Effects of macrophage-specific adiponectin expression on lipid metabolism in vivo

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The metabolic syndrome is associated with significantly increased risk for both cardiovascular disease and type 2 diabetes, in which abnormal lipid metabolism plays a key role and contributes to atherosclerotic plaque formation and diabetic dyslipidemia (10). The dyslipidemia that characterizes the metabolic syndrome includes elevations in serum triglycerides, apolipoprotein B (apoB), and small dense LDL particle concentration and a reduced level of HDL (14). In part, the dyslipidemia is generated by a combination of VLDL overproduction, decreased catabolism of apoB-containing lipoproteins, and increased catabolism of apoA-I in HDL (6); however, the biochemical factors or mechanisms that cause this dysregulation of lipoprotein metabolism are not fully clear.

Adiponectin is a serum protein that is secreted from adipose tissue (29), and the metabolic syndrome is epidemiologically associated with a low serum adiponectin level (40, 15). Serum adiponectin levels are positively correlated with LDL particle size, HDL cholesterol, and apoA-I but are negatively associated with serum triglyceride and apoB concentrations (18, 34, 38), which suggests that adiponectin may influence in lipid metabolism. A number of recent studies indicate that adiponectin may ameliorate dyslipidemia by reducing the hepatic production of apoB (31), increasing catabolism of VLDL and apoB (32) and accelerating apoA-I synthesis together with an enhancement of ATP-binding cassette transporter A1 gene expression (30), and by reducing apoA-I catabolism (44). Increased plasma adiponectin levels resulting from overexpression of adiponectin targeted to adipose tissue have been shown to decrease fasting plasma triglyceride levels (3), whereas disruption of the adiponectin gene results in a significant increase in plasma triglyceride level (20). Although genetic manipulation of the adiponectin gene did not affect plasma total cholesterol levels in these studies, it is not clear whether the alterations in adiponectin gene expression influence the distribution of cholesterol among lipoprotein fractions. The mechanism(s) by which adiponectin could regulate lipoproteins or cholesterol distribution in plasma is presently largely unknown.

Macrophages represent a heterogeneous population of phagocytic cells found throughout the body that originate from the mononuclear phagocytic system (28). These highly plastic cells arise from circulating myeloid-derived blood monocytes that have entered target tissues and gained the phenotypic and functional attributes corresponding to their tissue of residence. Recent attention has focused on the role of macrophages in metabolic diseases (4, 17). In obesity and insulin resistance, adipose tissue contains an increased number of resident macrophages that can constitute up to 40% of the cell population within an adipose tissue depot (45, 46). Macrophages secrete numerous important proinflammatory factors that may influence adiposity biology and lipid metabolism in other tissues.

We (42) and others (13, 35) have demonstrated that adiponectin inhibits macrophage foam cell formation and lipid accumulation by downregulating the expression of the scaveng-
ger receptor A (SR-A) and acyl-coenzyme A:cholesterol-acyltransferase 1 genes. Adiponectin also may inhibit both the inflammatory process and atherogenesis by suppressing both the migration of monocytes/macrophages and their transformation into macrophage foam cells in the vascular wall (35, 36). We have further demonstrated that expression of adiponectin in mouse macrophage cells can reduce macrophage foam cell formation and improve inflammatory responses in other tissues either through macrophage infiltration or an elevation in circulating adiponectin levels.

We now report results of additional studies conducted in mice made transgenic by selectively overexpressing human adiponectin in macrophages. These mice exhibit decrements in both low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) while increasing HDL cholesterol levels. Adiponectin overexpression in macrophages also altered systemic lipid metabolism in vivo by influencing both the circulating levels of inflammatory cytokines and also expression of critical genes related to lipid metabolism in other distal metabolically active tissues. These results indicate that adiponectin-producing macrophages can alter important lipid metabolic activities and inflammatory responses in other tissues either through macrophage infiltration or an elevation in circulating adiponectin levels.

MATERIALS AND METHODS

Animals. Mice made transgenic by the macrophage-specific expression of the human adiponectin gene were created and maintained as reported previously (26). Wild-type (WT) and transgenic mice were fed a high-fat diet (60% kcal% fat) from Research Diets (New Brunswick, NJ) for 16 wk and then euthanized at 20 wk of age and bled by cardiac puncture. Animals were housed in a specific pathogen-free facility with 12:12-h light-dark cycles. Only male mice were used for the experiments. Plasma was isolated from blood by centrifugation at 12,000 g for 10 min and stored at −80°C until analyses were performed, with the exception of using fresh plasma for analysis of lipoprotein cholesterol profiles (see below). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Animal Resources Program at the University of Alabama at Birmingham.

Peritoneal macrophage isolation and culture. Macrophages were isolated from the peritoneum of 20-wk-old WT or transgenic mice after an intraperitoneal priming injection with 2 ml of 5% (wt/vol) thioglycollate to elicit macrophage accumulation. Five days after the injection, mice were anesthetized with isoflurane and injected peritoneally with 5 ml of cold PBS containing 10 mM EDTA. The PBS lavage containing the peritoneal macrophages was aspirated, and the process was repeated three times for each mouse. Macrophages from each mouse were cultured separately overnight in medium (RPMI) containing 10% FBS, after which nonadherent cells were removed by aspiration and the remaining adherent cells were cultured an additional 6–7 days before they were used for the experiments.

Determination of mouse plasma lipoprotein cholesterol profiles. Briefly, nonemolized blood samples were obtained from control or transgenic mice by collecting blood in tubes containing EDTA (1 mg/ml blood) following decapitation. Plasma was then separated from red blood cells by centrifuging blood at 5,000 rpm in a microcentrifuge. Plasma lipoprotein cholesterol profiles were determined by the modified method to determine the lipoprotein cholesterol profile developed by Chung and colleagues (8, 9). This method involves 1) a short (150 min) single-spin density gradient ultracentrifugal separation of the major lipoprotein (VLDL, LDL, and HDL) fractions in 100 μl of plasma using a swinging bucket rotor (Model SW50; Beckman-Coulter, Palo Alto, CA), 2) continuous-flow online mixing of effluent from the density-gradient tube with an enzymatic cholesterol assay reagent (Pointe Scientific, Canton, MI), 3) continuous-flow online incubation of assay mixtures and online measurement of the absorbance of reaction color developed at 500 nm, and 4) computerized calculation of cholesterol level of each lipoprotein fraction after deconvolution of lipoprotein cholesterol profile.

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from ALPCO Diagnostics (Salem, NH). Total and high molecular weight (HMW) of adiponectin levels in mouse plasma were quantitatively analyzed according to the kit manufacturer’s instructions. The absorbance of 96-well plates was measured using a microtiter plate reader set at a wavelength of 492 nm.

Gene expression of lipid metabolic molecules and inflammatory cytokines in metabolic tissues. To determine the expression levels of genes encoding proteins that modulate lipid metabolism and inflammatory cytokines, total RNA was extracted from WT and transgenic mouse macrophages, adipose, and liver tissues using a commercially available reagent (TRIzol; Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 5 μg of total RNA using random hexamer primers according to the kit manufacturer’s instructions (Invitrogen). The complete reaction was cycled for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. A 1-μl aliquot of the reverse transcription reaction mixture was then used for quantitative real-time PCR analysis using an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). Reactions were carried out in triplicate in a total volume of 20 μl using the Brilliant SYBR Green QPCR Master Mix (Stratagene). The average starting quantity of fluorescence units from macrophages was used for analysis. Quantification was calculated using the starting quantity of the cDNA of interest relative to that of 18S ribosomal cDNA in the same sample.

Western blot analysis. Mouse liver tissues were homogenized in tissue lysis buffer containing freshly added protease inhibitor cocktail (Sigma Chemical, St. Louis, MO). Cell lysates (25 μg protein) were separated by SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred onto nitrocellulose membranes and incubated overnight at 4°C with blocking solution (5% nonfat milk in TBS buffer). The blocked membranes were incubated individually with the specific antibodies (1:5,000 dilution with 1% nonfat milk in TBS buffer) for 1 h at room temperature and washed three times with TBS buffer containing 0.1% Tween 20 for 15 min at room temperature with shaking. The secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was reacted with the primary antibody and washed as described above. Immunoreactive protein bands were detected using the Enhances Chemiluminescence Kit (New England Nuclear Life Science Products, Boston, MA).

Statistics. Experimental results are reported as means ± SE. Statistical analyses were conducted using the unpaired Student’s t-test assuming unequal variance unless otherwise indicated. Statistical significance was defined as P < 0.05.

RESULTS

Lipid concentration and lipoprotein distribution are altered in adiponectin transgenic mice. Our previous studies revealed that the body weight and adipose tissue masses did not differ significantly between transgenic and WT mice when the animals were fed normal chow but were significantly lower in transgenic mice when animals were fed a high-fat diet providing 60% of calories from fat (26). We have now analyzed circulating lipids and lipoproteins in high-fat-fed animals and observed that plasma cholesterol levels in adiponectin transgenic (Ad-TG) mice were reduced compared with WT mice by 21% (P < 0.05). The lower plasma total cholesterol levels in Ad-TG mice resulted from a significant decrease in both LDL...
(−34%, \( P < 0.05 \)) and VLDL cholesterol (−32%, \( P < 0.05 \)) concentrations compared with the levels in WT mice. However, the levels of HDL cholesterol were increased by 41% (\( P < 0.05 \)) in Ad-TG relative to WT (Fig. 1).

**Total and HMW adiponectin levels in plasma are increased in Ad-TG mice.** Considering that the changes in the lipid profile detected in our Ad-TG mice would probably result from plasma adiponectin level changes in these mice, we next decided to measure the total and HMW adiponectin levels in these mice. To quantitatively measure the levels of total and HMW adiponectin in plasma of control WT and adiponectin transgenic mice, ELISA analyses were performed with plasma from control WT and Ad-TG mice. As shown in Fig. 2, the data indicated that total adiponectin levels in Ad-TG mice were on average 53% (\( P < 0.05 \)) higher than those in WT mice and that HMW adiponectin levels in Ad-TG mice were on average 105% (\( P < 0.01 \)) higher than those in control WT mice.

**Fig. 2. Increased plasma total and high-molecular-weight (HMW) adiponectin in Ad-TG mice.** Control wild-type (WT) and Ad-TG mice were fed the high-fat diet for 20 wk, and total weight and HMW of adiponectin levels in mouse plasma were quantitatively analyzed using ELISA kits from ALPCO Diagnostics according to the manufacturer’s instructions. The absorbance of 96-well plates was measured using a microtiter plate reader set at a wavelength of 492 nm. Means ± SE from 3 separate experiments with triplicate samples (\( n = 12 \) for each group) are presented. *\( P < 0.05 \) and **\( P < 0.01 \).

Expression of genes involved in lipid metabolism and cytokines in peritoneal macrophages and adipose tissue of Ad-TG mice. We further examined the impact of macrophage adiponectin expression in Ad-TG mice on genes that mediate key metabolic and inflammatory processes in peritoneal macrophages and in adipose tissues. Genes selected for study included hormone-sensitive lipase (HSL), an enzyme involved in triglyceride hydrolysis; fatty acid-binding protein 4 (FABP4), which promotes lipid loading into cells; IL-10, an anti-inflammatory cytokine; and IL-6, a proinflammatory cytokine. Macrophages from Ad-TG mice exhibited increased expression of both the HSL and IL-10 genes and decreased expression of both the FABP4 and IL-6 genes compared with macrophages from WT mice (Fig. 3A). In adipose tissue, HSL gene expression was significantly increased in Ad-TG mice compared with WT, whereas the expression of both the FABP4 and IL-6 genes was significantly decreased (Fig. 3B). Unexpectedly, the gene expression of IL-10 was significantly lower in adipose tissue from Ad-TG mice compared with that observed in WT mice. Thus, the expression of adiponectin in mouse macrophages altered the expression of genes in both macrophages as well as in adipocytes in a manner that, on balance, would tend to promote lipid efflux and reduce inflammation.

**Fig. 3. Levels of expression of genes coding for enzymes that regulate lipid metabolism and inflammatory cytokine production in mouse peritoneal macrophages and adipose tissues.** Control WT and Ad-TG mice were fed the high-fat diet for 20 wk, and mouse peritoneal macrophages (A) and adipose tissues (B) were dissected and analyzed. Levels of expression of the genes coding for hormone-sensitive lipase (HSL), fatty acid-binding protein 4 (FABP4), IL-10, and IL-6 were determined using quantitative real-time PCR. Means ± SE from 3 separate experiments with triplicate samples (\( n = 6 \) for each group) are presented. *\( P < 0.05 \) and **\( P < 0.01 \).
Effects on genes involved in lipid and glucose metabolism in liver. Since circulating lipids and lipoproteins were altered in Ad-TG mice, we assessed the expression of genes involved in lipoprotein synthesis and catabolism in liver tissues from Ad-TG and WT mice fed the high-fat diet for 16 wk (Fig. 4). The expression levels of genes encoding the three major structural apoproteins of VLDL and HDL, namely apoA-I, apoB, and apoE, were increased significantly (average 25%, \(P < 0.01\)) in the liver tissues from Ad-TG mice compared with the levels in WT mice (Fig. 4A). In addition, levels of gene expression for the LDL receptor (LDLR) that binds and removes apoB-containing lipoproteins, ATP-binding cassette G1 (ABCG1) that regulates the maturation of nascent HDL, and HSL that regulates cellular triglyceride were all markedly elevated in liver tissue from Ad-TG mice compared with the levels in WT mice (Fig. 4B). We further examined whether the expression of sterol regulatory element-binding protein 1 (SREBP-1), proprotein convertase subtilisin/kexin type 9 (PCSK9), and hydroxymethylglutaryl-CoA reductase (HMGCR) genes would be affected in liver tissues of Ad-TG mice since these molecules play important roles in cholesterol homeostasis for lipid metabolism. As shown in Fig. 4C, all of these three genes in liver of Ad-TG mice were detected with significant downregulations; SREBP-1 was decreased on average 85% (\(P < 0.01\)), PCSK9 was decreased on average 53% (\(P < 0.01\)), and HMGCR was decreased on average 52% (\(P < 0.01\)) when expression of these genes was compared with the results from control WT mice. Since AMP-activated protein kinase (AMPK) is well known as a regulator in liver for stimulating fatty acid oxidation and reducing the level of SREBP-1 that is a key regulator of fatty acid synthesis in liver.

In liver, lipid metabolism is regulated in concert with glucose metabolism, where lipid oxidation and storage are highly linked to glucose turnover and utilization. In diabetes, one of the hallmarks is increased hepatic glucose production due to failure of insulin to inhibit hepatic gluconeogenesis. To further analyze the mechanisms for adiponectin-mediated alteration of lipid and glucose metabolism in liver, we examined the expression levels of three genes that encode critical enzyme regulators of gluconeogenesis: glucose-6-phosphatase (G6PC), fructose-1,6-bisphosphatase (Fbp1), and phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 5A). The expression levels of the G6PC and Fbp1 genes were significantly decreased in Ad-TG mice compared with levels in WT, whereas PEPCK gene expression was significantly increased. The functional consequences of these changes were not assessed. The decrease in Fbp1 would be predicted to result in reduced gluconeogenesis, whereas the increase in PEPCK would enhance gluconeogenesis. The reduction in G6PC would, in any event, reduce hepatic glucose output, perhaps redirecting the intracellular metabolism of glucose. These results do indicate that adiponectin-producing macrophages have the ability to alter expression of both lipid and carbohydrate metabolic genes in liver. To confirm and extend these results, tissue levels of G6PC and PEPCK proteins were also examined using Western blot analysis (Fig. 5B). Hepatic levels of G6PC and PEPCK proteins were decreased and increased, respectively, in Ad-TG compared with WT mice, in agreement with our studies of gene expression.

DISCUSSION

Adiponectin is naturally expressed and secreted exclusively from adipocytes. To investigate the mechanisms of adiponectin-mediated alterations of whole body metabolism in vivo, we
Our current data now demonstrate that adiponectin-modified macrophages can alter in vivo biochemical pathways and gene expression regulating lipid and lipoprotein metabolism and exert differential responses on pro- and anti-inflammatory cytokine production in adipose and liver tissues. Specifically, IL-6 is a proinflammatory cytokine that can upregulate the expression of macrophage colony-stimulating factor receptors on monocytes, thus promoting differentiation to macrophages and the induction of MCP-1 expression (7), whereas IL-10 in contrast is an anti-inflammatory cytokine that suppresses production of inflammatory cytokines by macrophages. We observed that expression of IL-6 was reduced and IL-10 was increased in macrophages from Ad-TG mice, whereas expression of both IL-6 and IL-10 was decreased in adipose tissue, compared with WT mice. These latter data may relate to the fact that there was a diminution in macrophage infiltration and inflammatory status in adipose tissues in the Ad-TG mice, which could explain a generalized decrease in cytokine production. Also, another main mechanism for regulating these metabolic alternations is probably related to the increased total and HMW adiponectin levels in these Ad-TG mice. Epidemiological evidence has indicated that plasma total adiponectin levels are reduced in patients with metabolic disease (2, 21, 37), and these relationships are more strongly related to a decrement in the HMW adiponectin form than the low-molecular-weight adiponectin form (22, 23).

The data pertaining to lipid and lipoprotein metabolism are novel. Substantial increases in HDL cholesterol and decrements in both VLDL and LDL cholesterol were observed in Ad-TG mice when compared with WT mice. These pronounced changes in circulating lipoproteins can be explained in part by alterations in hepatic gene expression that were induced by the adiponectin-expressing macrophages. Key functions of apolipoproteins include the regulation of lipoprotein-mediated transport and redistribution of lipids among various cells and tissues, their role as cofactors for enzymes of lipid metabolism, and the structural integrity of circulating lipoprotein particles. Virtually all apoA- and >90% of apoE are synthesized in liver and delivered by the liver to the plasma. apoAI is one of main components for HDL (19, 24), and apoE is a major constituent of VLDL and serves as a ligand for the LDL receptor, which is important for the receptor-mediated uptake of chylomicron and VLDL remnants by the liver (12, 27). Our data showed that expression of all three of these critical apolipoproteins was upregulated in liver from adiponectin transgenic mice compared with WT mouse tissue. In addition, we studied hepatic expression of the LDLR, which binds LDL on the cell surface and internalizes LDL cholesterol via endocytosis (1, 16, 25). We observed that liver tissue from Ad-TG mice expressed LDLR at a higher level than in control WT mice, which predictably would contribute to improved clearance of LDL from the circulation. The upregulation in LDLR is likely responsible in part for the low levels of VLDL and LDL in plasma from adiponectin transgenic mice compared with control WT mice, whereas the lower expression of SREBP-1 (upregulating the synthesis of sterols), PCSK9 (inducing LDLR degradation), and HMGCR (upregulating cholesterol biosynthesis) in Ad-TG mice provides further evidence for the critical role of LDLR in lipid metabolism. At the same time, HSL expression was increased, which, through increased lipolysis of triglycerides, would augment the availability of free

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**Fig. 5. Expression in mouse liver tissues of genes and proteins that regulate gluconeogenesis.**

A: comparison of WT and Ad-TG mice fed the high-fat diet for 20 wk. Mouse liver tissues were dissected and analyzed for the expression of genes that regulate gluconeogenesis. Quantitative real-time PCR was used to quantify the levels of expression of the glucose-6-phosphatase (G6PC), fructose-1,6-bisphosphatase (Fbp1), and phosphoenolpyruvate carboxykinase (PEPCK) genes. WT: total proteins were extracted from dissected liver tissues of the control WT (WT1 and WT2) or Ad-TG (Ad1 and Ad2) mice fed the high-fat diet for 16 wk. Protein expression levels were examined using Western blot analysis of G6PC and PEPCK. Results represented 1 of the 3 separate experiments. Means ± SE from 3 separate experiments with triplicate samples (n = 7 for each group) were presented. *P < 0.05 and **P < 0.01.

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developed a mouse model in which the adiponectin gene was specifically expressed in macrophages using a human scavenger receptor A-I gene (SR-AI) enhancer/promoter (26). Our recent studies have demonstrated that adiponectin expression by macrophages in Ad-TG mice can significantly decrease cholesterol and triglyceride accumulation in macrophages in vivo. The adiponectin “modified macrophages” affect other metabolically active tissues such as adipose, liver, and skeletal muscle tissues, where they promote favorable changes in multiple metabolic pathways. For example, compared with WT, Ad-TG mice display reduced production of proinflammatory monocyte chemotactrant protein-1 (MCP-1) and TNFα cytokines, enhanced glucose uptake, and more robust insulin signal transduction (26). When these adiponectin transgenic mice were crossed with the LDLR-deficient mice, fewer lipoprotein-loaded macrophage foam cells were found in atherosclerotic lesions, together with reduced plaque formation, in these double-crossed mice (26). Whereas there was no change in the distribution of macrophage subtypes (for example, from M1 to M2) in active metabolic tissues, total tissue content of macrophages was reduced in the Ad-TG transgenic mice (26).
fatty acids for resynthesis or export. It is also interesting to note that adiponectin-producing macrophages altered expression of genes regulating carbohydrate metabolism in hepatocytes, including effects on G6PC, Fbp1, and PEPCCK. PEPCCK is also a key enzyme in synthesis of glycerol-3-phosphate for controlling triglyceride turnover during glycogenesis, and studies have shown that glycogenesis is the primary pathway for triglyceride synthesis in adipose tissue and liver and that the regulation of glycogenesis by hormones and diet is a key element in controlling lipid metabolism in mammals (33, 39).

Cholesterol that is synthesized or deposited in peripheral tissues is returned to the liver in a process referred to as reverse cholesterol transport, in which HDL plays a central role (5). HDL is secreted by the liver or intestine in the form of nascent particles consisting of phospholipid and apoA1. Nascent HDL interacts with peripheral cells, such as macrophages, to facilitate the removal of excess free cholesterol, a process facilitated by the ABCG1 gene (11, 43). Thus, ABCG1 mediates the transport of cholesterol from cells to HDL (41). Interestingly, expression of both ABCG1 and HSL genes was observed to be upregulated in liver from adiponectin-macrophage transgenic mice, which would predictably lead to greater lipid efflux from hepatocytes.

The current data indicate that macrophages engineered to produce adiponectin can influence in vivo gene expression in adipose tissues in a manner that reduces inflammation and macrophage infiltration and in liver tissue in a manner that affects key glycoenergetic enzymes and impacts lipid/lipoprotein metabolism and the lipoprotein distribution. Regarding effects on lipoproteins, there is a significant increase in circulating HDL and decrements in LDL and VLDL. These observations, in combination with our earlier report showing reduced body weight, increased insulin sensitivity, and improved glucose homeostasis, underscores the potential of the manipulated macrophages to reverse all key attributes of the metabolic syndrome. It is not clear whether the resulting increase in adiponectin in the microenvironment of the macrophage is directly affecting the biology of cells in metabolically active organs or whether the adiponectin is influencing macrophage biology and interactions between macrophages and tissue cells. Alternatively, the previously observed increase in circulating adiponectin levels in Ad-TG mice (26) or HMW adiponectin in the microenvironment of the macrophage is adiponectin in the metabolic syndrome nexus of diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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