Lack of Association Between Adiponectin Levels and Atherosclerosis in Mice


Objective—Adiponectin is an adipocyte-derived, secreted protein that is implicated in protection against a cluster of related metabolic disorders. Mice lacking adiponectin display impaired hepatic insulin sensitivity and respond only partially to peroxisome proliferator-activated receptor γ agonists. Adiponectin has been associated with antiinflammatory and antiatherogenic properties; however, the direct involvement of adiponectin on the atherogenic process has not been studied.

Methods and Results—We crossed adiponectin knockout mice (Adn<sup>−/−</sup>) or mice with chronically elevated adiponectin levels (Adn<sup>−/−</sup>) into the low-density lipoprotein receptor–null (Ldlr<sup>−/−</sup>) and the apolipoprotein E–null (Apo<sub>E−/−</sub>) mouse models. Adiponectin levels did not correlate with a suppression of the atherogenic process. Plaque volume in the aortic root, cholesterol accumulation in the aorta, and plaque morphology under various dietary conditions were not affected by circulating adiponectin levels. In light of the strong associations reported for adiponectin with cardiovascular disease in humans, the lack of a phenotype in gain- and loss-of-function studies in mice suggests a lack of causation for adiponectin in inhibiting the buildup of atherosclerotic lesions.

Conclusion—These data indicate that the actions of adiponectin on the cardiovascular system are complex and multifaceted, with a minimal direct impact on atherosclerotic plaque formation in preclinical rodent models. (Arterioscler Thromb Vasc Biol. 2010;30:1159-1165.)

Key Words: atherosclerosis ■ diabetes mellitus ■ insulin resistance ■ metabolism ■ low-density lipoprotein receptor ■ adiponectin

The increasing prevalence of obesity and its association with the pathogenesis of cardiovascular disease has evoked great interest in understanding the impact of adipokines on vascular integrity, systemic inflammation, and atherosclerosis.1 Adipokines are factors secreted from adipose tissue that play a key role in systemic energy homeostasis. Many of them, including leptin, resistin, and retinol binding protein-4, are dysregulated in obesity.2 Similarly, proinflammatory cytokines tend to be upregulated in the obese state as well.3

Adiponectin is a secreted adipokine abundantly expressed in differentiated adipocytes.4 Adiponectin levels correlate negatively with body mass index and enhanced inflammation within the adipose tissue.5 In humans, adiponectin levels correlate inversely with risk factors for cardiovascular disease, including visceral adiposity, hyperlipidemia, and high-density lipoprotein (HDL) cholesterol levels, underscoring a potential value of adiponectin as a biomarker.6 Hotta et al demonstrated that adiponectin levels of diabetics with coronary artery disease are lower than those of diabetics without coronary artery disease or of nondiabetics.7 In contrast, high adiponectin levels were associated with a reduced risk for myocardial infarction.8 A number of additional epidemiological studies suggest a link between adiponectin and the pathologies of cardiovascular disease, although no conclusions were drawn about whether this relationship is correlative or causal.

Adiponectin may have antiatherogenic and antiinflammatory effects.9 Although adiponectin is frequently measured as a marker for cardiovascular disease in the clinical area, to date, only limited systematic studies have been published that address the direct effects of adiponectin on atherosclerosis in preclinical models. We therefore embarked on a comprehen-

Received on: August 13, 2009; final version accepted on: March 9, 2010.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.109.195826
sive analysis of commonly used atherosclerosis mouse models that were made adiponectin deficient or that have mildly elevated levels of adiponectin by means of overexpression of an adiponectin transgene.\textsuperscript{10,11} We exposed these mice to various diets to differentiate between correlative and causal effects of adiponectin. For example, we fed the mice a nonobesogenic diet intended to prevent excess adiposity or insulin resistance, yet causing significant hypercholesterolemia. Alternatively, we generated diet-induced obesity using a high-fat “Western style” diet. Interestingly, independently of the genetic or dietary modality we applied, we were unable to detect changes of atherosclerosis associated with adiponectin levels. Our observations may suggest a lack of direct causation for adiponectin in protecting against the infiltration of macrophages, cholesterol deposition, and necrosis within the atherosclerotic lesions.

Our studies also addressed the effects of peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$). Pharmacological activation of this nuclear receptor with a small molecule agonist is the single most potent means by which to induce adiponectin secretion.\textsuperscript{12} PPAR$\gamma$ activation improves insulin sensitivity and various cardiovascular risk factors. However, prolonged treatment with selective PPAR$\gamma$ agonists from the thiazolidinedione class is associated with increased adiposity and edema, and substantial concern has been raised regarding the safety of these drugs.\textsuperscript{13,14} Pro- and antiatherogenic roles have been attributed to PPAR$\gamma$ activation. In fact, Thorp et al recently demonstrated that pioglitazone exposure can increase macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions.\textsuperscript{15}

In this study, we analyzed adiponectin-dependent and -independent effects of chronic PPAR$\gamma$ agonism on atherosclerosis in mice. We examined lesion size in adiponectin and low-density lipoprotein (LDL) receptor–deficient models on long-term PPAR$\gamma$ agonist treatment. In our experimental paradigm, PPAR$\gamma$ activation did not reveal beneficial effects on atherosclerosis. In fact, we found a small but significant increase in the average area of lesions specifically within the brachiocephalic branch of the aorta, related to the agonist treatment.

**Methods**

Detailed information about the materials and methods used can be found in the supplemental data, available online at http://atvb.ahajournals.org.

**Animals and Diets**

Animals were maintained in a pathogen-free facility, and all experimental protocols were approved by the institutes for animal studies of the Albert Einstein College of Medicine and the University of Texas Southwestern Medical Center. Mice were group housed and were on an alternating 12-hour light and dark cycle under controlled environmental conditions (22°C to 25°C, 40% to 50% humidity) with free access to food and water. Adiponectin knockout mice were generated as described previously\textsuperscript{10} and backcrossed for at least 6 generations onto a C57BL/6J background in all studies, with exception of the first study, for which the mice were backcrossed 5 times. Adiponectin transgenic mice (Adn$^{75}$) were generated as described by Combs et al.\textsuperscript{11}

**Results**

**Adiponectin Deficiency Does Not Accelerate the Formation of Atherosclerotic Lesions in Lean, LDL Receptor–Deficient Mice**

The majority of our studies were performed using the LDL receptor knockout model ($Ldlr^{-/-}$).\textsuperscript{16,17} Adiponectin knockout mice ($Adn^{-/-}$)\textsuperscript{10} were crossed with $Ldlr^{-/-}$ mice to generate double knockout mice ($Ldlr/Adn^{-/-}$). Male mice were fed a low-fat diet (10% kcal from fat) containing 0.15% cholesterol, a regimen that causes substantial atherosclerotic lesions within 3 to 4 months but does not evoke metabolic complications such as obesity and insulin resistance.\textsuperscript{18} Total cholesterol levels were elevated to approximately 800 mg/dL in all groups, whereas adiponectin levels were not affected by the dietary intervention (Supplemental Table I). After 3 months on the diet, mice were lean and had normal glucose levels. The lack of adiponectin per se had no effect on total plasma lipids and lipoprotein distribution, as seen in $Adn^{-/-}$ mice in the absence of the $Ldlr^{-/-}$ mutation (Supplemental Figure I).

To assess the influence of adiponectin on the vasculature, we determined atherosclerosis at the aortic root and in the brachiocephalic artery (BCA). Macroscopic lesions were observed underneath most of the valve leaflets and near the aortic branches but were generally absent from the thoracic or abdominal aorta. As shown in Figure 1, the lesion area within the aortic root (Figure 1A) and the BCA (Figure 1B) was not affected by adiponectin (no drug; $Ldlr^{-/-}$ versus $Ldlr/Adn^{-/-}$), suggesting that adiponectin does not affect the atherogenic process. To exclude the possibility of a phenomenon unique to the $Ldlr^{-/-}$ model, we generated apolipoprotein E–deficient, adiponectin-deficient double knockout mice (Apoe$^{-/-}$/Adn$^{-/-}$). Total cholesterol was 116±17 mg/dL in wild-type versus 143±17 in Apoe$^{-/-}$ mice when fed a high-fat, high-cholesterol diet. Deletion of Apoe$^{-/-}$ dramatically increased plasma cholesterol levels (841±27 mg/dL in wild-type versus 808±60 in Apoe$^{-/-}$), independently of adiponectin levels. More importantly, as in the $Ldlr^{-/-}$ model, adiponectin deficiency had no effect on atherosclerosis in Apoe$^{-/-}$ mice (Supplemental Figure II).

**Pharmacological Induction of Adiponectin by PPAR$\gamma$ Agonism Does Not Prevent Atherosclerosis in Lean Mice**

PPAR$\gamma$ agonists have been evaluated in various long-term human studies for potential cardiovascular benefits, with mixed results.\textsuperscript{19,20} PPAR$\gamma$ activation causes an increase in plasma adiponectin levels, particularly the high molecular weight form.\textsuperscript{12} Selective PPAR$\gamma$ ligands of the thiazolidinedione class (troglitazone and rosiglitazone) have been shown to inhibit atherosclerosis in $Ldlr^{-/-}$ mice.\textsuperscript{21,22} To assess whether these effects may be secondary to an upregulation of adiponectin, we included 2 parallel groups that received a PPAR$\gamma$-selective, nonthiazolidinedione agonist
Adiponectin and Atherosclerosis: A Therapeutic Target for Metabolic Syndrome

Nawrocki et al

Adiponectin Deficiency Does Not Promote Atherosclerosis Secondary to Metabolic Disturbances in Mice

On the basis of our initial studies, we concluded that the manifestation of several aspects of the metabolic syndrome (ie, insulin resistance, increased adiposity and dyslipidemia) may be necessary to unravel a putative role of adiponectin in the atherogenic process. This assumption is in line with the fact that genetic ablation of adiponectin causes a mild metabolic phenotype unless challenged with a high-fat diet. We hypothesized that adiponectin may act indirectly through its overall effects on metabolic and inflammatory parameters. To address this question, we exposed Ldlr−/− mice to a high-caloric Western-type diet (WD; 42% kcal from fat, 42% kcal from carbohydrates, and 0.2% cholesterol). We included females because plasma adiponectin levels are higher in females than in males, and one can assume that the extent of lesion formation may be gender related. After 4 months on WD, all groups were obese and hyperglycemic (Supplemental Table II). Both genders developed severe hypercholesterolemia and hypertriglyceridemia independent of their respective adiponectin levels. Lipoprotein profiles were indistinguishable between Ldlr/Adn−/− and Ldlr−/− mice (data not shown). Adiponectin levels tended to be lower at the end of the study, consistent with an adiposity-associated suppression of adiponectin secretion (Supplemental Table II).

Atherosclerosis was assessed as cholesterol and cholesterol ester content in the aortic wall. This lipid extraction method has been validated and correlates well with the assessment of atherosclerotic lesions by en face lipid staining or intima/media thickness. The measurement considers surface area as well as volume and distribution of lesions, including cholesterol depositions that may be undetectable by visual methods. At the end of the study, all mice had increased body weight, hyperlipidemia, and elevated basal glucose levels, and there was no difference in the deposition of cholesterol in aortas of female and male Ldlr/Adn−/− and Ldlr−/− mice (Figure 2A and 2B).

In a separate cohort, we assessed lesion area by morphological methods. We analyzed cross-sectional and necrotic lesion area in the aortic roots of both female and male mice (Figure 2C and 2D). Note that plaque necrosis is associated with vulnerable plaques and culprit lesions in humans. None of the measurements revealed a correlation between the adiponectin genotype and morphology of the plaques. We concluded that (1) adiponectin does not directly suppress early stages of the atherogenic process, (2) a role of adiponectin may be masked by compensatory mechanisms that could have evolved as a consequence of a germ line deletion of adiponectin, or (3) adiponectin per se did not affect atherosclerotic lesion area or distribution. It is important to note that under these conditions (ie, nonobesogenic, low-fat diet), COOH treatment had no effect on overall plasma glucose levels or body weight. All groups gained similar amounts of body weight (average weight gain, 9.9 ± 1.3 g) over the course of the study. COOH treatment did not affect total or HDL cholesterol levels, whereas triglycerides were slightly elevated at the end of the study (COOH; Supplemental Table I).

**Figure 1.** Atherosclerosis in Ldlr−/− and Ldlr/Adn−/− mice fed a nonobesogenic diet supplemented with 0.15% cholesterol for 3 months. Mice were left untreated (no drug) or were dosed with a selective PPARγ agonist (COOH) in feed. Shown are lesion area within aortic root (A) and BCA (B). *P<0.05; n=12 to 18.

(COOH) at 30 mg/kg body weight per day in feed. As expected, COOH treatment increased plasma adiponectin levels in male mice approximately 4-fold over basal (Supplemental Table I). COOH has been shown previously to elicit a robust signature of hepatic PPARγ-responsive genes virtually identical to the profile obtained with rosiglitazone on gene expression analysis on microarrays.

Lesion area was similar within the aortic root of Ldlr−/− and Ldlr/Adn−/− mice treated with COOH (Figure 1A). Interestingly, we found a small but significant increase in average area of lesions within the BCA related to COOH treatment (Ldlr−/−; no drug versus COOH; Figure 1B). This effect was absent in Adn−/− mice (COOH, Ldlr−/− versus Ldlr/Adn−/−; Figure 1B), suggesting that this increase is dependent on the elevation of plasma adiponectin induced by PPARγ. Lesions within the BCA tend to be more advanced than lesions in other areas, and analysis of this region has been used mainly for qualitative purposes. Further investigation of the role of COOH treatment on the quality and quantity of lesions within the BCA was beyond the scope of this investigation and will be presented elsewhere. Lack of

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Adiponectin may affect later stages of the atherosclerotic process, such as plaque rupture or luminal thrombosis, which are not a component of these murine models.25

Elevated Adiponectin Levels Do Not Prevent Atherosclerosis

We have shown previously that adiponectin overexpression leads to a significant improvement of dyslipidemia in mice.5 The transgenic expression of an adiponectin deletion mutant (AdnTg) causes a moderate increase of steady state adiponectin concentrations in the circulation by approximately 2- to 3-fold over baseline, similar to levels achieved by activation of PPARγ.5,11 Adiponectin overexpression increases lipid clearance and lipoprotein lipase activity and improves the suppression of endogenous glucose production in the liver. We crossed AdnTg mice into the Ldlr−/− background (Ldlr/AdnTg) and determined atherosclerosis on a WD. Body weight, adiponectin, glucose levels, triglyceride, and total and HDL cholesterol after 3 months on the diet are shown in Supplemental Table III. On average, males gained 16.5 ± 0.9 g and females 11.4 ± 0.8 g independently of the genotype. Adiponectin levels were 3- to 3.5-fold higher in Ldlr/AdnTg mice than in Ldlr−/− mice and slightly elevated in females but not in males at the end of the study. Fasting glucose was significantly lower in Ldlr/AdnTg mice than in Ldlr−/− mice throughout the study (Supplemental Table III) and correlated with an improved response to an oral glucose challenge in both genders at the end of the study (females; Figure 3A, males; Figure 3B). Ldlr−/− mice were mildly glucose intolerant, and this defect was significantly improved in Ldlr/AdnTg mice. Before feeding of the WD, total cholesterol and triglyceride levels were significantly lower in Ldlr/AdnTg mice compared with Ldlr−/− mice, whereas HDL cholesterol was independent of the genotype (Supplemental Table III; end of study). In males, HDL cholesterol was significantly increased in Ldlr/AdnTg mice compared with Ldlr−/− mice, and a similar trend was observed in female mice.

Lipoprotein distribution and atherosclerosis were measured in a separate cohort of mice after 4 months on WD. In this study, we measured a trend in male mice and a significant reduction of total cholesterol in female Ldlr/AdnTg mice compared with Ldlr−/− mice (Figure 4A). Triglycerides were reduced significantly in both female and male Ldlr/AdnTg mice (Figure 4B). This reduction in total lipid levels was not seen in plasma samples collected from tail blood in the previous study (Supplemental Table III). The discrepancy may be explained by variations in sample preparation or among cohorts. The lipoprotein profile measured by size exclusion chromatography revealed reduced cholesterol levels in very-low-density lipoprotein and intermediate-density/low-density lipoprotein fractions from both female and male Ldlr/AdnTg mice compared with Ldlr−/− mice, whereas HDL fractions were unaffected (Figure 4C and 4E, females and males, respectively). Lower triglycerides were observed in female and male Ldlr/AdnTg mice and were mostly accounted for by very-low-density lipoprotein fractions (Figure 4D and 4F, females and males, respectively).

As before, atherosclerosis was quantified by the lipid extraction method. Despite an improved lipoprotein profile, we could not detect differences in cholesterol deposited in the aortic wall between Ldlr/AdnTg and Ldlr−/− (females; Figure 5A, males; Figure 5B). These results were confirmed by a histological assessment of lesions within the aortic root (n = 9) after staining with oil red O (Figure 5C). Lesion area was independent of plasma adiponectin levels in both female and male mice. Overexpression of adiponectin did not alter the number of macrophages in the lesion or the amount of

Figure 2. Adiponectin deficiency has no impact on atherosclerosis in mice fed a WD. Total cholesterol, free cholesterol, and cholesterol esters (CholEster) were measured from lipid extracts of aortas of Ldlr−/− and Ldlr/Adn−/− mice. A, Female mice; B, male mice. C, Lesion area and necrotic core area in aortic root of females. D, Lesion and necrotic core area in aortic root of males. Mean ± SEM; n = 11 to 14.

Figure 3. Overexpression of adiponectin improves glucose tolerance. Shown are female (A) and male (B) Ldlr/AdnTg and Ldlr−/− mice after feeding of WD for 4 months. An oral glucose tolerance test was performed after 4 hours of fasting. Circulating glucose was measured at times 0, 20, 40, 60, 120, and 180 minutes after an oral glucose challenge. Data are mean ± SEM; *P < 0.05; n = 9 to 13. AUC indicates area under the curve.
collagen deposition (Figure 5D and 5E). Positive staining for collagen was localized to regions surrounding the necrotic core in plaques from both Ldlr−/− and Ldlr/AdnTg mice. Visual analysis of the staining using a score from 0 to 10, with 10 being the highest intensity, revealed no significant differences within the genotypes (scores of 5.5±4.9 for Ldlr−/− mice and 3.4±2.8 for Ldlr/AdnTg mice). CD68-positive macrophages were localized throughout the plaques, with the greatest abundance near the subendothelial space (Figure 5E), but the content and distribution were unaffected by the genotype (scores of 5.3±3.2 for Ldlr−/− and 4.7±3.8 for Ldlr/AdnTg mice). Our data demonstrate that genetic elevation of adiponectin has no effect on the formation of lesions despite some beneficial effects on lipid clearance and plasma lipids in the Ldlr−/− atherosclerosis model.

Discussion
The role of adiponectin in cardiovascular disease has been met with considerable interest and examined in a large number of epidemiological studies. A picture has emerged suggesting that adiponectin plays an important role in cardiovascular disease in humans.31 This may reflect a compensatory upregulation of a beneficial factor in light of a generally disadvantageous cardiovascular environment, through a mechanism that is far from understood.

Studies in preclinical models also suggest that adiponectin is more than an innocent bystander and convenient marker for cardiovascular integrity. An association between low adiponectin and increased vascular thickening has been suggested on the basis of a mouse cuff-injury model.32 In vitro experiments attributed a potent anti-inflammatory role to the globular form of adiponectin, and Okamoto et al demonstrated that globular adiponectin suppressed lipopolysaccharide-induced cytokine release in macrophages and suppressed T-lymphocyte accumulation in atherogenesis in Apoe−/− mice.33 Our study is the first to address the function of the full-length form of adiponectin secreted in its native conformation. We are also the first to address the role of adiponectin in the Ldlr−/− mouse model, which develops atherosclerosis only after supplementation of high levels of dietary cholesterol.16 We used diets that cause substantial hypercholesterolemia but differentially affect the degree of adiposity, insulin resistance, and inflammation. Surprisingly, while observing the expected differential susceptibility to the diets with respect to insulin sensitivity, we failed to detect an effect on atherosclerosis in both genetic adiponectin gain- and loss-of-function mice. Our data differ from those of previous reports derived from the Apoe−/− model, which may be best explained by differences in the experimental paradigm, the form of adiponectin supplied, and the diets used.

As described previously,5 introduction of the AdnTg caused reduction of plasma lipids in our studies; however, mice on an Ldlr−/− background remained extremely hypercholesterolemic. We conclude that adiponectin plays an important role in the regulation of plasma lipids; however, the severity of hyperlipidemia in Ldlr−/− mice may prevent conclusions about the role of adiponectin under physiological conditions. Similarly, elevation of adiponectin levels by pharmacological means did not affect atherosclerosis in the Ldlr−/− model. In fact, COOH treatment caused a small but significant increase in lesion area in the BCA in males. PPARγ is highly expressed in macrophage-derived foam cells and has been implicated in the development of atherosclerosis. Most but not all preclinical studies described a reduction of atherosclerosis coinciding with an improvement of overall metabolic parameters.21,22,34 Another study suggested that pioglitazone increased macrophage apoptosis and plaque necrosis in non-diabetic Ldlr−/− mice.15 We chose to study lean, insulin-sensitive mice using a nonobesogenic diet in the hope of dissociating direct actions of adiponectin on the vascular wall from secondary effects related to a metabolic improvement. Notably, we did not observe any signs of reduction of atherosclerosis on treatment with COOH, despite a large number of samples.

Our findings underline potential limitations of the LDL receptor mouse model in reproducing associations that have been established in humans. Mice are generally resistant to plaque rupture and myocardial infarction. In our studies, genetic or pharmacological manipulation of adiponectin levels in traditional rodent models did not correlate with atherosclerosis, suggesting that adiponectin is not involved in advanced plaque progression.
Figure 5. Adiponectin overexpression does not inhibit macrophage infiltration, collagen deposition, or atherosclerosis. A and B, Aortic cholesterol content in Ldlr/AdnTg and Ldlr−/− mice. A, Female mice; B, male mice. Shown are total cholesterol, free cholesterol, and cholesterol ester (CholEster) content in aorta (n=7 to 11). C, Lesion area in aortic root (n=3). D, Collagen content by Masson’s trichrome stain (blue). E, CD68-macrophages (red, blue 4′,6-diamidino-2-phenylindole–positive nuclei).
Sources of Funding

The authors were supported by NIH grants R01-DK55758, R01-CA112023 (P.E.S.), 8UL1DE019584-03 (Jay Horton), and the University of Texas Southwestern Mouse Phenotyping Core (1PPL1DK081182-03), the Cincinnati Mouse Metabolic Phenotype Center (Patrick Tsao), as well as NIH grants P01-HL087123 and P01-HL054591 (L.T.). Dr Nawrocki was supported by a postdoctoral fellowship from the Swiss National Science Foundation and the American Heart Association (Heritage Affiliate; 0425800T). Dr Hofmann was supported by the American Heart Association (0655070N).

Disclosures

None.

References

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Arterioscler Thromb Vasc Biol. 2010;30:1159-1165; originally published online March 18, 2010;
doi: 10.1161/ATVBAHA.109.195826
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/6/1159

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**Supplement Material**

**Expanded Material and Methods**

**Animals and Diets**

The transgenic line was generated by expressing a deletion mutant of adiponectin under the control of the adipose specific enhancer/promoter of the aP2 gene (aP2-deltaGly). The mutant lacks 13 of the 22 Gly-X-Y repeats in the collagenous domain of adiponectin. Both strains were crossbred with C57BL/6J-Ldlr<sup>tm1Her</sup>, stock No. 002207 (Jackson Laboratory, Bar Harbor, Me). Subsequently, double heterozygous (Adn<sup>+/−</sup>/Ldlr<sup>+/−</sup>) offspring were intercrossed to obtain Ldlr/Adn<sup>−/−</sup> double knockout mice. Ldlr<sup>−/−</sup>/Adn<sup>Tg</sup> mice were generated by crossing Adn<sup>Tg</sup> males containing a single transgene with Ldlr<sup>−/−</sup> females and subsequently by crossing Adn<sup>Tg</sup>/Ldlr<sup>+/−</sup> males with Ldlr<sup>−/−</sup> males. Mice were genotyped as described before<sup>1,2</sup>. Apoe/Adn<sup>−/−</sup> mice generated by crossing Adn<sup>−/−</sup> mice with C57BL/6J-Apoe<sup>tm1Her</sup> mice, stock No. 002052 (Jackson Laboratory, Bar Harbor, Me).

All experiments were performed with littermate control groups. Eight week old mice were placed on various diets for the times indicated in the results and the figure legend: (1) Clinton-Cybulsky diet<sup>3</sup> (Research Diets D00110804), (2) Western style diet (Harlan Teklad 88137). For the Apoe/Adn<sup>−/−</sup> double KO study mice were fed a purified diet containing 35% kcal from fat, 45% kcal from carbohydrates and 1.25% cholesterol (Research diets D12336) for 12 weeks. The PPARγ agonist, 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (COOH) was a kind gift from Merck Research Laboratories (Rahway, NJ).

**Plasma analysis**

Plasma glucose determinations were performed from heparinized tail blood using glucose oxidase assay kits (FastBlue B, Sigma-Aldrich). Basal glucose was measured using glucoanalyzer blood glucose strips (MediSense Precision Xtra, Abbott Laboratories, Abbott...
Park, IL). For oral glucose tolerance tests mice were fasted for 4 hours before given an oral glucose load of 2.5 g glucose/kilogram body weight using a solution of 10% glucose. Blood was drawn at the indicated times and glucose concentrations were measured as above. Access to food was denied during the course of the study. Adiponectin was measured using a rat adiponectin RIA kit (Millipore). Levels of total cholesterol and triglycerides and HDL cholesterol were measured with standard enzymatic assays from tail bleeds (Infinity, Cholesterol and Triglyceride kit; Thermo Scientific, HDL-C kit from Wako Chemicals). Lipoprotein subclass profiles were measured by Liposcience Inc. using nuclear magnetic resonance spectroscopy. For lipoprotein analyses, EDTA plasma aliquots of blood collected by cardiac puncture from mice anesthetized with isofluorane were stored at 4°C until separation. Fifty microliters of EDTA plasma was pooled from 5 mice per group and 200 microliters were separated via FPLC size exclusion chromatography on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech). The column was equilibrated with PBS and run at a flow rate of 0.2 mL/min and 0.25 ml fractions were collected. Each fraction and pooled plasma samples from the same animals were assayed for total cholesterol and triglycerides as above.

**Aortic Cholesterol Measurements**

After collection of blood by cardiac puncture, the vasculature was gently perfused through the left ventricle with cold PBS and 3 mmol/L EDTA. Any branches and adipose tissue connected to the aorta was removed carefully and each aorta was excised distal to the aortic root to the right renal artery. The aortas were stored briefly on ice in PBS and then blotted dry, weighed, minced, and extracted with chloroform/methanol (2:1) according to the method of Folch et al. 4. The lipid extracts were prepared as described by 5. Extracts were dried down,
resuspended quantitatively in chloroform/methanol (2:1), and stored at -20°C until the time of assay. Total and free cholesterol levels in the aortic extracts were determined with by enzymatic fluorometric assays. Briefly, the solvent was evaporated from aliquots containing 1 to 16 nmol of cholesterol, and the lipid residue was resolubilized in 100 µL of reagent grade ethanol. Aliquots of cholesterol (Sigma-Aldrich) and cholesteryl oleate (Sigma-Aldrich) standard solutions prepared in chloroform/methanol (1:1) were treated similarly. To determine free cholesterol, samples and standards were incubated for 1 hour at 37°C in a total volume of 1.01 mL of 0.1 mol/L potassium phosphate buffer, pH 7.4, containing 0.03% Triton X-100 and 0.9 mmol/L sodium cholate. Cholesterol oxidase (0.18 U; Boehringer Mannheim), peroxidase (2 U; Boehringer Mannheim), and p-hydroxyphenylacetic acid (0.5 mg/mL; Aldrich) were added for an additional 1-hour incubation at 37°C. The fluorescent product was measured in a Spex FluoroMax (SPEX Industries, Inc) (excitation 325 nm, emission 415 nm) with acrylic UVT semimicrocuvettes (Evergreen Scientific). For total cholesterol determinations, cholesterol esterase (10 U; Calbiochem) was included in the first incubation step, and cholesteryl oleate was used as a standard. The cholesteryl ester in each sample was calculated by subtracting the value of free cholesterol from that for total cholesterol. Samples for each aorta were run in duplicate at 2 different concentrations. All values are expressed as nmol/mg wet tissue weight.

**Tissue preparation and morphometric analysis of lesions**

Atherosclerosis was determined at the aortic origin as well as in the brachiocephalic branch of the aorta. To quantify lesion area in the aortic root, formalin-fixed hearts were processed as previously described [6](http://atvb.ahajournals.org/cgi/content/full/23/10/1907-R17-135424#R17-135424). Briefly, serial 12 micron sections were cut from the origin of the
aortic valve leaflets, throughout the aortic sinus and stained with oil red O and counter-stained with hematoxylin. Mean lesion area was calculated from the analysis of digital images obtained from 5 sections/mouse, using the Image Pro Plus software. To quantify cross-sectional lesion area in the brachiocephalic artery, the Y-shaped piece of brachiocephalic artery was frozen in OCT and sectioned distal to proximal at 10 µm thickness, starting from the subclavian and carotid arteries. Digital images were taken from 6 equidistant (100-µm) oil red O stained sections located 200 to 700 µm from the branching point of the brachiocephalic into the carotid and subclavian arteries. Atherosclerotic lesions lumenal to the internal elastic lamina were quantified on one section per slide. Lesion size was calculated as the mean lesion area of six sections. Plaque necrosis was quantified by measuring the area of hematoxylin and eosin-negative acellular areas in the intima, as described previously. Boundary lines were drawn around these regions, and the area measurements were obtained using Image-Pro-Plus software (version 3.0; Mediacybernetics).

Histomorphological analysis of collagen was performed with Masson’s trichrome stain. Frozen sections were thawed and washed in distilled water. Slides were stepwise stained in Weigert's iron hematoxylin solution then Biebrich scarlet-acid fuchsin with extensive washing in dHOH between steps. They were differentiated in phosphomolybdic-phosphotungstic acid solution for 15 minutes then immediately transfered to aniline blue solution for an additional 15 minutes. After washing in dHOH, the slides were rapidly dehydrated in 95% ethyl alcohol. Slides were mounted in Permaslip and imaged using a bright field microscope. Macrophages were detected by indirect immunofluorescence using an antibody against the macrophage-specific antigen CD68. For anti-CD68 decoration, frozen sections were thawed and air-dried
overnight then permeabilized in cold acetone for 30 minutes. After blocking with normal goat serum, slides were incubated with anti-CD68 antibody (Santa Cruz, sc-7084) overnight at 4°C. Following washes in phosphate-buffered saline containing 0.1% triton-X-100 (PBS-T), slides were incubated with Alexa488 conjugated secondary antibodies for one hour at room temperature in the dark. Nuclei were counterstained with DAPI after extensive washes in PBS-T, mounted under cover slips and imaged with an Olympus BX61 fluorescent microscope. Staining score = 0 to 10 with ten being the highest intensity. Each value represents the mean score from duplicate or triplicate slides per mouse.

Statistical Analyses

Results are shown as mean ± SEM. For studies shown in Figures 1 and 2, comparisons between treatment groups and genotype were performed by using two-way Anova tests to determine differences between individual group means. For glucose measurements and all other studies, significance was determined using the Mann Whitney test comparing genotypes. Differences were deemed significant at P < 0.05.

References


Supplemental Figure I

Lipoprotein profile from male Adn−/− and wild type mice on regular chow diet. Plasma was pooled from 5 mice and subfractionated by gel filtration chromatography and triglyceride and cholesterol was measured in each fraction. VLDL, very low density lipoprotein, LDL/IDL, Intermediate and low density lipoprotein, HDL, high density lipoprotein.

Supplemental Figure II

Quantification of atherosclerotic lesion area the aortic root of male C57Bl6/J (WT), adiponectin knockout (Adn−/−), Apolipoprotein E knockout (Apoe−/−) and Apoe−/−/Adn−/− double knockout mice fed a high fat/high carbohydrate diet for 3 months. Lesion area was analyzed from 5 sections of each animal (n= 9 for WT and n=14 for Adn−/−; n = 3 for Apoe−/− and Apoe−/−/Adn−/−) at the level of the valves as described in the Methods Section. Data means are shown.
Supplemental Table I

<table>
<thead>
<tr>
<th></th>
<th>Ldlr&lt;sup&gt;-/-&lt;/sup&gt; (n = 9)</th>
<th>Ldlr/Adn&lt;sup&gt;-/-&lt;/sup&gt; (n = 12)</th>
<th>Ldlr&lt;sup&gt;-/-&lt;/sup&gt; (n = 14)</th>
<th>Ldlr/Adn&lt;sup&gt;-/-&lt;/sup&gt; (n = 14)</th>
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<tbody>
<tr>
<td>No drug</td>
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<tr>
<td>Adiponectin (µg/ml)</td>
<td>6.9 ± 1.2</td>
<td>8.3 ± 0.6</td>
<td>8.5 ± 0.4</td>
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<td>Weight (g)</td>
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<td>31.8 ± 0.8</td>
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<td>Fasting Glucose (mg/dL)</td>
<td>91.5 ± 7.0</td>
<td>83.5 ± 5.2</td>
<td>89.6 ± 6.7</td>
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<td>898 ± 69</td>
<td>802 ± 54</td>
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<td>Triglyceride (mg/dL)</td>
<td>395 ± 29</td>
<td>404 ± 37</td>
<td>525 ± 31&lt;sup&gt;^&lt;/sup&gt;</td>
<td>649 ± 57</td>
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<td>HDL-C (mg/dL)</td>
<td>208 ± 26</td>
<td>179 ± 22</td>
<td>212 ± 15</td>
<td>184 ± 14</td>
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</table>

* Ldlr<sup>-/-</sup> vs. Ldlr<sup>-/-</sup> COOH, p < 0.001
^ Ldlr<sup>-/-</sup> vs. Ldlr<sup>-/-</sup> COOH, p < 0.05
### Supplemental Table II

<table>
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<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ldlr/Adn&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ldlr/Adn&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>(n = 11)</td>
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<td>(n = 12)</td>
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<tr>
<td>Males</td>
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<td>Weight (g)</td>
<td>45.7 ± 2.1</td>
<td>43.4 ± 2.5</td>
<td>32.2 ± 2.0</td>
<td>29.8 ± 1.5</td>
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<td>Fasting Glucose (mg/dL)</td>
<td>200 ± 10</td>
<td>187 ± 8</td>
<td>192 ± 7</td>
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<td>Adiponectin (µg/ml)</td>
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<tr>
<td>Week 0</td>
<td>10.7 ± 1.2</td>
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<td>Week 16</td>
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<td>13.8 ± 1.0</td>
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<td>Total Cholesterol (mg/dL)</td>
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<td>1746 ± 156</td>
<td>1193 ± 62</td>
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<tr>
<td>Triglyceride (mg/dL)</td>
<td>564 ± 81</td>
<td>572 ± 81</td>
<td>256 ± 21</td>
<td>284 ± 63</td>
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<tr>
<td>HDL-C (mg/dL)</td>
<td>292 ± 13</td>
<td>298 ± 19</td>
<td>278 ± 13</td>
<td>274 ± 17</td>
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<tr>
<td>Females</td>
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## Supplemental Table III

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<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;/Adn&lt;sup&gt;tg&lt;/sup&gt;</th>
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<td>Weight (g)</td>
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<td>41.0 ± 1.8#</td>
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<td>Fasting Glucose (mg/dL)</td>
<td>182 ± 6</td>
<td>156 ± 6**</td>
<td>200.5 ± 11.9*</td>
<td>139.4 ± 8.1*</td>
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<td>Adiponectin (µg/ml)</td>
<td>12.5 ± 0.8</td>
<td>44.5 ± 4.6*</td>
<td>13.7 ± 1.3</td>
<td>46.0 ± 5.7*</td>
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<td>Total Cholesterol (mg/dL)</td>
<td>252 ± 17</td>
<td>204 ± 6**</td>
<td>1082 ± 110</td>
<td>906 ± 87</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>182 ± 21</td>
<td>91 ± 9**</td>
<td>415 ± 44</td>
<td>346 ± 34</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>99.7 ± 6.7</td>
<td>73.2 ± 10.0</td>
<td>76.8 ± 5.4</td>
<td>116.7 ± 9.8*</td>
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<tr>
<td><strong>End of Study</strong></td>
<td></td>
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<tr>
<td>Weight (g)</td>
<td>23.6 ± 0.9</td>
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<td>39.3 ± 1.8#</td>
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<tr>
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</table>

* Ldlr<sup>−/−</sup> vs. Ldlr<sup>−/−</sup>/Adn<sup>tg</sup>, p < 0.001
** Ldlr<sup>−/−</sup> vs. Ldlr<sup>−/−</sup>/Adn<sup>tg</sup>, p < 0.05
# Beginning of Study vs. End of Study, p < 0.05
Supplemental Figure I

- Cholesterol (mg/dl)
- Triglycerides (mg/dl)

WT vs. Adn^{-/-}

VLDL, IDL/LDL, HDL
Supplemental Figure II

Aortic root

Total Area (μm²)

WT  Adn⁻⁻  Apoe⁻⁻  Apoe⁻⁻/Adn⁻⁻