Original Article

Inhibition of ERK1/2 and Activation of LXR Synergistically Reduce Atherosclerotic Lesions in ApoE-Deficient Mice

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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 inhibitor synergizes LXR ligand–induced macrophage ABCA1 expression and cholesterol efflux. In this study, we determined whether the mitogen-activated protein kinase kinase 1/2 inhibitor (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;35:00-00. DOI: 10.1161/ATVBAHA.114.305116.)**

Key Words: atherosclerosis ■ extracellular signal-regulated map kinases ■ foam cells ■ hypertriglyceridemia ■ lipogenesis ■ liver X receptor

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

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Nonstandard Abbreviations and Acronyms	
AMPK-α	AMP-activated protein kinase α
CHD	coronary heart disease
ERK1/2	extracellular signal–regulated kinases 1/2
HDL	high-density lipoprotein
HFD	high-fat diet
HSL	hormone-sensitive lipase
LDL	low-density lipoprotein
LXR	liver X receptor
MEK1/2	mitogen-activated protein kinase kinases 1/2
mpk	milligram per kilogram of bodyweight
RCT	reverse cholesterol transport
VLDL	very low-density lipoprotein

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.[11] 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.[6] 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.[12] Mitogen-activated protein kinase kinases 1/2 (MEK1/2) are the upstream dual specificity ERK1/2 kinases and are prerequired 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.[12] 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.[13] Hypertriglyceridemia also seems central to the pathophysiology of dyslipoproteinemia in insulin resistance and type 2 diabetes mellitus.[14] Although LXR can play important roles in different diseases,[15] 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.[16] 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.

Results

U0126 Antagonizes T0901317-Induced Hypertriglyceridemia and Hepatic Lipid Accumulation

Wild-type mice were fed a high-fat diet (HFD: 0.5% cholesterol and 21% fat) or HFD-containing T0901317 (1 milligram per kilogram of bodyweight [mpk]) or U0126 (1, 3, and 9 mpk) alone or in combination for 2 weeks. After these regimens, serum lipid profiles and hepatic lipid content were assessed. Compared with normal chow, serum total cholesterol was significantly increased by HFD, and the increase was further enhanced by T0901317. U0126 moderately reduced HFD or HFD plus T0901317-induced cholesterol levels (Figure 1A). T0901317 elevated HFD-increased alanine aminotransferase and aspartate aminotransferase levels (Figure 1B), indicating that T0901317 caused a moderate liver injury. In contrast, U0126 decreased T0901317-induced aspartate aminotransferase and alanine aminotransferase, suggesting that U0126 provides some protection to the liver. Figure 1C shows that T0901317 further increased HFD-induced serum triglyceride levels. U0126, at 3 and 9 mpk, blocked T0901317-induced serum triglyceride levels.

In the liver, HFD or HFD plus T0901317 reduced liver color (Figure 1D), implying lipid accumulation. U0126 blocked the liver color change, suggesting that it attenuates T0901317-induced lipid accumulation. Oil Red O staining indicates that HFD induced hepatic lipid accumulation, and the accumulation was greatly enhanced by T0901317 (Figure 1E, top). U0126 reduced lipid accumulation induced by HFD or HFD plus T0901317 (Figure 1E, middle and right columns). The quantitative analysis of the triglyceride content in the total lipid extract of liver samples demonstrates similar results (Figure 1F). Thus, U0126 blocks T0901317-induced transient hypertriglyceridemia and hepatic lipid accumulation.

Combination of T0901317 and U0126 Synergistically Inhibits the Development of Atherosclerosis

To determine whether the combined T0901317 and U0126 can synergistically inhibit lesion development while eliminating hypertriglyceridemia, both male and female apoE deficient (apoE$^{-/-}$) mice were fed T0901317 (1 mpk) or U0126 (3 mpk) alone or in combination in HFD for 16 weeks. The reported dose ranges of T0901317 and U0126 used in animal models were 3 to 50 and 5 to 40 mpk, respectively.[7,9,17,18] During the treatment, we routinely determined food intake, water drinking, and bodyweight gain. We observed no difference between the control group and the groups receiving treatment.

At the end of the treatment, we assessed lesion development in en face aortas and aortic root cross-sections by Oil Red O staining. Figure 2A demonstrates that T0901317 inhibited en face aortic lesions by ≈43% and ≈28% in male and female mice, respectively. U0126 alone slightly affected lesions. However, the combination of T0901317 and U0126 inhibited lesions >60% that was much more than the additive results of T0901317 and U0126 alone, suggesting that U0126 can synergize the inhibition of atherosclerosis by T0901317.

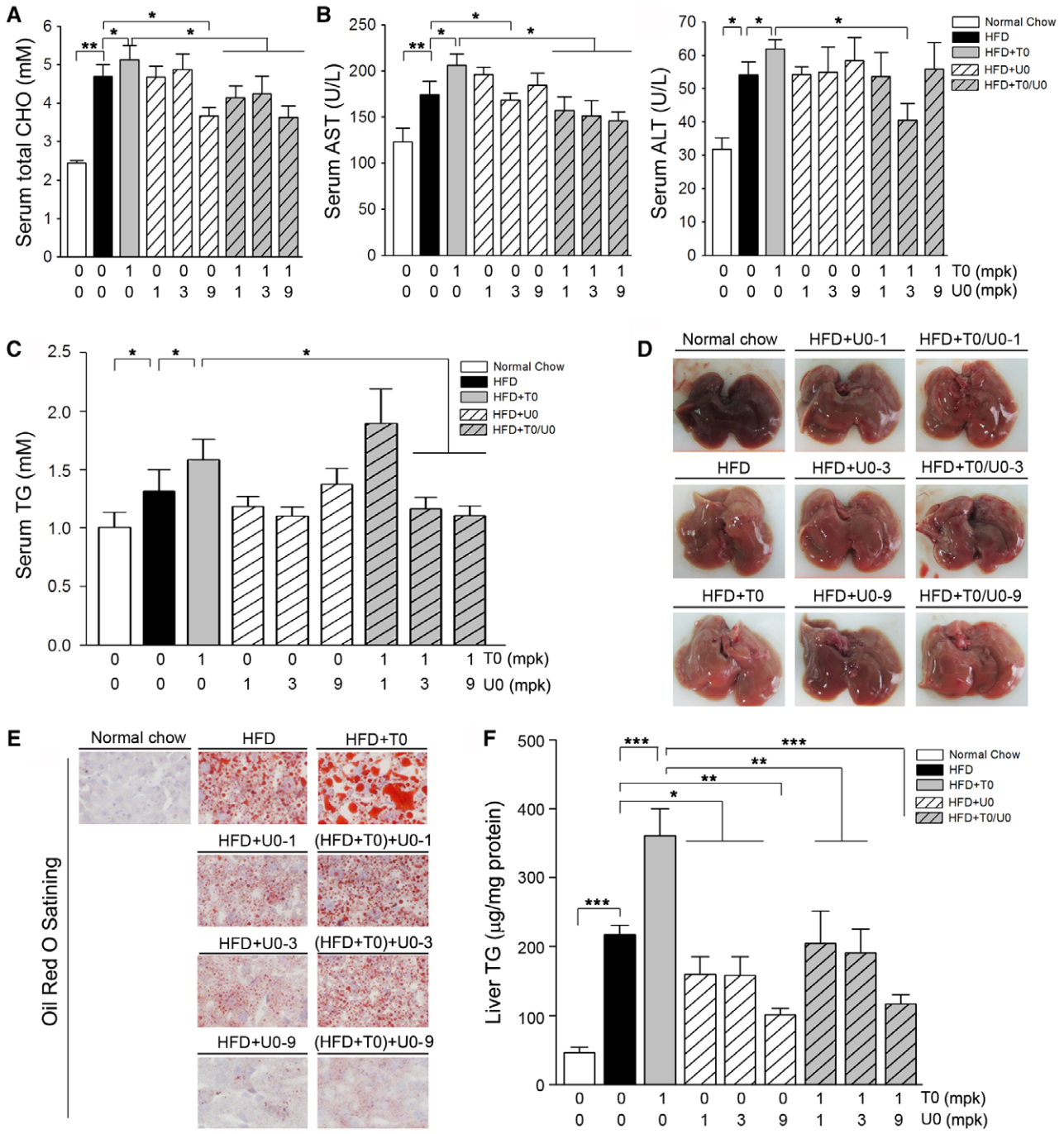

Figure 1. U0126 protects wild-type mice against T0901317-induced transient hypertriglyceridemia and hepatic lipid accumulation. Female wild-type mice (n=6) were fed normal chow or high-fat diet (HFD) or HFD containing T0901317 (T0) or U0126 (U0) alone or both for 2 weeks. The following assays were then completed. **A**–**C**, Serum total cholesterol (CHO), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride (TG). **D**, Liver photos. **E**, Oil Red O staining liver frozen sections. **F**, Hepatic TG content. **P*<0.05; ***P*<0.01; *****P*<0.001.

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 $(9.5\pm0.9)\times10^5$ μm^2, whereas the lesions were reduced to $(5.9\pm0.7)\times10^5$, $(6.50\pm0.5)\times10^5$, and $(1.2\pm0.2)\times10^5$ 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

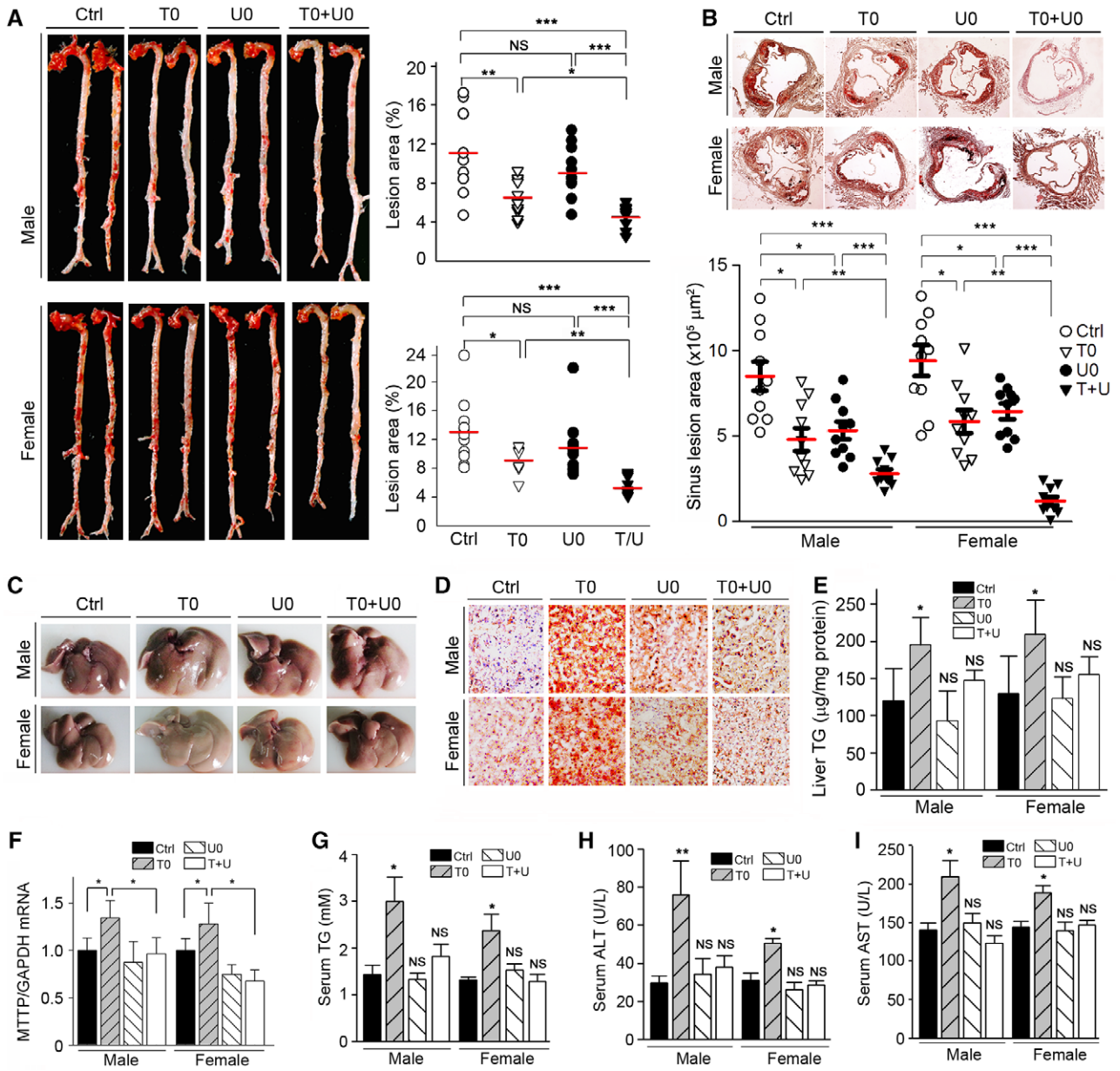

Figure 2. U0126 synergizes T0901317-inhibited atherosclerosis and blocks T0901317-induced hypertriglyceridemia and fatty liver. ApoE$^{-/-}$ mice (15 per group) were fed high-fat diet (HFD) or HFD containing T0901317 (T0 or T, 1 milligram per kilogram of body-weight [mpk]) or U0126 (U0 or U, 3 mpk) alone or both for 16 weeks. Serum, liver, and aorta were collected for the following assays. **A** and **B**, Lesions in en face aortas and aortic root cross-sections were determined by Oil Red O staining. **C**, Liver photos. **D** and **E**, Hepatic lipid content was determined by Oil Red O staining liver frozen sections and triglyceride (TG) quantitative analysis. **F**, Microsomal triglyceride transfer protein (MTTP) mRNA expression was determined by real-time reverse transcription polymerase chain reaction (n=6). **G–I**, Serum TG, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were determined by enzymatic methods. **P*<0.05; ***P*<0.01; ****P*<0.001 vs control in the corresponding group (n=15). NS indicates not significantly different.

doses used.[9,17] 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 (Figure 2E–2G). In addition, U0126

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.

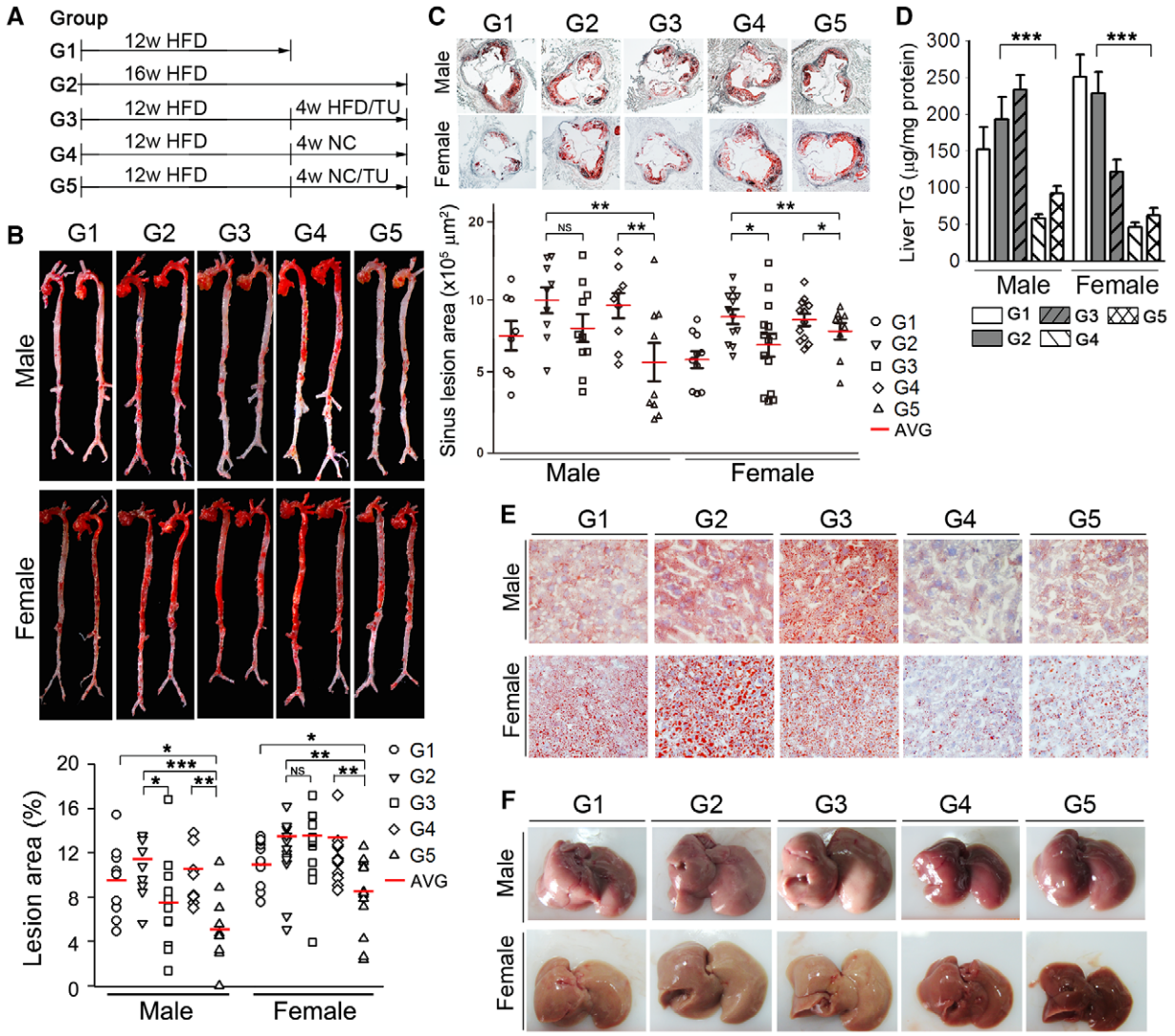

Figure 3. Regression of the advanced lesions by the combination of T0901317 (T0) and U0126 (U0) in normal chow. **A**, Experimental design: apoE$^{-/-}$ mice in 5 groups (15 per group) were scheduled for the indicated treatment; and the following assays were completed. **B** and **C**, Lesions in en 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-richest 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[^{3}H]-cholesterol–labeled macrophages followed by determination of excreted [^{3}H]-tracer into the feces. Figure 4C (left) demonstrates that T0901317 or U0126 alone or their combination increased [^{3}H]-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 metallopeptidase 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 (π-ERK1/2) in the aortic root (Figure III in the online-only Data Supplement). Similar π-ERK1/2 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.

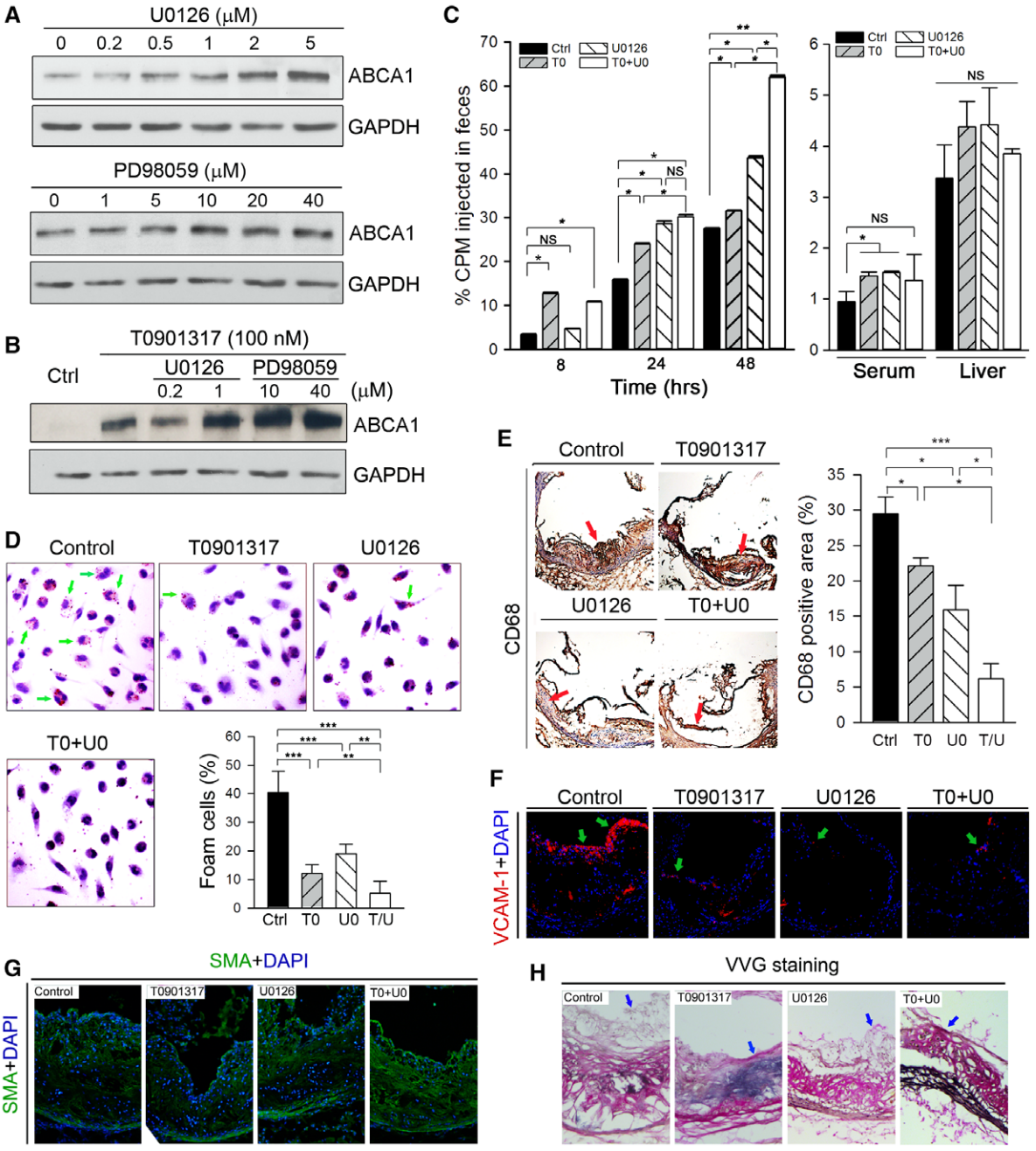

Figure 4. Mechanisms for synergistic reduction of atherosclerosis. **A** and **B**, Peritoneal macrophages isolated from apoE$^{-/-}$ mice were treated with mitogen-activated protein kinase kinase 1/2 inhibitors (PD98059 and U0126 [U0]) alone at the indicated concentrations or plus 100 nmol/L of T0901317 (T0) overnight followed by determination of ABCA1 expression by western blot. **C**, ApoE$^{-/-}$ mice (6 per group) received T0901317 (1 milligram per kilogram of bodyweight [mpk]) or U0126 (3 mpk) or both (1+3 mpk) contained in high-fat diet for 1 week followed by determination of in vivo reverse cholesterol transport with intraperitoneal injection of preradiolabeled macrophages. *P<0.05; **P<0.01; ***P<0.001, **D**, Peritoneal macrophages were collected from mice in Figure 2, fixed, and stained with Oil Red O and hematoxylin. The cells containing lipid droplets (>10 per cell) were considered as foam cells and >10 fields per sample were counted. Arrows indicate macrophage/foam cells. **E**, Aortic root sections were stained immunohistochemically with anti-CD68 antibody for determination of macrophage accumulation. **F**, Expression of VCAM-1 in aortic root sections was determined by immunofluorescent staining. Arrows indicate expression of vascular cell adhesion molecule-1 (VCAM-1) in the cap of lesions. **G** and **H**, The aortic root sections were conducted immunofluorescent staining with antismooth muscle actin (SMA) antibody and Verhoeff-Van Gieson (VVG) staining, respectively. Arrows indicate the cap of lesions. Ns indicates not significantly different.

Acyl-CoA:diacylglycerolacyltransferase 1 is the rate-limiting enzyme for liver triglyceride biosynthesis.[20] 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

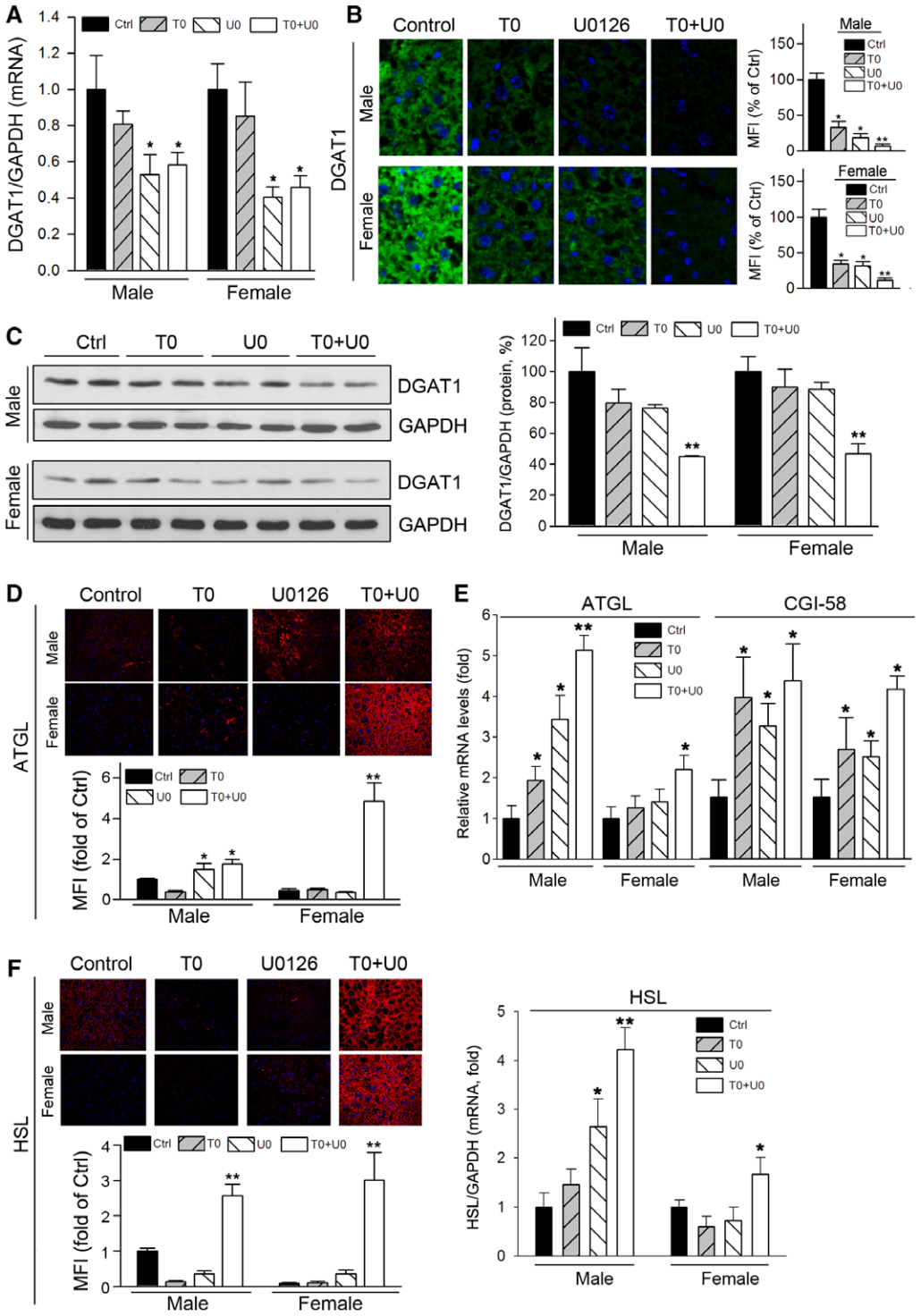

Figure 5. Combination of T0901317 (T0) and U0126 (U0) inhibits triglyceride (TG) biosynthesis and activates TG hydrolysis pathways. Total RNA, protein, and paraffin sections were prepared from the liver of mice used in Figure 2. Acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1; **A**), adipose triglyceride 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).

into diacylglycerol and fatty acid, the first and rate-limiting step in triglyceride hydrolysis although HSL can act on hydrolysis of diacylglycerol.[21,22] Adipose triglyceride lipase can be substantially activated by its coactivator, comparative gene identification-58.[23] 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

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 (π-AMPK-α), activates fatty acid β-oxidation.[25] Figure 6F shows T0901317 or U0126 alone or their combination increased π-AMPK-α protein and the ratio of π-AMPK-α to AMPK-α. Consequently, the phosphorylated acetyl-CoA carboxylase 1 (π-ACC1), a target of π-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 hepatic cell 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

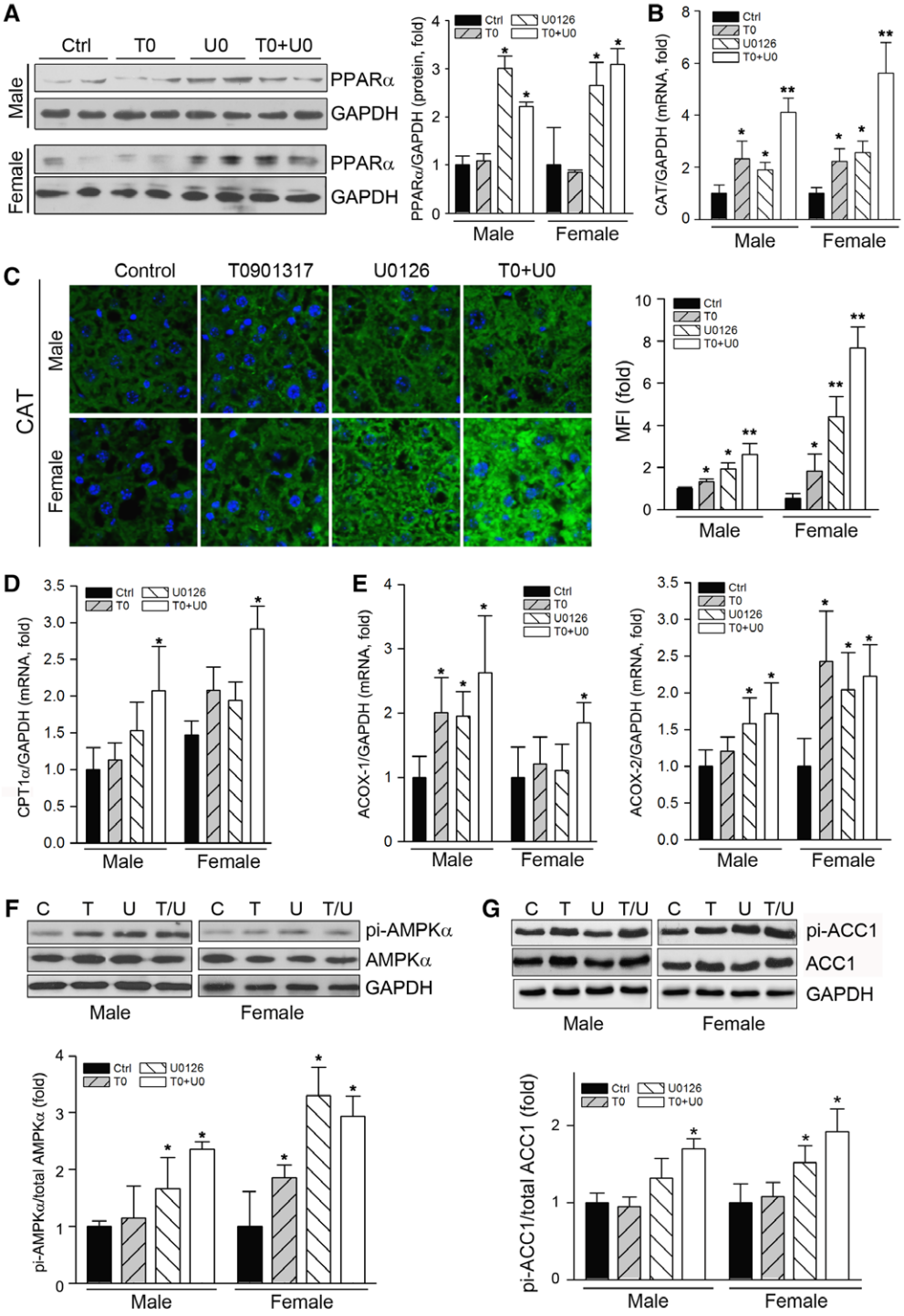

Figure 6. Combination of T0901317 (T0) and U0126 (U0) activates peroxisome proliferator–activated receptor-α (PPAR-α) and AMP-activated protein kinase α (AMPK-α) pathways. The samples in Figure 5 were used to complete the following assays. **A**, **F**, and **G**, PPAR-α, AMPK-α, π-AMPK-α, acetyl-CoA carboxylase 1 (ACC1), and π-AAC1 protein were determined by western blot. **B** and **C**, Carnitine acetyltransferase (CAT) mRNA and protein were determined by real-time reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescent staining. **D** and **E**, Carnitine palmitoyltransferase 1A (CPT1α), peroxisomal acyl-CoA A oxidase 1 (ACOX-1), and ACOX-2 mRNA were determined by real-time RT-PCR. **P*<0.05; ***P*<0.01 vs control in the corresponding group (n=6). MFI indicates mean of fluorescent intensity.

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 but it was terminated because of the adverse effects on the central nervous system.[27] LXR-623, in combination with simvastatin, synergistically induces regression of advanced arterial lesions in the rabbit model.[39] The regressive effect is ascribed to inhibition of some inflammatory molecules, such as MCP-1, COX-2, and tissue factor. However, LXR-623 at a high dose induces hypertriglyceridemia, but it had no effect on lesions, and activated expression of those inflammatory molecules.[39]

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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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.