages followed by determination of excreted [3H]-tracer into the feces. Figure 4C (left) demonstrates that T0901317 or U0126 alone or their combination increased [3H]-tracer 24 hours after cell injection. In the combined T0901317 and U0126 group, >60% of the injected radioactivity was excreted into feces 48 hours after cell injection, which may result in no increased radioactivity in the liver and serum (Figure 4C, right). In addition to macrophage ABCA1, adipose tissue ABCA1 expression was also activated by T0901317 or U0126 alone or by their combination (Figure IID in the online-only Data Supplement).

In vivo, HFD resulted in >1/3 peritoneal macrophages differentiated into foam cells (>10 lipid droplets per cell; Figure 4D). T0901317 or U0126 decreased foam cells to 12% or 19%, and their combination decreased them to <5% (Figure 4D), suggesting that the combination of T0901317 and U0126 has a synergistic inhibitory effect on foam cell formation. Expression of CD68, a marker for macrophages/foam cells, in aortic root was substantially inhibited by the combination of T0901317 and U0126, suggesting that the macrophage accumulation was reduced (Figure 4E). In addition, we determined that the combination of T0901317 and U0126 can reduce levels of some inflammatory molecules in serum, such as chemokine (C-C motif) ligand 1, C-reactive protein, and matrix metalloproteinase 9. In contrast, serum CCR-7, which plays an important role in lesion regression, was increased (Figure IIA in the online-only Data Supplement). In human umbilical vein endothelial cells, the combination of T0901317 and U0126 blocked oxLDL-induced MCP-1 and ICAM-1 expression (Figure IIB in the online-only Data Supplement). In human aortic smooth muscle cells, U0126 or in combination with T0901317 increased smooth muscle actin expression (Figure IIC in the online-only Data Supplement), implying that the treatment may also affect smooth muscle cell differentiation. All these effects can demonstrate additional antiatherogenic properties.

A long-term treatment of animals with U0126 alone or in combination with T0901317 had no effect on total ERK1/2 expression, whereas it slightly inhibited phosphorylated ERK1/2 (pERK1/2) in the aortic root (Figure III in the online-only Data Supplement). Similar pERK1/2 levels were determined in other tissues, indicating that the treatment can cause some inhibition on ERK1/2 activity in vivo.

Vascular cell adhesion molecule-1 facilitates the adhesion of monocytes to the endothelial layer, one of the initial steps in the accumulation of macrophages/foam cells in the arterial wall.19 Figure 4F demonstrates that T0901317 or U0126 alone or in combination can substantially inhibit vascular cell adhesion molecule-1 expression in the outer layer of the lesion area. Aortic root sections were stained with an anti-α-smooth muscle actin antibody. Figure 4G shows disordered layers of smooth muscle cells and reduced smooth muscle actin expression in the middle area of arterial wall in the control group. In contrast, the well-organized smooth muscle cell layers and even elevated smooth muscle actin expression were observed in the group treated with the combination of T0901317 and U0126, indicating that the structure and integrity of arterial wall were maintained. Figure 4H (Verhoeff-Van Gieson staining) also shows some destruction of the arterial wall, which was associated with the formation of necrotic cores and little collagen in the fibrous caps in the control group. However, the combination of T0901317 and U0126 increased both collagen and elastin contents in the arterial wall.

Mechanisms for Inhibition of Lipogenesis and Hypertriglyceridemia

We initially determined the effect of U0126 on the T0901317-activated sterol-responsive element binding protein 1c pathway. U0126 alone altered the expression of sterol-responsive element binding protein 1c, fatty acid synthase, stearoyl-CoA desaturase 1, and acetyl-CoA carboxylase 1 differently. For example, it activated fatty acid synthase but not stearoyl-CoA desaturase 1 expression. The combination of T0901317 and U0126 had similar effects as T0901317 alone (Figure IV in the online-only Data Supplement), suggesting that U0126 had little effect on the T0901317-activated fatty acid synthesis pathway at the transcriptional level.
Acyl-CoA:diacylglycerolacyltransferase 1 is the rate-limiting enzyme for liver triglyceride biosynthesis. Figure 5A to 5C show that T0901317 alone had little effect on acyl-CoA:diacylglycerolacyltransferase 1 mRNA expression, whereas it slightly decreased acyl-CoA:diacylglycerolacyltransferase 1 protein expression. U0126 alone moderately inhibited acyl-CoA:diacylglycerolacyltransferase 1 expression, and the inhibition was further enhanced by the combined T0901317. Both adipose triglyceride lipase and hormone-sensitive lipase (HSL) catalyze the hydrolysis of triglyceride.
into diacylglycerol and fatty acid, the first and rate-limiting step in triglyceride hydrolysis although HSL can act on hydrolysis of diacylglycerol. Adipose triglyceride lipase can be substantially activated by its coactivator, comparative gene identification-58. The immunofluorescent results show that T0901317 or U0126 alone had little effect, whereas their combination increased adipose triglyceride lipase expression (Figure 5D), which might be because of activation of

Figure 5. Combination of T0901317 (T0) and U0126 (U0) inhibits triglyceride (TG) biosynthesis and activates TG hydrolysis pathways. Total RNA, protein, and paraffin sections were prepared from the liver of mice used in Figure 2. Acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1; A), adipose triglyceride lipase (ATGL) and comparative gene identification-58 (CGI-58; E), and hormone-sensitive lipase (HSL; F) mRNA were determined by real-time reverse transcription polymerase chain reaction. B–D and F, Liver DGAT1, ATGL, and HSL protein were determined by immunofluorescent staining and western blot. *P<0.05; **P<0.01 vs control in the corresponding group (n=6). MFI indicates mean of fluorescent intensity of the images (n=6).
comparative gene identification-58 expression (Figure 5E). Similarly, the combination of T0901317 and U0126 increased HSL expression (Figure 5F). The inductive effect of the cotreatment on adipose triglyceride lipase and HSL expression was also confirmed by immunohistochemical assay (Figure VA in the online-only Data Supplement). However, the treatment had little effect on expression of monoacylglycerol lipase (Figure VB in the online-only Data Supplement).

To determine whether the combination of T0901317 and U0126 can activate the fatty acid oxidation pathway, we assessed the expression of the peroxisome proliferator–activated receptor α, a transcription factor regulating activities of all 3 free fatty acid oxidation systems (mitochondrial and peroxisomal β-oxidation and microsomal ω-oxidation).24 Figure 6A indicates U0126 alone or combined with T0901317 increased peroxisome proliferator–activated receptor-α expression. Expression of peroxisome proliferator–activated receptor-α target genes involved in β-oxidation, such as carnitine acetyltransferase, carnitine palmitoyltransferase 1A, and peroxisomal acyl-CoA oxidase 1/2 (ACOX-1/2), was also induced (Figure 6B–6E).

AMP-activated protein kinase α (AMPK-α), particularly its phosphorylated form (pi-AMPK-α), activates fatty acid β-oxidation.25 Figure 6F shows T0901317 or U0126 alone or their combination increased pi-AMPK-α protein and the ratio of pi-AMPK-α to AMPK-α. Consequently, the phosphorylated acetyl-CoA carboxylase 1 (pi-ACC1), a target of pi-AMPK-α, was slightly increased (Figure 6G), which indicates that the added U0126 may cause some attenuation of T0901317-induced fatty acid synthesis.

Finally, we determined the effects of the combination of T0901317 and U0126 on triglyceride or fatty acid metabolism pathways in human hepatocellular line, HepG2 cells. Figure VI in the online-only Data Supplement shows that T0901317 or U0126/PD98059 alone increased HSL and peroxisome proliferator–activated receptor-α expression, and the increase was further enhanced by cotreatment (Figure VIA and VIB in the online-only Data Supplement). T0901317 alone had little effect and did not influence MEK1/2 inhibitor–activated AMPK-α (Figure VIC in the online-only Data Supplement). These results imply that the prevention of hypertriglyceridemia by the combination of T0901317 and U0126 is species-independent, and it can occur in human cells.

Discussion

Hypercholesterolemia is a dominant risk factor for CHD. Increased LDL- and decreased HDL-cholesterol levels are specifically associated with increased prevalence of CHD. Statins reduce plasma LDL-cholesterol levels, whereas they mildly increase HDL-cholesterol levels, which can reduce cardiovascular events by 40%.26 However, other factors may also play important roles in the regulation of atherogenesis. In this study, we determined that T0901317 and U0126 did not ameliorate serum LDL- or HDL-cholesterol levels (Table II in the online-only Data Supplement) but maintained the integrity of arterial wall and inhibited vascular cell adhesion molecule-1 expression (Figure 4F–4H), which may prevent monocyte adhesion. Accordingly, macrophage accumulation in the lesion area was inhibited (Figure 4E). The synergistic induction of macrophage ABCA1 expression and RCT (Figure 4B and 4C) by the combination of T0901317 and U0126 can block foam cell formation (Figure 4D). These effects together resulted in the reduction of atherosclerosis (Figures 2 and 3). In addition, the combination of T0901317 and U0126 substantially reduced total and VLDL-cholesterol levels (Table II in the online-only Data Supplement). In the prevention study, whereas the combination plus a normal chow ameliorated total, LDL-, and VLDL-cholesterol levels (Table III in the online-only Data Supplement) in the regression study, these cholesterol-lowering effects can contribute to a reduction in atherosclerosis. Although T0901317 alone can complete some of the above antiatherogenic actions, it also promotes moderate liver injury and hypertriglyceridemia (Figures 1 and 2). GW3965, another nonsteroidal LXR ligand with less lipogenic effect than T0901317, has little effect on lesions at 1 mpk. At 10 mpk, GW3965 inhibits ≈50% sinus lesions, increases serum triglyceride levels, activates liver SREBP1, and has no effect on aortic CD68 expression, indicating that the accumulation of macrophages/foam cells in aortic root and hypertriglyceridemia still progresses.7 T0901317 inhibits lesions, whereas it induces hypertriglyceridemia both in a dose-dependent manner, suggesting a link between atherosclerosis inhibition and the severity of increased-lipogenesis by LXR ligands. The LXR-induced negative effects, including hypertriglyceridemia, result in disappointing outcomes using LXR ligands in clinical trial evaluations.27

Abnormalities of triglyceride metabolism are a hallmark of many clinical disturbances, such as type 2 diabetes mellitus, familial combined hyperlipidemia, dysbetalipoproteinemia, and severe hypertriglyceridemia, which are conferred to be increased risks for CHD.28 Triglyceride can be associated with atherogenic remnant particles and apoC-III (a proinflammatory and proatherogenic apolipoprotein).29,30 The triglyceride-rich lipoproteins, such as VLDL or chylomicron remnants, can promote atherosclerosis independently of LDL cholesterol. These remnant species can be subject to macrophage uptake that eventually leads to foam cell formation. The endothelial accumulation of triglyceride-rich lipoprotein remnants can generate numerous proatherogenic responses that enhance recruitment of leukocytes, produce endothelial- and macrophage-derived inflammatory proteins, and can result in endothelial injury. Clinically, patients with type 2 diabetes mellitus and metabolic syndrome are commonly associated with a combined dyslipidemia characterized by hypertriglyceridemia, low HDL-cholesterol levels, and accumulation of chylomicron and VLDL remnant particles, and they are at significant risk for CHD.31

Triglyceride accumulation is also a major risk factor for development of fatty liver. Hepatic lipid content depends on de novo synthesis, delivery, incorporation or export of triglyceride as VLDL, and use. Thus, any abnormality in these physiological processes can contribute to the development of fatty liver. LXR ligands stimulate fatty acid biosynthesis resulting in severe hepatic triglyceride accumulation, fatty liver, and hypertriglyceridemia that hampers their application.
for treatment of atherosclerosis. Two strategies for such treatment have been proposed, but the progress is slow: (1) use selective LXR-β modulators that activate macrophage ABCA1 expression and RCT with acceptable lipogenic effects. The problem with this approach is that high homology between LXR-α and LXR-β and high conserved sequence of the DNA-binding motif exist among target genes, and (2) inactivation of LXR-α. The problem with this approach is that LXR-α induces hepatic lipogenesis, but LXR-α deficiency in the liver decreases RCT, and cholesterol removal...
will increase atherosclerosis.32 The agents inhibiting LXR-α and LXR-α-dependent lipogenesis do not enhance RCT.33,34 A few studies have reported that activation of LXR can regulate RCT or atherosclerosis in an ABCA1- or macrophage-independent manner. T0901317 increases RCT to a similar degree in wild-type mice injected with radiolabeled wild-type macrophages or LXR-deleted macrophages, whereas it had little effect on LXR-deficient mice injected with wild-type macrophages.35 Genetic deletion of hepatic LXR-α expression results in decreased RCT, cholesterol catabolism, and excretion and increased atherosclerosis that was still inhibited by T0901317.36 Treatment of LDLR−/− mice receiving ABCA1−/− ABCG1−/− bone marrow transplantation with T0901317 reduces atherosclerosis.37 Therefore, these studies suggest that other mechanisms than macrophage ABCA1 expression can participate in LXR-activated RCT or LXR-inhibited atherosclerosis.

We determined that U0126 eliminated T0901317-induced undesired effects by mechanisms in which U0126 alone or in combination with T0901317 inhibited triglyceride biosynthesis, whereas it activated triglyceride hydrolysis and fatty acid oxidation pathway (Figures 5 and 6). U0126 alone activated macrophage ABCA1 expression and RCT (Figure 4). To further determine whether the high fat can enhance hypercholesterolemia-induced atherosclerosis, we fed apoe−/− mice a normal chow containing cholesterol (0.5%) or fat (21%) alone or both for 16 weeks and observed that a cholesterol-enriched diet alone can increase serum total and LDL-cholesterol levels and atherosclerotic lesions. Fat alone had a little effect on lesions, but it clearly enhanced the development of hypercholesterolemia-induced lesions (data not shown).

Atherosclerosis is a potentially reversible disease. T0901317 at a high dose can induce regression of early stage aortic en face lesions in LDL receptor–deficient mice, but the sinus lesions and hypertriglyceridemia continue to progress.38 LXR-623 was the first compound in a clinical trial (data not shown). U0126 alone acti-
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In conclusion, our study shows for the first time that the combined LXR ligand and MEK1/2 inhibitor reduce atherosclerosis by multiple mechanisms, including the protection of arterial integrity, prevention of macrophage/foam cell formation, and enhancement of RCT. Meanwhile, MEK1/2 inhibitor blocks LXR ligand–induced lipogenesis, fatty liver, and hypertriglyceridemia which can substantially enhance the antiatherogenic properties of the LXR ligand (Figure VI in the online-only Data Supplement). Our study suggests that the combination of MEK1/2 inhibitor and LXR ligand can function as a novel therapy to synergistically reduce atherosclerosis while eliminating several LXR–induced deleterious effects.

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Disclosures
None.

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Combination Therapy of Antiatherosclerosis

Significance

Atherosclerosis is one of the causes of coronary heart disease. Expression of macrophage ABCA1 enhances reverse cholesterol transport to reduce atherosclerosis. Liver X receptor (LXR) ligand induces ABCA1 expression, thereby reducing atherosclerosis. However, the induction of severe hepatic lipogenesis, fatty liver, and hypertriglyceridemia limits application of LXR ligands to treat atherosclerosis. Herein, we found that the combined LXR ligand (T0901317) and mitogen-activated protein kinase kinase 1/2 inhibitor (U0126) reduced atherosclerosis in proatherogenic apoE-deficient mice in a synergistic manner but had little LXR ligand-induced undesired effects. The reduction of atherosclerosis is completed by multiple mechanisms, including induction of macrophage ABCA1 expression and reverse cholesterol transport, maintenance of the arterial wall integrity, and inhibition of macrophage accumulation in aortas. The combination of T0901317 and U0126 inhibited triglyceride biosynthesis, whereas it activated triglyceride hydrolysis and fatty acid oxidation pathways, thereby preventing hypertriglyceridemia. Our study suggests that this combination can function as a novel therapy to treat atherosclerosis without LXR-induced deleterious effects.
Inhibition of ERK1/2 and Activation of LXR Synergistically Reduce Atherosclerotic Lesions in ApoE-Deficient Mice
Yuanli Chen, Yajun Duan, Xiaoxiao Yang, Lei Sun, Mengyang Liu, Qixue Wang, Xingzhe Ma, Wenwen Zhang, Xiaoju Li, Wenquan Hu, Robert Q. Miao, Rong Xiang, David P. Hajjar and Jihong Han

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## II. Supplemental Material

Table II. Effect of treatment with T0901317 or U0126 alone or in combination on serum cholesterol levels in apoE⁻/⁻ mice

<table>
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<th>Treatment</th>
<th>Ctrl</th>
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<th>U0126</th>
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<td>Total-cholesterol</td>
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<td>LDL-cholesterol</td>
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<td>9.2 ± 1.6</td>
<td>8.2 ± 1.8</td>
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<td>VLDL-cholesterol</td>
<td>8.6 ± 1.2</td>
<td>12.8 ± 1.8*</td>
<td>9.5 ± 0.9</td>
<td>3.5 ± 1.1**</td>
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<td><strong>Females</strong></td>
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<tr>
<td>Total-cholesterol</td>
<td>17.6 ± 3.0</td>
<td>21.9 ± 1.4*</td>
<td>18.2 ± 1.4</td>
<td>14.5 ± 2.8'</td>
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<td>4.4 ± 0.5</td>
<td>2.9 ± 0.9</td>
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<td>LDL-cholesterol</td>
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<td>VLDL-cholesterol</td>
<td>6.0 ± 1.8</td>
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<td>5.1 ± 0.8</td>
<td>5.6 ± 0.9**</td>
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* Both male and female apoE⁻/⁻ mice received the treatment as indicated in Figure 2. Serum samples were used to determine total cholesterol, VLDL-, LDL- and HDL-cholesterol levels (mM). The results are expressed as mean ± SEM [n=15 or n=5 (for VLDL-C only)]. *: P<0.05 vs. control; **: P<0.01 vs. T0901317 alone by One-Way Anova [n=15 or 5 (for VLDL-C)].
Table III. Combination of T0901317 and U0126 does not influence normal chow-ameliorated serum cholesterol levels in apoE−/− mice with advanced lesions.

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<td>4.5±0.3$§§</td>
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</tr>
<tr>
<td>Total-cholesterol</td>
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<td>19.9±1.3</td>
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<td>3.0±0.2#§§</td>
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<td>6.6±1.2</td>
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<td>1.7±0.3#§§</td>
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¶: Both male and female apoE−/− mice received treatment as indicated in Fig. 3A. Serum samples were used to determine total cholesterol, VLDL-, LDL- and HDL-cholesterol levels (mM). The results are expressed as mean ± SEM. #: vs. Gr 1 (12 w HFD); §: vs. Gr 2 (16 w HFD); $: vs. Gr 3 (12 w HFD followed by 4 w HFD/TU), P<0.05, by One-Way Anova [n=15 or 5 (for VLDL-C)].
Figure I. Induction of ABCA1 expression in peritoneal macrophages and aortic root cross sections

A: The aortic root cross sections of Figure 2 were used to determine ABCA1 and CD68 expression by immunohistochemical staining; B: the quantitative analysis of ABCA1 expression relative to CD68 expression in Figure IA was conducted. *: vs. control, P<0.05 (n=3); C: peritoneal macrophages used in Figure 4C (RCT) were collected at the end of the experiments. Total cellular RNA was extracted and used to determine ABCA1 expression by real time RT-PCR. *: vs. control, P<0.05 (n=6).
Figure II. The combination of T0901317 and U0126 affects inflammatory and atherogenic-related gene expression

A: serum samples of male apoE−/− mice used in Figure 2 were collected for assay using the “Antibody Arrays” by RayBiotech, Inc. The relative changes in CCL1, CRP, MMP9 and CCR7 were presented; B: HUVEC received the indicated treatment overnight in serum-free M199 medium. Expression of ICAM-1 and MCP-1 was determined by real time RT-PCR, respectively; C: HASMC received the indicated treatment overnight in serum-free DMEM:F12 (1:1) medium. Expression of SMA was determined by Western blot; D: adipose tissue was collected from apoE−/− mice used in Figure 4C, and used to determine ABCA1 and SR-BI expression by Western blot. NS: no significant difference, *: P<0.05, **: P<0.01 vs. control (n=6).
Figure III. U0126 alone or in combination with T0901317 moderately reduces pi-ERK1/2 expression but have no effect on ERK1/2 expression.

The aortic root cross sections of Figure 2 were used to assess expression of pi-ERK1/2 and ERK1/2 by immunofluorescent staining with anti-pi-ERK1/2 and ERK1/2 antibodies, respectively.
Figure IV. U0126 does not influence T0901317-induced hepatic fatty acid biosynthesis
Total RNA was extracted from the mice used in Figure 2. Expression of SREBP1c, FASN, SCD and ACC1 mRNA was determined by real time RT-PCR using the primers listed in Table S1. *: P<0.05 vs. control in the corresponding group (n=6).
Figure V. Induction of ATGL and HSL by the combination of T0901317 and U0126

The paraffin sections and total RNA were prepared from liver samples of mice used in Figure 2, respectively. A: expression of ATGL and HSL was determined by immunohistochemical staining (left panel) and the density of images was quantified (right panel); B: MGLL mRNA expression was determined by real time RT-PCR (n=6).
Figure VI. The combination of T0901317 and U0126 activates pathways for TG hydrolysis and fatty acid oxidation in HepG2 cells

HepG2 cells, a human hepatic cell line, at 95% confluence received the indicated treatment overnight in serum-free DMEM medium. Expression of HSL (A), PPARα (B), AMPKα and pi-AMPKα (C) was determined by Western blot, respectively.
Figure VII. The hypothetical model depicts the multiple mechanisms by which the combined T0901317 and U0126 reduce the development of atherosclerosis and fatty liver formation in mice.

Pathways 1, 2: LXR inhibits lesion development by activating ABCA1 expression and RCT. LXR also induces lipogenesis and hypertriglyceridemia which attenuates its anti-atherogenic properties; Pathways 3, 4: inhibition of ERK1/2 activates ABCA1 expression. It also inhibits TG biosynthesis and activates TG hydrolysis and fatty acid oxidation which reduces LXR-induced side effects; Pathways 5, 6: the combination of T0901317 and U0126 inhibits VCAM-1 expression which can reduce adhesion of circulating monocytes, an important step for the development of atherosclerosis. The synergistic activation of macrophage ABCA1 expression results in enhanced RCT which inhibits macrophage accumulation and formation of foam cells in the arterial wall. Together, the combination of T0901317 and U0126 synergistically reduces atherosclerosis and inhibits the development of a fatty liver.
MATERIALS AND METHODS

Reagents
Rabbit anti-CD68 (Cat#: sc-9139), α-SMA (Cat#: sc-53142), DGAT1 (Cat#: sc-26173), CAT (Cat#: sc-22683), ATGL (Cat#: sc-67355), HSL (Cat#: sc-25843) and PPARα (Cat#: sc-9000) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-ABCA1 (Cat#: NB400-105) AMPKα (Cat#: NBP2-22127) and SR-BI (Cat#: NB400-104) polyclonal antibodies were purchased from Novus Biologicals (Littleton, CO). Rabbit anti-ERK1/2 (Cat#: 9102S) and phospho-ERK1/2 (p-ERK1/2, Cat#: 9101S) polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-phospho-AMPKα (p-AMPKα, Cat#: ab131357) polyclonal antibody was purchased from Abcam (Cambridge, MA). Rabbit anti-goat IgG (whole molecule)-FITC antibody (Cat#: F2016), goat anti-rabbit IgG (whole molecule)-FITC antibody (Cat#: F0382), goat anti-mouse IgG (whole molecule)-FITC antibody (Cat#: F0257) and goat anti-rabbit IgG (H+L), F(ab) fragment-Rhodamine antibody (Cat#: SAB3700969) were purchased from Sigma-Aldrich (St Louis, MO). Triglyceride assay kit (Cat#: 290-63701) was purchased from Wako Chemicals (Neuss, Germany). PD98059 (Cat#: P4313) and U0126 (Cat#: U0126) were purchased from LC Laboratories (Woburn, MA). T0901317 (Cat#: 71810) was purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma-Aldrich except where indicated.

Cell culture
HepG2, a human hepatocellular carcinoma cell line, was purchased from ATCC (Rockville, MD) and cultured in complete DMEM medium containing 10% fetal calf serum (FCS), 50 μg/mL of penicillin/streptomycin and 2 mmol/L glutamine. HUVECs (human umbilical vein vascular endothelium cells) were purchased from ATCC (Rockville, MD) and cultured in M199 medium supplemented with 10% FCS, 50 μg/mL endothelial cell growth supplement, 100 μg/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells at passages of 3 to 5 were used to conduct experiments. HASMC (human aortic smooth muscle cells) were kindly provided by Dr. XL Zheng from Calgary University, Canada, and cultured in DMEM:F12 (1:1) medium supplemented with 10% FCS, 50 μg/mL of penicillin/streptomycin and 2 mmol/L glutamine. Both HepG2 and HASMC at ~90% confluence were switched into serum-free medium before treatment.

Animals and in vivo studies
The protocol for in vivo studies was approved by the Ethics Committee of Nankai University and conforms to the Guide for the Care and Use of Laboratory Animals published by the NIH. C57BL/6 wild type mice and apoE deficient (apoE−/−) mice with same background as the wild type mice were originally obtained from The Jackson Laboratory (Bar Harbor, Maine) and then bred in the Animal Center of Nankai University.
To study the effects of U0126 on a high-fat diet (HFD: 0.5% cholesterol and 21% fat) or HFD plus T0901317-induced transient hepatic lipogenesis, hypertriglyceridemia and liver injury, female wild type mice (~8-week-old) were randomly divided into 9 groups (6 mice/group), and fed normal chow or HFD or HFD containing T0901317 [1 mg/day/kg body weight (mpk)] or U0126 (1 or 3 or 9 mpk) alone or T0901317 plus U0126 for 2 weeks.

To study the inhibitory effects of T0901317 or U0126 or in combination on the development of atherosclerotic lesions and fatty liver, both male and female apoE−/− mice (~8-week-old) were divided into 4 groups (15 mice/group). They were fed HFD or HFD containing T0901317 (1 mpk) or U0126 (3 mpk) alone or in combination for 16 weeks.

To study the regressive effects of the combination of T090317 and U0126 on advanced lesions, both male and female apoE−/− mice (~8-week-old) were pre-fed a HFD for 12 weeks. The mice were then divided into 5 groups (15 mice/group) and received the following treatment for 4 more weeks except group 1, which was terminated at the time of division for sample collection. The following experimental design was used: group 2, HFD; group 3, HFD containing T0901317 + U0126 (1 + 3 mpk); group 4, normal chow; and group 5, normal chow containing T0901317 plus U0126 (1 + 3 mpk).

At the end of the experiments, all of the mice were anesthetized and euthanized in a CO2 chamber followed by collection of mouse aorta, liver, peritoneal macrophages and blood samples. Blood was kept for about 2 h at room temperature (RT). After centrifugation for 20 min at 2,000 g, the serum was transferred into a new test tube and kept at -20°C. TG, total cholesterol, HDL-C, LDL-C, AST and ALT levels were analyzed using commercially available enzymatic kits purchased from BioSino (Beijing, China). To determine VLDL-C, the density of serum pooled from 3 mice was adjusted to 1.019 g/mL with NaBr solution and fractioned by ultracentrifugation. The fraction with density less than 1.019 g/mL was collected, and the cholesterol content was assessed with a commercially available enzymatic kit.

**Determination of serum chemokines and cytokines by “Antibody Arrays”**

Serum samples were prepared from mice used in the prevention study, and then shipped to RayBiotech, Inc. for determination of serum chemokines and cytokines by the assay of “Antibody Arrays” (G-Series: Glass Slide Antibody Arrays). The relative changes in CCL1, CRP, MMP9 and CCR7 were presented.

**Determination of hepatic lipid content**

After weighed and photographed, a piece of the liver was removed and frozen sections were prepared by a standard procedure. The sections were stained with freshly prepared Oil Red O solution (3 mg/mL in 60% isopropanol) for 45 min. After staining, the sections were rinsed with 60% isopropanol, and then the nuclei were
stained with alum haematoxylin solution for 30 sec. The sections were finally rinsed with distilled water and mounted in Glycerin Jelly mountant. Images of all sections were obtained with a Leica DM5000B microscope. To quantify TG content, a piece of liver (~50 mg) was homogenized in ~1.1 mL 1xPBS. A portion of the homogenate (100 μL) was saved for determination of protein content which was used to normalize TG levels; 1 mL homogenate was then used to extract total lipids followed by TG quantitative analysis.

Collection of aortas and assessment of lesions

Aortas were collected as follows: after euthanasia, the mouse vasculature was perfused with 1xPBS. The entire aorta extending 5 to 10 mm below bifurcation of the iliac arteries, including the subclavian right and left common carotid arteries, was removed. The aorta was fixed in 4% paraformaldehyde/PBS. After removal of outside connecting tissue and fat, the aorta was stained with Oil Red O solution and the en face lesions were quantified using a computer assisted image analysis protocol. The lesions were expressed as mean percent of lesion area in the aorta ± SEM. To determine the sinus lesions in aortic root, 5 μm frozen sections of the aortic root were prepared and also stained with Oil Red O solution. Images of the sections were obtained with a microscope. The total morphometric lesion area was determined with a computer assisted image analysis protocol and expressed as μm²/section.

Verhoeff-Van Gieson (VVG) staining

The 5 μm cross sections of aorta root were processed VVG staining as follows: the sections were first doused in Verhoeff’s working solution [A mixture of 20 mL 5% hematoxylin in alcohol, 8 mL 10% ferric chloride in water, and 8 mL Weigert’s iodine solution (2% potassium iodine and 1% iodine in water)] for 1 h and then rinsed in distilled water. After incubation in 2% ferric chloride solution for 1 min, the sections were subject to a 5% sodium thiosulfate bath for 1 min. The sections were then counter stained in Van Gieson’s solution (1% aqueous acid fuchsin in saturated aqueous picric acid) for 3 min. Finally the sections were dehydrated very quickly through 95% alcohol, 2 changes of 100% alcohol, cleared in xylene, and then mounted with resinous mounting medium. All images were captured with a Leica microscope (Wetzlar, Germany).

Collection of peritoneal macrophages and determination of foam cells

Mouse abdomen peritoneal macrophages were collected by lavage with 1xPBS. The cells were plated on cover slips in a 24-well plate. After attached, the cells were fixed and stained with Oil Red O solution and then with hematoxylin. Cells containing lipid droplets (>10/cell) were considered as foam cells, and at least 10 fields per sample were counted.

Immunohistochemical and immunofluorescent staining

To determine CD68 and ABCA1 expression in lesion areas, the aortic root cross sections were permeabilized with 0.5% (v/v) Triton X-100 for 10 min, blocked with 2%
bovine serum albumin (BSA) for 2 h at RT, and then incubated with primary antibody (CD68, 1:200; ABCA1, 1:500) overnight at 4°C respectively. After removal of the primary antibody by washing with PBS, the sections were incubated with biotin-conjugated goat anti-rabbit IgG for 15 min at RT. After washing with PBS, the sections were incubated in a HRP-conjugated avidin solution for 20 min followed by adding the developing solution. After development, the sections were stained with hematoxylin solution for nuclei, and then mounted under cover slides with permount. The images of cross sections were viewed and photographed using a Leica microscope, and the density of images was quantified by segmentation color-threshold analysis using morphometry software (IP Lab, Scanalytics, Rockville, MD) as described[1,2].

Expression of vascular cell adhesion molecule-1 (VCAM-1), phospho-ERK1/2 (p-ERK1/2), ERK1/2, and α smooth muscle actin (SMA) in lesion area was determined by immunofluorescent staining as follows: after permeabilization and blocking as described above, the aortic root cross sections were incubated with primary antibody (VCAM-1, 1:250; ERK1/2, p-ERK1/2 and SMA, 1:500) overnight at 4°C. After washing, the sections were incubated with the secondary antibody conjugated with either fluorescein isothiocyanate (FITC) or Rhodamine (1:1,000 dilution) for 2 h at RT. All sections were then stained with DAPI solution for nuclei.

To determine ATGL and HSL expression in the liver, a piece of liver was fixed in 4% paraformaldehyde followed by embedding in paraffin. The 5 μm paraffin sections were collected using a standard procedure. The sections were initially deparaffinised and hydrated. The antigen retrieval was obtained by heating the sections in a sodium citrate solution (0.01 M, pH 6.0) for 20 min in a 95°C water bath. The sections were then blocked with goat serum for 15 min followed by incubation with rabbit anti-ATGL or HSL polyclonal antibody (1:200 dilution) overnight at 4°C. After removal of primary antibody by washing with PBS, the sections were incubated with biotin-conjugated goat anti-rabbit IgG for 15 min at RT. After washing with PBS, the sections were incubated in a HRP-conjugated avidin solution for 20 min followed by adding the developing solution. After development, the sections were stained with hematoxylin solution for nuclei and then mounted under cover slides with permount. The images were obtained and quantified as described above.

Expression of ATGL, CAT, DGAT1 and HSL in the liver was determined by immunofluorescent staining with the 5 μm paraffin liver sections as described above. The mean fluorescence intensity (MFI) of all the immunofluorescent images was calculated as described[3].

**Determination of RCT**

The in vivo RCT was determined as described[4, 5]. Briefly, peritoneal macrophages isolated from un-treated apoE−/− were radiolabeled in suspension in serum-free RPMI 1640 medium containing 50 μg/mL acetylated LDL (acLDL) and 2 μCi/mL [3H]-cholesterol for 24 h, washed twice, equilibrated in suspension in RPMI
1640 with 0.2% BSA for 4 h, spun down and re-suspended in serum-free RPMI 1640 before immediate injection. ApoE−/− mice (6 mice/group) were fed HFD or HFD containing T0901317 (1 mpk) or U0126 (3 mpk) alone or in combination for one week. The mice were then i.p. injected with the above radiolabeled macrophages (~2x10^6 cells/mouse containing 1.2x10^6 cpm) and transferred into metabolic chambers. Feces from individual mouse were collected at 8, 24 and 48 h after the cell injection. At the end of the experiment (48 h after the cell injection), the mice were anesthetized and euthanized in a CO2 chamber. Samples of blood (for serum isolation) and liver were collected. After lipid extraction, the radioactivity in samples of feces, liver and serum was determined with a liquid scintillation counting. The values are expressed as a percentage of total injected [3H]-cholesterol.

Western blot

Peritoneal macrophages were collected from ~8-week-old female apoE−/− mice, and they received treatment in serum-free medium. HepG2 and HASMC cells also received treatment in serum-free medium. After treatment, total cellular protein was extracted from peritoneal macrophages, HepG2 cells, and HASMC cells using a cellular lysis buffer[6], respectively.

A piece of liver or white adipose tissue (~30 mg) was homogenized in the same lysis buffer. The supernatant of the homogenate was retained as tissue protein extract. After determination of protein content, expression of ABCA1 in macrophages, HSL, PPARα, pi-AMPKα and AMPKα in HepG2 cells, AMPKα, pi-AMPKα, ACC1, pi-ACC1, DGAT1 and PPARα in the liver, and ABCA1 and SR-BI in adipose tissue were determined by Western blot[6], respectively.

Real time RT-PCR

After treatment, peritoneal macrophages, HUVECs or a piece of liver was lysed or homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) to extract total cellular RNA[6]. The cDNA was then synthesized with 1 µg total cellular RNA using a reverse transcription kit purchased from New England Biolabs (Ipswich, MA). Real time PCR was performed using a SYBR green PCR master mix from Bio-Rad (Los Angeles, CA) with the primers listed in Table I. Expression of ACC1, ACOX-1/2, ATGL, CAT, CGI-58, CPT1α, DGAT1, FASN, HSL, MGLL, MTTP, PPARα, SCD-1 and SREBP1c mRNA in mouse liver, ICAM-1 and MCP-1 in HUVECs, and ABCA1 mRNA in peritoneal macrophages were normalized by the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Statistical analysis

Data were generated from at least three independent experiments. Values are represented as mean ± SEM. All the data were initially subject to a normal distribution analysis with SPSS software (1-sample K-S of non-parametric test). The in vivo RCT data in Figure 4C were analyzed by repeated measured analysis. All other data were analyzed by a parametric statistics, post-hoc test of one-way ANOVA if the data were
in normal distribution. A difference was considered to be statistically significant at $P<0.05$.

**References**


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ACC1: acetyl-CoA carboxylase 1; ACOX-1/2: peroxisomal acyl-coenzyme A oxidase 1 or 2; ATGL: adipose triglyceride lipase; CAT: carnitine acetyltransferase; CGI-58: comparative gene identification-58; CPT1α: carnitine palmitoyltransferase 1A; DGAT1: acyl-CoA:diacylglycerol acyltransferase 1; FASN: fatty acid synthase; HSL: hormone-sensitive lipase; ICAM-1: intercellular cell adhesion molecule-1; PPARα: peroxisome proliferator-activated receptor α; SCD-1: stearoyl-CoA desaturase 1; SREBP1c: sterol-responsive element binding protein 1c; MCP-1: monocyte chemotactic protein 1; MTPP: microsomal triglyceride transfer protein; MGLL: monoglyceride lipase.