Activation of Adiponectin Receptor Regulates Proprotein Convertase Subtilisin/Kexin Type 9 Expression and Inhibits Lesions in ApoE-Deficient Mice

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Objective—The reduced adiponectin levels are associated with atherosclerosis. Adiponectin exerts its functions by activating adiponectin receptor (AdipoR). Proprotein convertase subtilisin kexin type 9 (PCSK9) degrades LDLR protein (low-density lipoprotein receptor) to increase serum LDL-cholesterol levels. PCSK9 expression can be regulated by PPARγ (peroxisome proliferator–activated receptor γ) or SREBP2 (sterol regulatory element-binding protein 2). The effects of AdipoR agonists on PCSK9 and LDLR expression, serum lipid profiles, and atherosclerosis remain unknown.

Approach and Results—At cellular levels, AdipoR agonists (ADP355 and AdipoRon) induced PCSK9 transcription/ expression that solely depended on activation of PPAR-responsive element in the PCSK9 promoter. AdipoR agonists induced PPARγ expression; thus, the AdipoR agonist-activated PCSK9 expression/production was impaired in PPARγ deficient hepatocytes. Meanwhile, AdipoR agonists transcriptionally activated LDLR expression by activating SRE in the LDLR promoter. Moreover, AMP-activated protein kinase α (AMPKα) was involved in AdipoR agonist-activated PCSK9 expression. In wild-type mice, ADP355 increased PCSK9 and LDLR expression and serum PCSK9 levels, which was associated with activation of PPARγ, AMPKα and SREBP2 and reduction of LDL-cholesterol levels. In contrast, ADP355 reduced PCSK9 expression/secretion in apoE-deficient (apoE−/−) mice, but it still activated hepatic LDLR, PPARγ, AMPKα, and SREBP2. More importantly, ADP355 inhibited lesions in en face aortas and sinus lesions in aortic root in apoE−/− mice with amelioration of lipid profiles.

Conclusions—Our study demonstrates that AdipoR activation by agonists regulated PCSK9 expression differently in wild-type and apoE−/− mice. However, ADP355 activated hepatic LDLR expression and ameliorated lipid metabolism in both types of mice and inhibited atherosclerosis in apoE−/− mice.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1290-1300. DOI: 10.1161/ATVBAHA.117.309630.)

Key Words: adiponectin ◼ adiponectin receptors ◼ AMP-activated protein kinases ◼ atherosclerosis ◼ peroxisome proliferator-activated receptors

Adiponectin is mainly produced by and secreted from adipocytes/adipose tissues. Therefore, a high concentration of adiponectin can be determined in plasma.1,2 Reduction of circulating adiponectin levels has been demonstrated to be associated with obesity-related diseases, such as diabetes mellitus and atherosclerosis.1,3 Furthermore, both clinical and basic research studies indicate the antiatherogenic properties of adiponectin.1 For instance, adiponectin decreases type A scavenger receptor expression in human monocyte-derived macrophages, thereby reducing foam cell formation.4 High plasma adiponectin concentrations may reduce the risk of myocardial infarction in men; therefore, compared with those in age- and body mass index–matched control subjects, plasma adiponectin levels can be found lower in patients with coronary heart disease.6 In animal models, high expressing human adiponectin suppresses the development of atherosclerosis in apoE-deficient (apoE−/−) mice by multiple mechanisms, such as reducing expression of vascular cell adhesion molecule-1 and SRA (scavenger receptor type A) in lesion areas.7 However, controversial results have been reported that lack of adiponectin expression has little effect on atherosclerosis in low-density lipoprotein receptor...
Although the functions of adiponectin and AdipoR on atherosclerosis have been investigated, it remains unknown whether AdipoR activation by agonists, such as ADP355, can regulate PCSK9 expression/function and cholesterol metabolism to influence the development of atherosclerosis. In this study, we determined the effect of AdipoR activation by ADP355 and AdipoRon on PCSK9 expression/secretion, LDLR expression, and the involved mechanisms with hepatocytes in vitro. We further determined the effect of ADP355 on hepatic LDLR and PCSK9 expression and serum PCSK9 levels, and lipid profiles in both wild-type and apoE−/− mice and atherosclerotic lesions in apoE−/− mice.

Materials and Methods
The detailed Materials and Methods is available in the online-only Data Supplement.

Results
AdipoR Agonists Induce PCSK9 Expression Transcriptionally in a PPARγ-Responsive Element–Dependent Manner
Hepatocytes is a major cell type producing and secreting PCSK9.20 To determine whether AdipoR activation by agonists can regulate PCSK9 expression, we treated human hepatic cell line, HepG2 cells with 2 AdipoR agonists, ADP355 and AdipoRon, at different concentrations overnight followed by determination of PCSK9 protein expression. The results in Figure 1A and 1C indicate that ADP355 and AdipoRon induced PCSK9 expression in a concentration-dependent manner. The time-course study suggests that AdipoR agonists induced PCSK9 expression quickly after treatment (Figure 1B and 1D), and the induction lasted for 24 hours after treatment. In addition, we treated primary hepatocytes isolated from wild-type (C57BL/6) mice with ADP355 and AdipoRon at different concentrations overnight. Similarly, both ADP355 and AdipoRon increased PCSK9 expression in murine primary hepatocytes (Figure 1E and 1F), which indicates that the induction of PCSK9 expression by AdipoR agonists is not species dependent.

Associated with increased PCSK9 protein levels, PCSK9 mRNA expression was increased by ADP355 (Figure 1G), which suggests that activation of PCSK9 expression might occur at the transcriptional level. Human PCSK9 promoter contains both PPARγ-responsive element (PPRE; AGGGCAgAGGCCG, from −369 to −357) and sterol regulatory element (SRE; GTGGCGTGA, from +17 to +25). Therefore, activation of PPARγ or SREBP2 (sterol regulatory element–binding protein 2) can induce PCSK9 transcription. To determine the effect of AdipoR agonists on PPRE or SRE activity in the PCSK9 promoter, we constructed a normal human PCSK9 promoter and a promoter with mutation of either PPRE or SRE. The cells transfected with PCSK9 promoter DNA were treated with ADP355 overnight followed by determination of promoter activity. As shown in Figure 1H, ADP355 enhanced normal PCSK9 promoter activity (left panel), which confirms the activation of PCSK9 transcription by AdipoR agonists. Similar to the normal promoter, treatment of the SRE-mutated

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Nonstandard Abbreviations and Acronyms

| AdipoR | adiponectin receptor |
| AMPK | AMP-activated protein kinase |
| apoE−/− | apoE deficient |
| LDL-C | low-density lipoprotein-cholesterol |
| LDLR | low-density lipoprotein receptor |
| PCSK9 | proprotein convertase subtilisin/kexin type 9 |
| PPAR | peroxisome proliferator–activated receptor |
| PPRE | PPAR-responsive element |
| SRE | sterol regulatory element |
| SREBP2 | sterol regulatory element–binding protein 2 |
PCSK9 promoter with ADP355 still enhanced its activity (Figure 1H, middle panel), suggesting that SRE is not involved in AdipoR agonist-induced PCSK9 transcription. In contrast, the PPRE mutation impaired the activation of PCSK9 promoter by ADP355 (Figure 1H, right panel). Therefore, activation of PPARγ, not of SREBP2, plays an important role in the induction of PCSK9 expression by AdipoR agonists.

To further define the role of PPRE in AdipoR agonist-induced PCSK9 transcription, we conducted a ChIP (chromatin immunoprecipitation) assay to determine the effect of AdipoR agonists on the binding of PPARγ protein with the PPRE or SREBP2 protein with the SRE, in the PCSK9 promoter. As shown in Figure 1I, treatment of HepG2 cells with ADP355 increased the binding of PPRE with PPARγ protein (left panel) while had little effect on the binding of SRE with SREBP2 protein (right panel). Taken together, Figure 1 demonstrates that AdipoR activation by agonists induces PCSK9 transcription in a PPRE-dependent manner, but not in an SRE-dependent manner.
Activation of PCSK9 Expression by AdipoR Agonists Depends on Induction of PPARγ Expression

Our above results indicate that PPARγ can play an important role in the induction of PCSK9 expression by AdipoR agonists. Therefore, we determined PPARγ expression in response to AdipoR agonist treatment. Figure 2A shows that ADP355 and AdipoRon increased PPARγ protein expression in a concentration-dependent manner in HepG2 cells. Similarly, both ADP355 and AdipoRon induced PPARγ protein expression in primary hepatocytes (Figure 2B). Moreover, ADP355 and AdipoRon increased PPARγ mRNA expression (Figure 2C), which indicates that AdipoR agonists may activate PPARγ transcription. Indeed, we determined that ADP355 increased PPARγ promoter activity (Figure 2D).

Because AdipoR agonists induced PPARγ and PCSK9 expression simultaneously (Figures 1 and 2), and the induction of PCSK9 expression solely depended on PPRE activity (Figure 1H and 1I), we anticipate that PPARγ is the transcriptional factor controlling AdipoR agonist-induced PCSK9 expression, and lack of PPARγ expression can impair the functions of AdipoR agonists on PCSK9 expression. Therefore, we transiently transfected HepG2 cells with scrambled siRNA and PPARγ siRNA and determined that reduced PPARγ

![Figure 2.](image)

* Figure 2. Activation of proprotein convertase subtilisin kexin type 9 (PCSK9) production by ADP355 (ADP) and AdipoRon (AR) depends on PPARγ (peroxisome proliferator–activated receptor γ) expression. A and B, HepG2 cells or primary hepatocytes isolated from wild-type mice were treated with ADP355 or AR at the indicated concentrations overnight. Expression of PPARγ protein was determined by Western blot. C, HepG2 cells (left) or primary hepatocytes isolated from wild-type mice (right) were treated with ADP at the indicated concentrations or ADP (25 ng/mL) and AR (5 μmol/L) overnight. PPARγ mRNA expression was determined by real-time RT-PCR. *P<0.05 vs control (n=3). D, 293T cells were transfected with DNA for the PPARγ promoter and Renilla luciferase. After transfection and indicated treatment overnight, activity of firefly and Renilla luciferases in the cellular lysate were determined. *P<0.05 vs control (n=3). E, HepG2 cells in 6-well plates were transfected with scrambled siRNA or PPARγ siRNA (50 nmol/L) and then treated with ADP355 at the indicated concentrations overnight. F–H, Primary hepatocytes isolated from PPARγfl/fl and HepPPARγKO mice, respectively, were treated with ADP355 and AR at the indicated concentrations overnight. Expression of PPARγ protein (E) and PCSK9 protein (E and G) was determined by Western blot. Expression of PPARγ mRNA (F) and PCSK9 mRNA (H) was determined by real-time RT-PCR. *P<0.05, **P<0.01 (n=3). I, Confluent PPARγfl/fl and HepPPARγKO hepatocytes in serum-free medium were treated with ADP355 (25 ng/mL) or AR (5 μmol/L) overnight, respectively. PCSK9 in culture medium was determined using a PCSK9 ELISA assay kit. *P<0.05. ns indicates not significant difference (n=6).
expression abolished ADP355-induced PCSK9 expression (Figure 2E). Next, we isolated hepatocytes from mice with liver-specific PPARγ deficient (HepPPARγ KO) mice and littermate (PPARγ<sup>fl/fl</sup>) mice (Figure 2F) and treated cells with ADP355 or AdipoRon overnight. Similar to that in HepG2 cells and primary hepatocytes isolated from wild-type mice...
(Figure 1), treatment of cells isolated from PPARγfl/fl mice with ADP355 and AdipoRon increased PCSK9 mRNA and protein levels. However, neither ADP355 nor AdipoRon had effect on PCSK9 expression in cells isolated from HepPPARγ KO mice (Figure 2G and 2H). Consistently, PCSK9 secretion was enhanced by ADP355 or AdipoRon in hepatocytes isolated from PPARγfl/fl mice, but not from HepPPARγ KO mice (Figure 2I). Taken together, the results in Figure 2 suggest that induction of PCSK9 expression/secretion by AdipoR agonists is mainly determined by the induction of PPARγ expression.

**AdipoR Agonists Induce LDLR Expression in HepG2 Cells**

PCSK9 reduces LDLR levels by enhancing LDLR protein degradation.21 To determine the effect of AdipoR agonist-induced PCSK9 expression on hepatic LDLR expression, we assessed LDLR protein levels in response to AdipoR agonist treatment by Western blot and immunofluorescent staining. In contrast to what we expected, both ADP355 and AdipoRon increased LDLR protein expression (Figure 3A through 3C), indicating that LDLR expression is not affected by AdipoR agonist-induced PCSK9 expression, or AdipoR agonists induce LDLR expression by a different pathway.

To determine it in detail, we assessed the effects of AdipoR agonists on LDLR mRNA expression and promoter activity. Figure 3D shows that LDLR mRNA expression was induced by both ADP355 and AdipoRon. LDLR is also a target gene of SREBP2.22 Therefore, we constructed a normal murine or human LDLR promoter and the promoter with SRE mutation (pNLDR-SREmut or pHLDR-SREmut), respectively. Compared with normal LDLR promoter, SRE mutation substantially reduced the basal activity of LDLR promoter, indicating the critical role of SRE in LDLR transcription (Figure 3E). More importantly, the results in Figure 3E show that ADP355 and AdipoRon

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**Figure 4.** Induction of proprotein convertase subtilisin kexin type 9 (PCSK9) expression by ADP355 (ADP) and AdipoRon (AR) is related to activation of AMP-activated protein kinase α (AMPKα). A, Expression of AdipoR1 and AdipoR2 in the stable AdipoR1 and AdipoR2 knockdown HepG2 (shAR1i and shAR2i) cells was determined by real-time RT-PCR. "P<0.01 vs control (shNSi) cells (n=3). B, shNSi, shAR1i, or shAR2i cells received indicated treatment overnight. Expression of PCSK9, LDLR (low-density lipoprotein receptor), AMP-activated protein kinase α (AMPKα), and pi-AMPKα was determined by Western blot. C and D, HepG2 cells were treated with ADP355 and AR at the indicated concentrations overnight. Expression of AMPKα and pi-AMPKα was determined by Western blot. AICAR was used as a positive control. E, HepG2 cells were treated with AICAR or metformin (Met) at the indicated concentrations overnight followed by determination of PCSK9, AMPKα, pi-AMPKα, and PPARγ protein expression. F, CRISPR-Ctrl (clustered regulatory interspaced short palindromic repeat-associated 9) and CRISPR-AMPKα1 HepG2 cells were treated with ADP355 at the indicated concentrations overnight followed by determination of AMPKα1, PPARγ, and PCSK9 protein expression. G and H, Primary hepatocytes isolated from PPARγfl/fl and HepPPARγ KO mice were treated with AICAR or Met at the indicated concentrations overnight. Expression of PCSK9 protein (G) and mRNA (H) was determined by Western blot and real-time RT-PCR, respectively. "P<0.05 vs control (n=3).
increased normal LDLR promoter activity while had little effect on the activity of LDLR promoter with SRE mutation. Therefore, AdipoR agonists increased LDLR expression by activating SRE in the LDLR promoter. Indeed, the results of ChIP assay suggest that the interaction between SREBP2 protein and SRE was enhanced by ADP355 and AdipoRon treatment (Figure 3F).

Furthermore, the results in Figure 3G demonstrate that ADP355 and AdipoRon increased both precursor and mature form of SREBP2 protein, suggesting that the enhanced SREBP2 expression and maturation contributed to the induction of LDLR expression.

**AMPK Pathway Is Involved in AdipoR Agonist-Induced PCSK9 Expression**

To determine the role of adiponectin receptors in the agonist-induced PCSK9/LDLR expression, we prepared the AdipoR1 or AdipoR2 knockdown (shAR1i or shAR2i) cells. Compared with control cells (shNSi), the induction of PCSK9 or LDLR by ADP355 was attenuated in shAR1i cells, whereas it was slightly affected in shAR2i cells (Figure 4B), indicating the important role of AdipoR1 in agonist-regulated PCSK9/LDLR expression.

Although it has been reported that adiponectin activates AMPK pathway through AdipoR1 activation,11 not much is known whether AMPK pathway is reversely involved in adiponectin functions. Confirming the effect of adiponectin, we determined that both ADP355 and AdipoRon increased phosphorylated AMPKα (p-AMPKα; Figure 4C and 4D), and the increase depended on expression of AdipoR1, not of AdipoR2 (Figure 4B). To investigate the role of AMPKα pathway in regulation of PCSK9 expression, we treated cells with AICAR (5-aminimidazole-4-carboxamide 1-β-D-ribofuranoside) and metformin, 2 pharmacological activators of AMPKα. As expected, they increased PCSK9 and PPARγ expression in HepG2 cells (Figure 4E). Furthermore, we constructed an HepG2 cell line with deficiency of AMPKα1 expression by clustered regulatory interspersed short palindromic repeat (CRISPR)-associated 9 (Cas9) method (CRISPR-AMPKα1). Compared with the corresponding control cells (CRISPR-Ctrl), we determined that the

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Administration of ADP355 to wild-type mice induces proprotein convertase subtilisin kexin type 9 (PCSK9) and LDLR (low-density lipoprotein receptor) expression in the liver and improves serum lipid profiles. Wild-type mice in 2 groups (8 mice per group) were intraperitoneally injected with vehicle (scrambled peptide) and ADP355 (1 mg/kg bodyweight per day), respectively, for 3, 10, or 30 d. At the end of experiment, both liver and serum samples were individually collected and used for the following assays. A, Expression of PCSK9, p-AMPKα, AMPKα, PPARγ, LDLR, and SREBP2 (sterol regulatory element-binding protein 2) proteins in the liver was determined by Western blot. B and C, Serum PCSK9 levels and lipid profiles were determined by ELISA (B) and enzymatic methods (C), respectively. *P<0.05, **P<0.01 vs control in the corresponding groups (n=8). HDL-C indicates high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; T-CHO, total cholesterol; and TG, triglyceride.
induction of PCSK9 and PPARγ expression by ADP355 was abolished in the cells lacking AMPKα1 expression (Figure 4F). Taken together, AMPKα activity is also involved in AdipoR agonist-induced PPARγ/PCSK9 expression.

We next treated primary hepatocytes isolated from PPARγfl/fl and HepPPARγ KO mice with AICAR and metformin, respectively, and determined that both increased PCSK9 protein and mRNA expression in the cells isolated from PPARγfl/fl mice, whereas having little effect on cells collected from HepPPARγ KO mice (Figure 4G and 4H). These results further confirm the important role of AMPKα in regulation of PCSK9 expression, which is related to PPARγ expression.

**AdipoR Agonist Induces PCSK9 and LDLR Expression and Ameliorates Lipid Profiles in Wild-Type Mice**

To determine the physiological relevance of AdipoR activation on PCSK9 expression, wild-type mice on normal chow were intraperitoneally injected with scrambled peptide (control) or ADP355 for different periods (3, 10, or 30 days). Compared with control mice, ADP355 treatment did not cause differences, such as food intake and bodyweight gain, to the animals. After treatment, expression of PCSK9, AMPKα, pi-AMPKα, PPARγ, LDLR, and SREBP2 proteins in the liver was determined by Western blot. Interestingly, the short-term ADP355 treatment (3 days) had little effect on expression of forementioned molecules (Figure 5A, left panel). Serum PCSK9 levels and lipid profiles were not influenced either (Figure 5B and 5C, left panels). However, when ADP355 treatment to mice was extended to 10 or 30 days, expression of PCSK9 and LDLR was increased, which was associated with activation of AMPKα, SREBP2, and PPARγ (Figure 5A, middle and right panels). Meanwhile, serum PCSK9 levels were also increased after 10 or 30 days of treatment (Figure 5B, middle and right panels).

Although ADP355 increased liver PCSK9 expression and serum PCSK9 levels in wild-type mice, we observed that

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**Figure 6.** Administration of ADP355 to apoE−/− mice inhibits atherosclerosis. ApoE−/− mice in 2 groups (10 mice per group) were intraperitoneally injected with scrambled peptide (control) or ADP355 (1 mg/kg bodyweight per day), respectively, for 12 wk. At the end of experiment, aorta, liver, and serum samples were individually collected and used for the following assays. **A and B,** Lesions in en face aortas and sinus lesion in aortic root were determined by Oil-Red-O staining. **C and D,** Expression of CD68 and SMA (smooth muscle actin) in aortic root sections was determined by immunofluorescent staining. **E,** Serum proprotein convertase subtilisin kexin type 9 (PCSK9) levels were determined by an ELISA kit. **F,** Expression of PCSK9, pi-AMP-activated protein kinase-α (pi-AMPKα), AMPKα, PPARγ (peroxisome proliferator-activated receptor γ), LDLR (low-density lipoprotein receptor), and SREBP2 (sterol regulatory element-binding protein 2) proteins in the liver was determined by Western blot. *P<0.05; **P<0.01 vs control in the corresponding group (n=10).
ADP355 treatment for 10 or 30 days decreased serum total cholesterol and LDL-C levels (Figure 5C, middle and right panels). In addition, we determined the effects of ADP355 on serum apolipoprotein levels. ADP355 had no effect on serum apoA1 and apoB levels at all 3 time points of treatment, and apoE levels after 3 and 10 days of treatment. However, it substantially increased serum apoE levels after 30 days of treatment (Figure I in the online-only Data Supplement). Taken together, although AdipoR activation by agonists induced PCSK9 expression, it increased LDLR expression by activating SREBP2, reduced LDL-C levels, and increased apoE levels in wild-type mice after a long-term treatment, suggesting the amelioration of cholesterol metabolism.

ADP355 Inhibits Atherosclerosis in apoE−/− Mice

Although ADP355 induced PCSK9 expression/secretion in wild-type mice (Figure 5A and 5B), it still activated hepatic LDLR expression (Figure 5A) and ameliorated cholesterol metabolism (Figure 5C; Figure I in the online-only Data Supplement), which implies that ADP355 may inhibit atherosclerosis. To determine it, apoE−/− mice on high-fat diet were intraperitoneally injected with scrambled peptide (control) or ADP355 for 12 weeks. We determined that ADP355 treatment inhibited en face aortic lesions by one third (12.3% in the control group versus 8.2% in the ADP355 group; Figure 6A). The similar reduction of sinus lesions in aortic root by ADP355 treatment was observed (8.67×10^5 μm^2 in the control group versus 5.6×10^5 μm^2 in the ADP355 group; Figure 6B).

CD68 is a marker for macrophages/foam cells. The results of immunofluorescent staining demonstrate that CD68 expression in aortic root cross section was moderately inhibited by ADP355 (Figure 6C), suggesting an inhibition of macrophage/foam cells accumulation in arterial wall. Meanwhile, ADP355 had little effect on SMA (smooth muscle actin; Figure 6D) and CD31 levels (Figure II in the online-only Data Supplement), indicating that the aortic vascular integrity was not influenced by ADP355 treatment.

In contrast to the induction of PCSK9 expression/secretion in wild-type mice, ADP355 decreased serum PCSK9 levels (Figure 6E) and hepatic PCSK9 expression in apoE−/− mice (Figure 6F). Meanwhile, similar to wild-type mice, LDLR expression in apoE−/− mouse liver was increased, which might be contributed by activation of SREBP2, PPARγ and AMPKα, and inhibition of PCSK9 expression (Figure 6F). The results of lipid profile assay in the Table show that ADP355 treatment reduced serum LDL-C while increasing high-density lipoprotein-cholesterol (HDL-C) levels. Taken together, these results suggest that ADP355 inhibits atherosclerosis by multiple mechanisms.

**Discussion**

The mature PCSK9 binds with LDLR protein to form a complex to direct LDLR protein toward lysosome for degradation. Thus, PCSK9 inhibits LDLR expression at a post-transcriptional level and plays a critical role in cholesterol homeostasis. In addition to SREBP2, we have reported that PPARγ is another transcription factor activating PCSK9 transcription because a PPRE exists in the PCSK9 promoter. In the current study, at cellular levels, we determined that PCSK9 expression can be induced by AdipoR agonists, and the induction was completed by activating PPARγ pathway. The induction of PCSK9 expression/secretion was also observed in wild-type mice. Meanwhile, AdipoR agonists activated SRE in the promoter of LDLR, but not of PCSK9, which resulted in a direct induction of LDLR expression by AdipoR agonists and surpassed the facilitation of LDLR degradation by increased PCSK9. Therefore, the long-term ADP355 treatment decreased serum total and LDL-C levels, but increased apoE levels in wild-type mice. More importantly, the long-term ADP355 treatment inhibited atherosclerosis in apoE−/− mice, suggesting the physiological relevance of AdipoR agonists.

AdipoR1 transmits signals mainly through AMPK and PPARγ coactivator-1α, whereas AdipoR2 acts through PPARα-related pathways. Consistently, we observed that similar to adiponectin, both ADP355 and AdipoRon activated AMPK in HepG2 cells in an AdipoR1-, not in an AdipoR2-dependent manner (Figure 4B through 4D). Furthermore, we found that AMPK activation was involved in AdipoR agonist-induced PCSK9 expression based on the observations that AMPK activators (AICAR and metformin) enhanced PPARγ and PCSK9 expression (Figure 4E), whereas lack of AMPK expression impaired the inductive effects of AdipoR agonists on PPARγ and PCSK9 expression (Figure 4F). Reciprocally, AMPK agonists had no effect on PCSK9 expression in cells lacking PPARγ expression (Figure 4G and 4H). Taken together, our study suggests that PPARγ plays a central role in AdipoR agonist-induced PCSK9 expression. However, we still observed that PCSK9 basal level was slightly increased in PPARγ deficient hepatocytes, which implies that multiple regulatory mechanisms may influence PCSK9 expression.

The effects of PPARγ activation on adiponectin production have been investigated. Thiazolidinediones, the synthetic PPARγ ligands, can increase adiponectin production both in vitro and in vivo. In addition, PPARγ activation by thiazolidinediones restores tumor necrosis factor-α inhibited adiponectin production in adipocytes. Adiponectin exhibits anti-inflammatory effect including inhibition of the tumor necrosis factor-α–induced gene expression in human aortic endothelial cells. In human monocytes or monocyte-derived macrophages and dendritic cells, adiponectin induces the expression of anti-inflammatory cytokines, interleukin-10 and interleukin-1RA, but inhibits macrophage interferon-γ expression. PPARγ activation by thiazolidinediones also demonstrates anti-inflammatory effects. In our study, we determined

**Table.** ADP355 Ameliorates Lipid Profiles in apoE−/− Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ADP355</th>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>32.69±6.16</td>
<td>32.27±5.63</td>
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<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>3.74±0.27</td>
<td>4.16±0.38*</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>15.02±1.87</td>
<td>13.14±1.83†</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.82±0.35</td>
<td>1.59±0.30</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>32.45±1.69</td>
<td>33.36±1.37</td>
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</table>

The lipid profiles of mice in Figure 6 were determined by enzymatic methods, respectively. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein.

*P<0.01 vs control (n=10).
†P<0.05.
that AdipoR agonists induced PPARγ expression indicating the cross-regulation between PPARγ and adiponectin, which may impact the common effects of PPARγ and adiponectin, such as anti-inflammation.

Activation of PPARα by agonists, such as fenofibrate, can ameliorate lipid and metabolic abnormalities in patients by reducing plasma triglyceride levels and increasing high-density lipoprotein-cholesterol levels. The effects of PPARα agonists on PCSK9 have also been determined with controversial results. Fenofibrate decreased plasma PCSK9, total and LDL-C levels in diabetic patients.28 However, other studies indicated that treatment of patients with fibrate increased plasma PCSK9 levels.29,30 We also determined that fenofibrate had little effect on PCSK9 expression in HepG2 cells (data not shown). Adiponectin has been reported to activate expression of PPARα and PPARα target genes in different tissues.3,10,31 In our study, we obtained several lines of evidence to demonstrate that induction of PCSK9 expression/production by AdipoR agonists is completed through activation of PPARγ pathway. Besides activation of PPRE in the PCSK9 promoter (Figure 1H) and PPARγ expression (Figure 2A through 2D), we observed that induction of PCSK9 expression/secretion by ADP355 was impaired in cells with reduced or knockout of PPARγ expression (Figure 2E through 2I). Furthermore, we determined that activation of PCSK9 expression by AMPK activators was also attenuated by deficiency of PPARγ expression (Figure 4G and 4H).

LDLR expression, a critical molecule in LDL-C metabolism, is regulated by SREBP2, which is initially synthesized as an inactive precursor. After cleavage, the mature form of SREBP2 translocates into the nuclei, binds to SRE in the LDLR promoter to activate LDLR transcription.32 Similarly, SREBP2 activates PCSK9 transcription because of an SRE in the PCSK9 promoter.33 Therefore, stains activate LDLR and PCSK9 transcription simultaneously, which results in the resistance to LDL-C-lowering effect of statins in dyslipidemic patients.34 In our study, AdipoR agonists activated PCSK9 expression through PPARγ activation that may enhance LDLR degradation. Meanwhile, AdipoR agonists enhanced SREBP2 expression/maturation thereby activating LDLR transcription (Figure 3). In contrast, activation of SREBP2 pathway by AdipoR agonists seemed to have little effect on PCSK9 transcription (Figure 1H). Therefore, the induction of LDLR transcription can surpass the LDLR degradation and eventually lead to reduction of LDL-C levels by ADP355 treatment in wild-type mice (Figure 5C). In apoE−/− mice, ADP355 treatment also activated SREBP2 and LDLR expression (Figure 6F), which was associated with increased HDL-C and decreased LDL-C levels (Table). Interestingly, in contrast to wild-type mice, ADP355 decreased PCSK9 expression/secretion in apoE−/− mice (Figure 6E and 6F). The different regulations of PCSK9 expression by ADP355 between wild-type and apoE−/− mice might be related to apoE expression. Although whether apoE is able to activate PCSK9 expression has not been reported, deficiency of PCSK9 expression decreases apoE-containing HDL subfractions and reduces cholesterol efflux capacity of the serum.35 In our study, we determined that long-term ADP355 treatment substantially increased serum apoE levels in wild type mice (Figure 1A in the online-only Data Supplement). These findings imply that the interactions between PCSK9 and apoE need further investigation to elucidate. However, reduction of PCSK9 expression/secretion by ADP355 treatment can support the antiatherogenic properties of adiponectin selectively in apoE−/− mice, not in LDLR-deficient mice.35 Besides amelioration of lipid profiles, expression of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase, in human umbilical vein endothelial cells was increased by ADP355 (Figure III in the online-only Data Supplement), indicating that AdipoR agonists can protect endothelial cells against oxidative stress. AdipoR agonists also decreased the levels of mitochondrial superoxide levels in primary hepatocytes isolated from PPARγ−/− and HepPPARγ KO mice (Figure IV in the online-only Data Supplement), which partially depends on PPARγ expression. Taken together, multiple biological actions of ADP355 make contribution to its antiatherogenic properties.

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Disclosures

None.

References

AdipoR agonists induce hepatic proprotein convertase subtilisin kexin type 9 (PCSK9) expression/secretion in wild-type mice.

The induction of PCSK9 expression is related to activation of AMP-activated protein kinase \( \alpha \) (AMPK\( \alpha \)) and PPAR\( \gamma \) receptor–activated receptor \( \gamma \).

AdipoR agonists induce hepatic LDLR (low-density lipoprotein receptor) expression by activating sterol regulatory element (SRE).

AdipoR agonists inhibit PCSK9 expression but activate LDLR expression and ameliorate cholesterol metabolism, in apoE-deficient mice, that results in inhibition of atherosclerosis.
Activation of Adiponectin Receptor Regulates Proprotein Convertase Subtilisin/Kexin Type 9 Expression and Inhibits Lesions in ApoE-Deficient Mice

Lei Sun, Xiaoxiao Yang, Qi Li, Peng Zeng, Ying Liu, Lipei Liu, Yuanli Chen, Miao Yu, Chuanrui Ma, Xiaoju Li, Yan Li, Rongxian Zhang, Yan Zhu, Qing Robert Miao, Jihong Han and Yajun Duan

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## II Supplemental Materials

### Table I. Sequences of the primers for real time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Backward</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPCSK9</td>
<td>AGTTGCCCCCATGTCGACTAC</td>
<td>GAGATACACCTCCACCAGGC</td>
</tr>
<tr>
<td>mPCSK9</td>
<td>TATGAAGAGCTGATGCTCGC</td>
<td>CACAATGTAAGTTCTCGGCA</td>
</tr>
<tr>
<td>hPPARγ</td>
<td>TTCAGAAATGCCTTGAGTGG</td>
<td>AGCTTCTCTTCTCGGCTTG</td>
</tr>
<tr>
<td>mPPARγ</td>
<td>ATGTCTCAACATGCCATCAGTT</td>
<td>GCTCGCAGATCAGCAGACTCT</td>
</tr>
<tr>
<td>hLDLR</td>
<td>AGGAGACGTGCTTGTCTGTC</td>
<td>CTGAGCCGTGGTCTGCACT</td>
</tr>
<tr>
<td>hAdipoR1</td>
<td>AAACTGGCAACATCTGGACC</td>
<td>GCTGTGGGGAGCAGTAGAAG</td>
</tr>
<tr>
<td>hAdipoR2</td>
<td>ACAGGCAACATTTGGACACA</td>
<td>CCAAGGAACAAAAACTTCCA</td>
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<tr>
<td>hSOD1</td>
<td>TAATGCTTCCCCACACCTTC</td>
<td>CTAGCAGGTATGGCGGACGA</td>
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<tr>
<td>hGSH-PX</td>
<td>TCTCTTCTTCTTGGCGTTC</td>
<td>CGGGACTACAACCAGATGAA</td>
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<tr>
<td>hCAT</td>
<td>ACGGCCCCTACTGTAATAA</td>
<td>AGATGCACTGGAGGAG</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>GGTGGTCTCTCCTGACTTCAACA</td>
<td>GTTGCTGTAGCCAAATTCGGTG</td>
</tr>
<tr>
<td>mGAPDH</td>
<td>ACCCAGAAGACTGTGGAATGG</td>
<td>ACACATTGGGAGTAGAACA</td>
</tr>
</tbody>
</table>

h: homo sapiens; m: mus musculus; PCSK9: proprotein convertase subtilisin kexin 9; PPARγ: peroxisome proliferator-activated receptor γ; LDLR: low-density lipoprotein receptor; AdipoR1: adiponectin receptor 1; AdipoR2: adiponectin receptor 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SOD1: superoxide dismutase 1; GSH-PX: glutathione peroxidase; CAT: catalase.
Table II. Sequences of the primers for promoter constructs

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Forward</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>CACGCTCGAGTTTGGATAGCA GTAAC</td>
<td>ACGTAAGCTTTAGGGTTCTATG CTGA</td>
</tr>
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<td>PCSK9</td>
<td>AGGGTACCCTGGACATCAAAA GCAAGC</td>
<td>AGCTCGAGCGCAGCGGTGGA AGGTG</td>
</tr>
<tr>
<td>PCSK9-SREmut</td>
<td>ATGGGGGTCTCTGAGATCCGTGT CTGGC CGCCCA</td>
<td>TGGGGGCGCGCAGACACGGGAT CTCAGAGCCCCAT</td>
</tr>
<tr>
<td>PCSK9-PPREmut</td>
<td>TGCAGGGTGCATAAA CAAGTTG T TCACGGAGGAGGC</td>
<td>GCCTGGAACCCCTCAGATGAA Ac AACTTGTATGCAACCCTGCA</td>
</tr>
<tr>
<td>mLDLR</td>
<td>GCCTGACCCTGAGGAGGAAT TTGAGGA</td>
<td>ACCTCGAGCGAGACAGC AGATGACC</td>
</tr>
<tr>
<td>mLDLR-SREmut</td>
<td>TGAAGATT TTTTGAAGAAATCAACGG CATTCAGACTCC</td>
<td>GGGAGGAGTCTGCAATGCC GTGATTTCAAAAAAT</td>
</tr>
<tr>
<td>hLDLR</td>
<td>GCCTGACCCTTTTGAGGAC AGAGGACA</td>
<td>ACCTCGAGGAGTGTATGCCTCTTC AACTTATTC</td>
</tr>
<tr>
<td>hLDLR-SREmut</td>
<td>TGAAGACATTTGAAATCAACGG CACTGCAAAACTCC</td>
<td>GGGAGGAGTTGCAAGTG GCC GTGATTTTCAATTGT</td>
</tr>
</tbody>
</table>

The sequences of normal PPRE and SRE in human PCSK9 promoter are: AGGGCAgAGGCCG and GTGGCGTGA, respectively. The sequences of SRE of human and murine LDLR are ATCACCCCAc and ATCACCCCAT, respectively. The underlined letters are mutated nucleotides.

Table III. Sequences of the primers for ChIP assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Backward</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCSK9-SRE</td>
<td>TTCCCTCTGCGCGTAATCTG</td>
<td>CAGACCCTGAACTGAACGGC</td>
</tr>
<tr>
<td>PCSK9-PPRE</td>
<td>ACGTCTTTTGCAAAAACTAAAACCTG</td>
<td>GTTTCTGTGGTGACCTTATG</td>
</tr>
<tr>
<td>LDLR-PPRE</td>
<td>TCGAAGGACTGGAGTGGAAA</td>
<td>GACCTGCTGTTGTCCTAGCTG</td>
</tr>
</tbody>
</table>
Figure I. Determination of serum apoE, apoB, and apoA1 levels in wild type mice after ADP355 treatment

Wild type mice in two groups (8 mice/group) were i.p. injected with vehicle (scrambled peptide solution) and ADP355 solution (1 mg/kg/day), respectively, for 3, 10 or 30 days. At the end of experiment, serum samples were collected and used to determine apoE (A), apoB (B) and apoA1 (C) levels by ELISA. **: P<0.01 vs. control in the corresponding groups (n=8).
Figure II. Administration of ADP355 to apoE<sup>−/−</sup> mice has little effect on endothelial layer in aortas
ApoE<sup>−/−</sup> mice in two groups (~10 mice/group) were i.p. injected with vehicle (scrambled peptide solution) and ADP355 solution (1 mg/kg/day), respectively, for 12 weeks. At the end of experiment, aorta samples were collected and the thoracic aortic sections were used to conduct immunohistochemical staining with anti-CD31 antibody. NC: negative control, no primary antibody was added.
Figure III. ADP355 treatment enhances expression of antioxidant enzymes in HUVECs

Human umbilical vein endothelial cells (HUVECs) in 6-well plates were transfected with scrambled siRNA, AdipoR1 siRNA or AdipoR2 siRNA alone, or their combination (50 nmol/L of each), and then treated with ADP355 (100 ng/mL) overnight. Expression of AdipoR1 or AdipoR2 in transfected HUVECs (A), and expression of SOD1, GSH-PX and CAT (B) in the cells were determined by real time RT-PCR. *: P<0.05 vs. control cells (n=3); C: Nitric oxide (NO) in cellular lysate of HUVECs was determined by the assay kit.
Figure IV. ADP355 and AdipoRon reduce mitochondria superoxide levels partially through PPARγ pathway
Primary hepatocytes isolated from PPARγfl/fl and HepPPARγ KO mice, respectively, were treated with ADP355 (100 ng/mL) or AdipoRon (10 μmol/L) overnight. Superoxide levels (A, B) in the mitochondria were determined by MitoTracker Red (red) staining. Cellular total ROS levels (C) were determined by an assay kit. MFI indicates mean of fluorescent intensity of the images. *: P<0.05 vs. control cells (n=3).
MATERIALS AND METHODS

Reagents

The mimic adiponectin peptide, ADP355 (Cat# 04010026162), was synthesized by China Peptides Co., Ltd. (Suzhou, China). AdipoRon (Cat# SML0998) was purchased from Sigma-Aldrich (St Louis, MO). PCSK9 polyclonal antibody (Cat# 10240) was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-PPARγ polyclonal antibody (Cat# 16643-1-AP) was purchased from Proteintech Group (Chicago, IL). Rabbit anti-LDLR (Cat# NBP1-06709) and SREBP2 (Cat# NBP1-71880) polyclonal antibodies were purchased from Novus Biologicals (Littleton, CO). Rabbit anti-AMPKα (Cat# 2532S) and phosphorylated AMPKα (pi-AMPKα) (Cat# 2535S) polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-SMA (Cat# sc-130617), CD68 (Cat# sc-20060) and GAPDH (Cat# sc-25778) polyclonal antibody and FITC-conjugated goat anti-rabbit IgG (Cat# sc-2054) were purchased from Santa Cruz Biotechnology (Dallas, Texas). Mouse PCSK9 ELISA assay kit (Cat# SEK50251) was purchased from Sino Biological (Beijing, China). Mouse apoE (Cat# E-EL-M0135c), apoB (Cat# E-EL-M0132c) and apoA1 (Cat# E-EL-M0130c) ELISA assay kits were purchased from elabscience (Wuhan, China). ROS (Cat# S0033) and total NO assay kits (Cat# S0023) were purchased from Beyotime (Nantong, China). MitoSOX Red (Cat# M36008) was purchased from Invitrogen (Carlsbad, CA).

Cell culture and preparation of stable transfected cell lines

The human hepatic cell line, HepG2 cells, was purchased from ATCC (Manassas, VA), and cultured in complete DMEM medium (10% FBS, 50 μg/mL penicillin/streptomycin and 2 mmol/L glutamine). The cells at 90% confluence were switched into serum-free medium before the indicated treatment. Human embryonic kidney 293T cell line was also purchased from ATCC and cultured in complete DMEM containing 10% FBS and 50 μg/mL of penicillin/streptomycin.

Mouse primary hepatocytes were isolated by a collagenase perfusion method. Briefly, after anesthetized the midline laparotomy was performed, and the inferior vena cava was cannulated with an angiocatheter. The liver was then perfused with 1 mL heparin (320 U/mL), 40 mL solution I (Krebs solution containing 0.1 mmol/LM EGTA) and 30 mL solution II (Krebs solution containing 2.74 mmol/L CaCl2 and 0.05% collagenase I) at 37° C, sequentially. The perfused liver was then passed through a 38 μm screening size filter by flushing with cold DMEM medium. The isolated hepatocytes were collected after centrifuge for 5 min at 50 g, re-suspended with DMEM medium and plated in 6-well plates. The viability of the isolated hepatocytes was ~90%
which was determined by the method of trypan blue exclusion.

HepG2 cells were infected with lentivirus expressing non-targeting shRNAi (shNSi) or shRNAi targeting AdipoR1 (shAR1i) or AdipoR2 (shAR2i). The sense sequences for shAR1 and shAR2 are 5′-GCCCAACCAGCACTTTACTAT-3′ and 5′-GCTCTTCTCTAAACTGGATTA-3′, respectively. The stable shNSi, shAR1i and shAR2i transfected cell lines were established by drug selection using puromycin (3 µg/mL).

**Generation of a CRISPR-Cas9-mediated AMPKα1 knockout HepG2 cell line**

An AMPKα1 genome knockout HepG2 cell line was generated using the clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) technology[1]. Guide RNA was designed to target exon 2 of AMPKα1 by an online CRISPR Design Tool (http://tools.genome-engineering.org). The sequences of guide oligos are: top, 5′-CACCAGTTGGCAAACATGAATTGAC-3′; and bottom, 5′-AAACGTCAATTCATGTTTGCCAAC-3′. The underlined letters represent the restriction site of BbsI. After annealed, the oligo duplex was ligated into pSpCas9 (BB)-2A-Puro vector (Addgene plasmid ID: 48139, pre-digested with BbsI) to generate Cas9-AMPKα1. HepG2 cells were then transfected with plasmid DNA for Cas9-AMPKα1 and pSpCas9 vector, respectively. The transfected cells were plated in 96-well plates with the limiting dilution and cultured in the medium containing puromycin (3 µg/mL) for screening AMPKα1 knockout. After 3 weeks of the 1st round selection, the formed mono clones were subjected to the 2nd round selection. The mutation was confirmed by both PCR and DNA sequencing, and lack of AMPKα protein expression was determined by Western blot. The cells lacking of AMPKα1 expression and the corresponding control cells were defined as CRISPR-AMPKα1 cells and CRISPR-Ctrl cells, respectively.

**In vivo study**

The protocol for animal study 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. Wild type (C57BL/6) mice, Alb-Cre mice and apoE deficient (apoE−/−) mice were purchased from the Animal Center of Nanjing University (Nanjing, China). To generate specific hepatocyte PPARγ deficient mice, the homozygous floxed (+/+) PPARγ mice crossbred with the Alb-Cre transgenic mice. Therefore, the control mice are flox+/−/Cre−/− and the specific hepatocyte PPARγ knockout mice are flox+/−/Cre+/−. The primary hepatocytes isolated from these two types of mice are defined as PPARγflox cells and HepPPARγ KO cells, respectively.

To determine the effect of ADP355 on PCSK9 expression in vivo, male wild type mice (~8 weeks old) fed normal chow were divided into two groups (8
mice/group), and i.p. injected with vehicle (scrambled peptide solution) and ADP355 peptide solution dissolved in PBS (1 mg/kg bodyweight/day) for 3, 10 or 30 days, respectively. Mice were then sacrificed with euthanasia in a CO₂ chamber followed by collection of liver samples individually to determine hepatic protein expression by Western blot. Blood samples were collected for determination of serum PCSK9, apoAl, apoB and apoE levels by ELISA assay kits, and lipid profiles by enzymatic methods with an automatic biochemical analyzer (Model 7020; Hitachi, Tokyo, Japan).

To study the effects of ADP355 on atherosclerosis, male apoE⁻/⁻ mice (~8 weeks old) fed a high-fat diet (HFD: 0.5% cholesterol and 21% fat) were divided into two groups (10 mice/group), and i.p. injected with vehicle (scrambled peptide solution) and ADP355 peptide solution dissolved in PBS (1 mg/kg bodyweight/day) for 12 weeks, respectively. At the end of the experiment, all mice were sacrificed with euthanasia in a CO₂ chamber followed by collection of mouse aorta, liver and blood samples. Liver samples were used to determine hepatic protein expression by Western blot. Blood samples were collected for determination of serum PCSK9 levels by an ELISA assay kit, and lipid profiles by enzymatic methods. Aortas were collected as described[2] and stained with Oil Red O solution. Lesions in en face aortas were quantitatively determined by technicians who were blinded of the treatment using a computer-assisted image analysis method (Photoshop CS3) and expression as percent lesion area. To determine sinus lesions in aortic root, frozen sections of aortic root were prepared and then stained with Oil Red O solution as described[2]. 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. The SMC content or macrophage/foam cell accumulation was determined by immunofluorescent staining aortic cross sections with anti-SMA antibody or anti-CD68 antibody. The endothelial cells in the aorta were determined by immunohistochemical staining with anti-CD31 antibody.

Western blot, immunofluorescent staining, and real time RT-PCR

After treatment, cellular or tissue proteins were extracted and used to determine PCSK9, LDLR, SREBP2, PPARγ, AMPKα and pi-AMPKα protein expression by Western blot. LDLR expression in HepG2 cells were also determined by immunofluorescent staining[3].

To determine expression of PCSK9, PPARγ and LDLR mRNA, total RNA was extracted from cells[3]. The cDNA was synthesized with 1 μg total RNA using the reverse transcription kit (New England Biolabs, Ipswich, MA). The real time RT-PCR was conducted with SYBR Green Master Mix (Bio-Rad, Los Angeles, CA) and the primers listed in Table I.
Inhibition of PPARγ, AdipoR1 and AdipoR2 expression by siRNA

The siRNA was purchased from Santa Cruz Biotechnology (Dallas, TX). HepG2 or HUVEC cells were transfected with siRNA of PPARγ, AdipoR1 or AdipoR2, or scrambled siRNA using Lipofectamine RNAiMAX (Invitrogen). After 24 h transfection, cells were switched to complete medium and continued culture for 36 h followed by the indicated treatment.

Preparation of plasmid DNA and determination of PCSK9, PPARγ and LDLR promoter activity

Human PPARγ, PCSK9 and LDLR promoters and murine LDLR promoter were constructed by PCR with genomic DNA isolated from HepG2 cells and mouse liver, and the primers listed in Table II, respectively. The PCR product was digested followed by ligation with pGL4 luciferase reporter vector. The product was then transformed into E. coli to amplify. The human PCSK9 promoter with PPRE or SRE mutation (pPCSK9-PPREmut or pPCSK9-SREmut), and human or murine LDLR promoter with SRE mutation (pmLDLR-SREmut or phLDLR-SREmut) were constructed using the Phusion site-directed mutagenesis kit with pPCSK9, pmLDLR or phLDLR DNA and primers with the corresponding PPRE or SRE mutation (Table II), respectively. Activity of promoters was determined using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI)[4].

Chromatin immunoprecipitation (ChIP) assay for DNA binding activity

After treatment, cells were subjected to ChIP assay as described[3]. Immunoprecipitation was conducted using anti-PPARγ or anti-SREBP2 polyclonal antibody or normal IgG followed by PCR with the primers listed in Table III.

Detection of intracellular mitochondria superoxide levels

MitoSOX™ Red mitochondrial superoxide indicator is a novel fluorogenic dye for highly selective detection of superoxide levels in the mitochondria of live cells. Mouse primary hepatocytes were incubated with MitoSOX Red (5 μmol/L) for 10 min at 37°C, protected from light followed by gently wash 3 times with warm HBSS buffer. The cells were stained with DAPI solution for nucleus. Images of the slides were obtained with a fluorescence microscope (Leica). The mean fluorescence intensity (MFI) of all the immunofluorescent images was calculated as described[5].

Determination of cellular ROS and NO levels

After treatment, the reactive oxygen species (ROS) levels in mouse primary hepatocytes were determined using a ROS assay kit purchased from Beyotime (Nantong, China) as described[6]. NO levels in HUVECs were determined by the NO assay kit according to the manufacturer’s instructions.
Data analysis

All experiments were repeated at least 3 times, and the representative results were presented. Data were presented as mean ± standard errors and analyzed by Student’s t-test using Prism (GraphPad Software, Inc.). P<0.05 was considered significant (n≥3).

References