Dietary Cholesterol Worsens Adipose Tissue Macrophage Accumulation and Atherosclerosis in Obese LDL Receptor–Deficient Mice

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Objective—Chronic systemic inflammation accompanies obesity and predicts development of cardiovascular disease. Dietary cholesterol has been shown to increase inflammation and atherosclerosis in LDL receptor–deficient (LDLR−/−) mice. This study was undertaken to determine whether dietary cholesterol and obesity have additive effects on inflammation and atherosclerosis.

Methods and Results—LDLR−/− mice were fed chow, high-fat, high-carbohydrate (diabetogenic) diets without (DD) or with added cholesterol (DDC) for 24 weeks. Effects on adipose tissue, inflammatory markers, and atherosclerosis were studied. Despite similar weight gain between DD and DDC groups, addition of dietary cholesterol increased insulin resistance relative to DD. Adipocyte hypertrophy, macrophage accumulation, and local inflammation were observed in intraabdominal adipose tissue in DD and DDC, but were significantly higher in the DDC group. Circulating levels of the inflammatory protein serum amyloid A (SAA) were 4.4-fold higher in DD animals and 15-fold higher in DDC animals than controls, suggesting chronic systemic inflammation. Hepatic SAA mRNA levels were similarly elevated. Atherosclerosis was increased in the DD-fed animals and further increased in the DDC group.

Conclusions—Obesity-induced macrophage accumulation in adipose tissue is exacerbated by dietary cholesterol. These local inflammatory changes in adipose tissue are associated with insulin resistance, systemic inflammation, and increased atherosclerosis in this mouse model. (Arterioscler Thromb Vasc Biol. 2008;28:685-691)

Key Words: atherosclerosis ■ inflammation ■ obesity ■ lipoproteins ■ diet

Chronic systemic inflammation, evidenced as modest low-grade increases in circulating levels of the inflammatory molecules C-reactive protein (CRP) and serum amyloid A (SAA), is a key feature of the obese state,1-3 particularly in visceral obesity.4,5 Adipocyte hypertrophy accompanying obesity is associated with macrophage accumulation in adipose tissue in human subjects.6,7 These macrophages produce cytokines, which cause insulin resistance and signal the liver to produce inflammatory molecules such as CRP and SAA as part of a chronic inflammatory response.8 Similar changes occur in obese mice,6,9 a species in which SAA functions as the main inflammatory molecule, because CRP levels are not regulated by inflammation.10 Although dietary cholesterol has long been thought to increase atherosclerosis risk in humans,11 the mechanism by which this occurs is unclear. Plasma cholesterol levels only increase slightly in response to cholesterol feeding in humans,12 compared to levels occurring in several animal species.13-15 Therefore, dietary cholesterol has been postulated to have a lipid-independent effect on atherosclerosis and cardiovascular disease.16 We previously demonstrated that the addition of a small amount (0.15%) of dietary cholesterol to a Western diet resulted in increased circulating SAA and increased atherosclerosis in LDL receptor–deficient mice (LDLR−/−), independent of an effect on plasma lipids and lipoproteins.17 Because increased SAA levels signify a chronic inflammatory state in mice, these findings suggested that inflammation induced by dietary cholesterol might contribute to atherogenesis in this mouse model.

The present study was undertaken to determine whether dietary cholesterol has an additive role in inducing inflammation, insulin resistance, and atherosclerosis in the setting of diet-induced obesity. To this end, we chose to induce obesity by feeding a diabetogenic diet rich in saturated fat and refined carbohydrate. This diet has previously been shown to cause obesity, hyperglycemia, and increased atherosclerosis in LDLR−/− mice.18 This strain is particularly susceptible to obesity-associated changes induced by the diabetogenic diet, because similar changes were not observed in apoE-deficient mice fed this diet.19 We added 0.15% cholesterol to the diabetogenic diet as in our previous study of LDLR−/− mice.17
Our observations indicate that the addition of cholesterol to a diabetogenic diet leads to a striking increase in macrophage accumulation in intraabdominal adipose tissue, evidence of increased insulin resistance and chronic systemic inflammation, and increased atherosclerosis in this mouse model.

Methods

Animals and Diet

Eight-week-old male LDLR<sup>−/−</sup> mice bred onto a C57BL/6 background were placed on 1 of 3 diets for 24 weeks: a diabetogenic diet high in fat and carbohydrate (DD; BioServ No.F1850); diabetogenic diet with 0.15% w/w total cholesterol (DDC; BioServ No.F4997), or standard rodent chow diet (Ch) providing 4% calories as fat. The diabetogenic diet provides 35.5% calories as fat and 36.6% as carbohydrate (supplemental Table I, available online at http://atvb.ahajournals.org). Animals were housed in cages with microisolator filter tops, maintained on a 12-hour light/dark cycle in a temperature-controlled room, and given free access to food and water. At sacrifice, harvested tissues were snap-frozen at ~70°C or fixed with 10% neutral-buffered formalin and embedded in paraffin wax. All experimental procedures were undertaken with approval from the Institutional Animal Care and Use Committee of the University of Washington.

Analytic Procedures

After 24 weeks of diet-feeding, mice were fasted for 4 hour before drawing blood on the day of sacrifice. Plasma insulin was assayed using a commercially available kit (Linco Research Inc). Triglycerides and cholesterol were assayed in plasma and fast-phase liquid chromatography (FPLC) fractions using colorimetric assay kits. Levels of lipoproteins were analyzed by FPLC as described previously. Circulating SAA levels were measured by enzyme-linked immunosorbent assay (ELISA) in plasma and in FPLC fractions from individual mice, as described previously. This assay measures SAA isoforms 1 and 2, derived from the liver. Insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), calculated using the formula, blood glucose (mMol/L) × insulin concentration (mU/L)/22.5. Glucose tolerance testing was performed after a 4-hour fast by intraperitoneal injection of 20% glucose (1.5 mg/g body weight); blood was drawn from the retroorbital plexus at 0, 30, 60, and 120 minutes for blood glucose measurements using a glucometer (One Touch Ultra, Lifescan Inc).

Immunohistochemistry

Single-label immunohistochemistry was performed on adipose tissues and aortic sinuses using procedures described previously in detail. Macrophages were detected using a rat monoclonal antibody against Mac2 (titer 1:2500, Cedarlane Laboratories). SAA was detected using a rabbit polyclonal antiserum raised against recombinant human SAA1 (titer 1:500, kind gift of Dr Frederick de Beer, University of Washington). The extent of atherosclerosis was measured using the en face technique as described previously. The content of adiponectin, a diabetogenic diet leads to a striking increase in macrophage accumulation in intraabdominal adipose tissue, evidence of increased insulin resistance and chronic systemic inflammation, and increased atherosclerosis in this mouse model.

Atherosclerosis Quantification

The extent of atherosclerosis was measured using the en face technique as described previously. Aortic root analysis on paraffin-embedded sections of the heart were analyzed for atherosclerotic lesion size, as described previously.

Statistical Analyses

Data are expressed as means±SD unless noted otherwise. Mean values were compared using ANOVA with Bonferroni post hoc testing for parametric data using GraphPad Prism program (Version 3.03, GraphPad Software Inc). Data not normally distributed were analyzed using the Kruskal-Wallis test with Dunn post test. P<0.05 was considered statistically significant. Multiple regression analysis was performed using the GraphPad InStat program (Version 3.05, GraphPad Software Inc).

Results

Obesity, Insulin Resistance, and Hyperlipidemia Are Increased in LDLR<sup>−/−</sup> Mice Fed a Diabetogenic Diet, and Worsened in the Presence of Added Cholesterol

LDLR<sup>−/−</sup> mice fed a diabetogenic diet (DD) showed marked increases in weight over 24 weeks compared to chow fed controls (DD, 52.5±0.9 g versus chow, 30±0.6 g, P<0.01, Figure 1A), as has been shown previously. Addition of cholesterol to the diabetogenic diet (DDC) resulted in a similar weight gain (52.3±0.9 g, P<0.01 versus chow), but was not significantly different compared to DD-fed animals. Fasting glucose was higher in the DD and DDC groups compared to chow-fed animals (P<0.001), but did not differ between the obese groups (Figure 1B). Assessment of glucose homeostasis revealed that animals in both DD and DDC groups developed equal and significant glucose intolerance compared to chow diet fed controls (data not shown). Animals in both DD and DDC groups demonstrated hyperinsulinemia (Figure 1C). However, plasma insulin levels were significantly higher in the DDC group (P<0.03), suggesting that these animals were the most insulin resistant. The calculated HOMA-IR index of the DD group was significantly higher than that of chow-fed animals (P<0.001; Figure 1D). The HOMA-IR value was highest in the DDC group (P<0.001 versus chow and P<0.05 versus DD), suggesting that this group was the most insulin resistant.

Hypertriglyceridemia and hypercholesterolemia developed in both groups of diabetogenic diet-fed animals compared to chow-fed controls (Figure 2A and 2B). Triglyceride levels
were higher compared to chow-fed animals \((P<0.001)\), but were not significantly different between the DD or DDC groups (Figure 2A). However, there was a small but statistically significant increase in plasma cholesterol in the DDC group compared to the DD-fed animals \((P<0.05, \text{Figure } 2B)\).

In addition, lipoprotein profiles of animals in the DDC group revealed lower HDL cholesterol and higher VLDL cholesterol levels compared to the DD group (Figure 2C).

**Intraabdominal Adipose Tissue Characteristics Are Altered in LDLR\(^{-/-}\) Mice by the Addition of Cholesterol to the Diabetogenic Diet**

To determine changes in adipose tissue morphology and gene expression that occur with obesity and cholesterol feeding, epididymal (intraabdominal) and inguinal (subcutaneous) adipose tissues harvested at the time of sacrifice were studied. Because macrophages in adipose tissue have been implicated in the pathogenesis of obesity-induced insulin resistance, we used immunohistochemistry to identify the presence of macrophages within adipose tissue. Staining with an antibody to macrophage Mac2 showed that the diabetogenic diets induced a significant increase in plasma cholesterol in the DDC group compared to the DD-fed animals \((P<0.05, \text{Figure } 2B)\). In addition, lipoprotein profiles of animals in the DDC group revealed lower HDL cholesterol and higher VLDL cholesterol levels compared to the DD group (Figure 2C).

**Local Inflammation Is Present in Intraabdominal Adipose Tissue in LDLR\(^{-/-}\) Mice Fed a Diabetogenic Diet Without or With Cholesterol**

Cytokines secreted by adipose tissue macrophages can induce a local inflammatory response. Therefore we studied the extent of local adipose tissue inflammation by measuring expression of genes involved in inflammation by real-time quantitative RT-PCR. Although TNF\(\alpha\) mRNA levels were...
significantly increased in epididymal adipose tissue in both obese animal groups compared to controls (P < 0.05; Figure 4C), levels were significantly higher in the DDC animals (P < 0.05). SAA3, the extrahepatic isoform of SAA produced by adipocytes and macrophages in mice, is a locally synthesized inflammatory molecule with chemoattractant properties. SAA3 gene expression was significantly increased in the DDC group (P < 0.05; Figure 4D) compared with DD. A similar pattern was also noted for the chemoattractant gene, MCP-1 (Figure 4E). Inguinal adipose tissue showed no evidence of altered TNFα production (Figure 4H), but there was evidence of significantly elevated SAA3 in the DDC group (Figure 4I), and of MCP-1 mRNA levels in both DD and DDC animals (Figure 4J). Because expression levels of SAA3 suggested local inflammation in the adipose tissue depots, we performed immunohistochemical analysis for SAA within epididymal and inguinal fat depots (data not shown). In the epididymal fat pad, stained areas for SAA were 3-fold higher in the DD animals (P < 0.05) and 9-fold greater in the DDC group (P < 0.01) compared to chow-fed controls. SAA-stained areas were significantly higher in the DDC compared to DD group (P < 0.05). Inguinal adipose tissue did not show increased SAA staining, this being in contrast to SAA3 mRNA expression (Figure 4I). These findings could reflect rapid tissue degradation of SAA3, lack of translation of mRNA in inguinal adipose tissue, or lack of retention of SAA3 by proteoglycans, which were not detected in inguinal fat. Overall, however, these results suggest inflammatory changes occurring mainly at the level of the epididymal adipose tissue in these mice.

**Atherosclerosis Is Increased in the Presence of Diet-Induced Obesity, Insulin Resistance, and Elevated SAA Levels and Worsened by Dietary Cholesterol**

We next assessed atherosclerosis and found that mice fed the diabetogenic diets for 24 weeks had significantly increased atherosclerotic lesion area by en face analysis as compared to chow-fed controls. There was a significant increase in atherosclerotic area in the DDC group compared to control (P < 0.001) and DD animals (P < 0.05; Figure 6A). Similarly, maximal cross-sectional area of lesions in the aortic sinus was significantly greater in mice fed the DDC diet compared to either DD or chow fed groups (Figure 6B). Immunohistochemistry of aortic root lesions showed significantly increased macrophage (Mac2 antibody stained) areas within lesions in the DDC group (P < 0.001 versus chow and P < 0.05 versus DD; Figure 6C). This staining pattern is similar to that seen in the epididymal adipose tissue where the greatest Mac2 staining was noted in the DDC adipose tissue (Figure...
3A and 3C). Staining with anti-SAA antibody revealed increased SAA immunostaining in the DDC group compared to chow (P<0.001) and DD (P<0.05; Figure 6D).

To further study the contribution of variables such as plasma cholesterol, insulin, and SAA to atherosclerosis in these 2 groups of animals, we performed a multiple regression analysis. Although cholesterol (P=0.36) and insulin levels (P=0.074) did not predict the development of atherosclerosis, logSAA (P<0.002) was a predictor of atherosclerotic lesion areas in the obese animal groups, suggesting the slight increase in cholesterol levels noted in the DDC animals compared to the DD group did not play a major role in the increased atherosclerosis in this setting. Lipoproteins also did not contribute to atherosclerosis in this regression model.

**Discussion**

Obesity is associated with increased insulin resistance, and a predisposition to diabetes and cardiovascular disease in humans. Obesity also is associated with chronic, low-grade, systemic inflammation in both mice and humans. Our study confirmed that consumption of diabetogenic diets (high in both fat and carbohydrates) led to obesity, insulin resistance, low-grade inflammation, and accelerated atherosclerosis in LDLR-/- mice.

We previously reported that the addition of a small amount of cholesterol to a Western-type diet was associated with chronic systemic inflammation, as evidenced by a modest increase in circulating levels of the inflammatory protein, SAA, and increased atherosclerosis. Therefore, we questioned whether dietary cholesterol would further increase inflammation and atherosclerosis in an obese, insulin resistant mouse model. Although addition of cholesterol to the diabetogenic diet increased systemic inflammation beyond that seen with the diabetogenic diet alone, we were surprised to find markedly increased inflammatory changes in adipose tissue. The addition of dietary cholesterol led to the accumulation of significantly more macrophages in epididymal adipose tissue, despite both obese groups gaining an equivalent amount of weight. Increased F4/80 mRNA provides additional evidence for macrophage accumulation, and increased TNF and SAA3 mRNA suggests more local inflammation. Interestingly, the inflammatory changes were limited to epididymal (intraabdominal) adipose tissue and were not observed in inguinal (subcutaneous) fat, despite increased inguinal fat mRNA levels of MCP-1 and SAA3, both known chemotactic factors.

Inflammatory changes in epididymal adipose tissue were accompanied by insulin resistance, as evidenced by increased plasma insulin levels and HOMA-IR values, greatest in the group fed the diabetogenic diet with added cholesterol. This observation is consistent with the notion that adipose tissue inflammation contributes to insulin resistance.

Local inflammation in epididymal adipose tissue in obese mice was accompanied by increased circulating levels of SAA, a marker of systemic inflammation, these changes...
being significantly greater in mice fed the diabetogenic diet with added cholesterol. Whether these hepatic changes resulted from direct exposure of the liver to chylomicrons/chylomicron remnants with increased cholesterol content, to adipose tissue-derived cytokines, or both, is unknown. However, it seems likely that the hepatic inflammatory response is mediated by adipose tissue-derived inflammatory cytokines for at least 2 reasons. First, cytokines such as TNFα and interleukin (IL)-6 have been strongly implicated in the upregulation of hepatic SAA synthesis. Second, the majority of hepatic blood flow derives from the portal circulation that drains intraabdominal adipose depots. Moreover, no detectable inflammatory response was seen in subcutaneous adipose tissue, further implicating factors derived from intraabdominal fat as the source of the hepatic inflammatory response.

Atherosclerosis was increased in both groups of obese mice, more so in animals that received added dietary cholesterol. Interestingly, macrophage staining in aortic sinus lesions also was greatest in the DDC group, similar to observations in epididymal adipose tissue. Multiple variable analysis of cholesterol, lipoproteins, insulin, and SAA showed that only logSAA significantly predicted atherosclerosis in this model, similar to a recent observation in cholesterol-fed rabbits. These findings suggest that low-grade inflammation, and specifically, an increase in SAA levels, might play a causal role in atherogenesis. Moreover, SAA immunostaining of aortic lesions also was greatest in the DDC group. As shown previously, some SAA was present on lipoproteins other than HDL, the lipoprotein on which most SAA has been thought to be transported through plasma. All SAA isoforms have proteoglycan-binding domains, and we previously have suggested that the presence of SAA on lipoproteins (HDL and non-HDL) might facilitate their retention by vascular proteoglycans. The presence of SAA on HDL could render these lipoproteins proatherogenic, and SAA on VLDL or LDL might further increase the retention of these atherogenic lipoproteins, thereby increasing their ability to promote atherogenesis.

Our multivariate analysis also suggests that the increased atherosclerosis in the DDC group was not simply attributable to the slightly greater degree of hypercholesterolemia. However, there was a marked redistribution of lipoproteins in the DDC animals with greater VLDL cholesterol compared to the DD animals, which had greater LDL cholesterol. Therefore, the contribution of this altered lipoprotein distribution to the increased atherosclerosis in the DDC group cannot be eliminated.

Our findings are consistent with a “two-hit” hypothesis for the pathogenesis of inflammation and atherosclerosis in this mouse model. First, weight gain resulting from consumption of a “diabetogenic” diet leads to adipocyte expansion and macrophage accrual in adipose tissue, which appears to be depot-specific and limited to intraabdominal adipose depots. Moreover, no detectable inflammatory response was seen in subcutaneous adipose tissue, further implicating factors derived from intraabdominal fat as the source of the hepatic inflammatory response.

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additional macrophage infiltration into intraabdominal adipose tissue is unclear at this time. One possibility is that cholesterol, or perhaps oxysterols formed by oxidation of dietary cholesterol,33 are delivered directly to adipose tissue by chylomicrons or chylomicrons remnants formed after lipolysis of chylomicron triglycerides by adipose tissue lipoprotein lipase. This could be a potential source of adipose cytotoxicity, which has been postulated as a cause of macrophage accumulation in adipose tissue.34

Clinical implications of our study are particularly relevant to patients with familial hypercholesterolemia, a condition in which mutations in the LDL receptor cause significant hypercholesterolemia. In these patients, the accumulation of visceral fat has been shown to be associated with higher insulin levels and an increased cardiovascular risk,35 similar to findings in our mouse model.

In summary, the addition of dietary cholesterol to an obesity-inducing diabetogenic diet results in inflammatory changes beyond those seen with obesity alone. These changes appear to be attributable, at least in part, to increased accumulation of macrophages in adipose tissue and may have implications for the pathogenesis of insulin resistance, inflammation, and atherosclerosis in obese human subjects.

Sources of Funding
This work was supported by an American Diabetes Association Mentor-based Postdoctoral Fellowship award (S.S.), an American Heart Association Fellowship award (C.Y.H.), NIH grants HL030086, DK002456, DK035818.

Disclosures
None.

References
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Arterioscler Thromb Vasc Biol. 2008;28:685-691; originally published online January 31, 2008; doi: 10.1161/ATVBAHA.107.157685

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/28/4/685

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SUPPLEMENTAL FIGURE I

A

![Bar chart showing relative gene expression of CD11c across different conditions.](image)

(i) Chow

(ii) DD

(iii) DDC

B

![Immunohistochemical images of muscle tissues.](image)
Supplemental Figure I legend

(A) Increased expression of CD11c mRNA, a marker of the alternative pathway of macrophage activation, in epididymal adipose tissue of obese LDLR-/- mice.  (B) Immunohistochemical localization of CD11c+ cells in epididymal adipose tissue. Sections from epididymal fat pads from (i) chow, (ii) DD and (iii) DDC groups of mice were stained with anti-CD11c antibody (titer 1 in 10, Serotec, Raleigh, NC). Legend: chow (open bars), DD (hatched bars) and DDC (solid bars). *p<0.05, **p<0.01. Values represent means±SD.
Supplemental Table I

Composition of the diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Composition</th>
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<tbody>
<tr>
<td>Chow</td>
<td>Carbohydrate 68%, Protein 24%, Fat 4%, Fiber 4%</td>
</tr>
<tr>
<td>Diabetogenic diet (DD)</td>
<td>Carbohydrate 36.3%, Fat 35.5% as lard*, Protein 20%, Fiber 0%</td>
</tr>
<tr>
<td>Bioserv F1850</td>
<td></td>
</tr>
<tr>
<td>Diabetogenic diet +cholesterol (DDC) BioServ F4997</td>
<td>As in DD, with 0.15% total cholesterol</td>
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</tbody>
</table>

All diets also contained: casein, methionine, vitamin and mineral mix.

* Lard adds small amounts of cholesterol (0.03%) – Source: USDA National Nutrient Database (http://www.nal.usda.gov/fnic/foodcomp/search/).