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Obesity Promotes Inflammation in Periaortic Adipose Tissue and Angiotensin II–Induced Abdominal Aortic Aneurysm Formation

Sara B. Police, Sean E. Thatcher, Richard Charnigo, Alan Daugherty, Lisa A. Cassis

Objective—Obesity promotes macrophage infiltration into adipose tissue and is associated with increases in several cardiovascular diseases. Infusion of angiotensin II (AngII) to mice induces formation of abdominal aortic aneurysms (AAAs) with profound medial and adventitial macrophage infiltration. We sought to determine whether obesity promotes macrophage infiltration and proinflammatory cytokines in periaortic adipose tissue surrounding abdominal aortas and increases AngII-induced AAAs.

Methods and Results—Hypertrophied white adipocytes surrounded abdominal aortas, whereas brown adipocytes surrounded thoracic aortas of obese mice. mRNA abundance of macrophage proinflammatory chemokines and their receptors were elevated with obesity to a greater extent in abdominal compared to thoracic periaortic adipose tissue. Periaortic adipose tissue explants surrounding abdominal aortas of obese mice released greater concentrations of MCP-1 and promoted more macrophage migration than explants from thoracic aortas. Male C57BL/6 mice were fed a high-fat (HF) diet for 1, 2, or 4 months and then infused with AngII (1000 ng/kg/min) for 28 days. AAA incidence increased progressively with the duration of HF feeding (18%, 36%,and 60%, respectively). Similarly, AngII-infused *ob/ob* mice exhibited increased AAAs compared to lean controls (76% compared to 32%, respectively, $P<0.05$). Infusion of AngII to obese mice promoted further macrophage infiltration into periaortic and visceral adipose tissue, and obese mice exhibiting AAAs had greater macrophage content in visceral adipose tissue than mice not developing AAAs.

Conclusions—Increased macrophage accumulation in periaortic adipose tissue surrounding abdominal aortas of AngII-infused obese mice is associated with enhanced AAA formation. **(*Arterioscler Thromb Vasc Biol.* 2009;29:1458-1464.)**

Key Words: obesity ■ angiotensin II ■ abdominal aortic aneurysm

Obesity, especially in the form of abdominal adipose deposition, increases the risk of cardiovascular-related mortality.[1,2] Virtually all arteries, including the aorta, are surrounded by significant amounts of perivascular adipose tissue. Recent studies suggest that perivascular adipose tissue may be a source of inflammatory cytokines or adipokines, contributing to a strong association between obesity and vascular diseases.[3–5] Importantly, a role for macrophage infiltration into perivascular adipose tissue and increased elaboration of proinflammatory cytokines as an initiator of vascular disease has been suggested[3,5–7] but not extensively examined.

Abdominal aortic aneurysms (AAAs) are a common vascular disease that affects 4% to 9% of the adult male population and account for at least 15 000 deaths per year in the United States.[8] There are no proven pharmacological treatments for AAAs, and therapeutic choices are restricted to surgery. Clinical risk factors for AAA formation include tobacco use, male sex, age (>60 years), and family history.[9–11] As far back as 1969, population-based studies examining the etiology of AAA formation demonstrated that the development of AAAs was associated with increased body weight.[12,13] Recently, a population study of greater than 12 000 men confirmed an index of obesity (waist circumference and waist-to-hip ratio) independently associates with AAA formation.[14] Approximately two-thirds of the United States adult population is overweight or obese (as defined by BMI 25 to 29.9 or >30, respectively[15]). With obesity at epidemic proportions and AAA incidence on the rise, it is important to understand mechanisms by which excess adiposity may predispose to AAA formation. Moreover, because obesity predisposes to several disorders of the metabolic syndrome, including insulin resistance, hypertension, and dyslipidemias, it is important to define whether these metabolic disturbances contribute to enhanced AAA risk in obese patients.

We have previously demonstrated that chronic infusion of AngII to hyperlipidemic mice results in aneurysm formation in suprarenal abdominal aortas of ≥80% of male mice.[16,17] The earliest cellular change noted after initiation of AngII infusion to apolipoprotein E–deficient mice was medial accumulation of macrophages in AAA-prone areas.[18] Macrophage accumulation was also pronounced in the adventitia of suprarenal aortas after AngII-infusion,[13] consistent with periadventitial entry of leukocytes into the vascular wall. Currently, mechanisms for the regional localization of AAAs to the suprarenal

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region of abdominal aortas from AngII-infused mice are unknown.

Previous studies demonstrated that deficiency of CCR2 in bone marrow–derived stem cells reduced AngII-induced atherosclerosis and AAAs, demonstrating a critical role for macrophages in vascular pathologies elicited by infusion of AngII.[19] Thus, manipulations or conditions influencing macrophage recruitment to the vascular wall would be anticipated to have marked effects on AngII-induced AAA formation. In the current study, we hypothesized that obesity-induced increases in macrophage infiltration and proinflammatory cytokine expression in periaortic adipose tissue surrounding abdominal aortas enhances AngII-induced AAAs. In addition, we defined whether obese mice exhibiting AngII-induced AAAs exhibit enhanced macrophage infiltration to adipose tissue. Finally, we used different models of obesity or hyperlipidemia to dissect out mechanisms contributing to enhanced AAA risk with obesity.

Methods

Animals and Diets

Male C57BL/6 mice (2 months of age; the Jackson Laboratory, Bar Harbor, Me) were fed either normal laboratory diet (5% kcal as fat for 4 months) or a high-fat diet (HF; D12492, 60% kcal from fat; Research Diets) for 1, 2, or 4 months before, and during, infusion of AngII. Mice were staggered into the experimental design for HF feeding such that all mice were 6 months of age at study end point. Mice in the normal diet group were age-matched to the longest duration of HF feeding (4 months) to control for effects of aging. A group of male $LDLr^{-/-}$ mice (2 months of age, from a breeding colony of mice originally obtained from the Jackson Laboratory) were fed the HF diet for 4 months before and during infusion of AngII. In separate studies, male leptin-deficient *ob/ob* mice and heterozygous *ob/+* littermates (n=35 per strain; 2 months of age) were obtained from the Jackson Laboratory and were fed normal laboratory diet ad libitum before, and during, infusion of either saline or AngII. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

AngII Infusion

AngII (1000 ng/kg/min) or saline was infused by subcutaneously implanted Alzet minipumps (Model 2004) for 28 days as described previously.[17,20,21]

Blood Pressure Measurements

Systolic blood pressure was measured on conscious restrained mice using tail cuff systems as described previously.[21]

Other Methods

For measurements of serum and plasma components, glucose tolerance tests, quantification of AAAs, histological analysis of periaortic adipose tissue, quantification of mRNA abundance in periaortic adipose tissue, macrophage migration using transwell assays, flow cytometric analysis of macrophage infiltration into visceral adipose tissue, and immunostaining in aneurysm tissue sections please the Supplemental Methods (available online at http://atvb.ahajournals.org).

Statistical Analyses

Data are presented as the mean and SEM. For statistical analyses performed on individual data sets, please see the supplemental Methods.

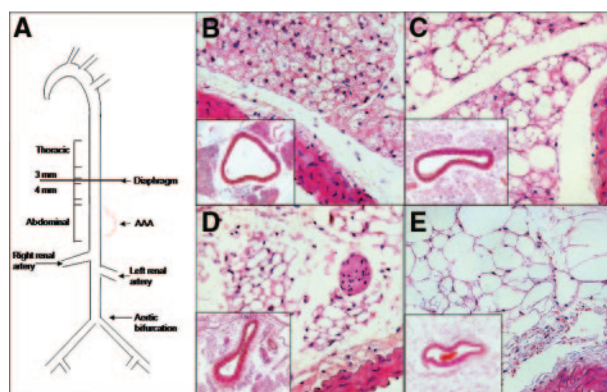

Figure 1. Regional differences in adipocyte morphology in periaortic adipose tissue surrounding thoracic versus abdominal aortas of lean and obese mice. Top, Panel A illustrates aortic regions with intact periaortic adipose tissue (thoracic and abdominal) dissected for analyses from lean (*ob/+*) and obese (*ob/ob*) mice. Thoracic (B and C) and abdominal (D and E) aortic sections (5 μm) from *ob/+* (B and D) and *ob/ob* (C and E) mice were stained with hematoxylin and eosin (H&E). Adipose tissue surrounding thoracic aortas was composed of multilocular brown adipocytes (B: 40×; insets: 10×). In contrast, adipocytes surrounding abdominal aortas were primarily unilocular (C: 40×; insets: 10×). With obesity (C and F), both brown and white adipocytes surrounding aortas were hypertrophied.

Results

Obesity Promotes Macrophage Infiltration and Proinflammatory Cytokine Expression in Periaortic Adipose Tissue: Differences Between Abdominal Versus Thoracic Periaortic Adipose Tissue

Periaortic adipose tissue surrounding thoracic aortas differs in gross appearance from that surrounding abdominal aortas (Figure 1). Thoracic aortas from lean *ob/+* mice were surrounded by multilocular brown adipocytes in close proximity to aortic adventitia (Figure 1). In contrast, abdominal aortas were surrounded by a mixture of cells, with a preponderance of unilocular white adipocytes (Figure 1). In tissue sections from *ob/ob* mice, adipocytes surrounding thoracic and abdominal aortas were markedly hypertrophied but retained their adipocyte phenotype (Figure 1).

Given morphological differences in adipocytes surrounding thoracic versus abdominal aortas, we examined effects of obesity (4 months of HF feeding) on markers of macrophage activation states, chemokine release, and macrophage migration in periaortic adipose tissue from these different aortic regions. We used mice with HF-fed obesity, rather than obesity from leptin deficiency, to contrast proteins expressed to a differing extent in brown (UCP-1) versus white (leptin) adipocytes. In LF-fed mice, mRNA abundance of leptin was similar in periaortic adipose tissue surrounding thoracic compared to abdominal aortas (ΔΔCt: thoracic, 0.42±0.16; abdominal 0.56±0.16). However, obesity resulted in a greater fold induction of leptin mRNA abundance in abdominal (47-fold; Figure 2) than thoracic periaortic adipose tissue (16-fold). Similarly, UCP-1 mRNA abundance was similar in periaortic adipose tissue surrounding thoracic versus abdominal aortas of LF-fed mice (ΔΔCt: thoracic, 0.73±0.10; abdominal, 0.61±0.08). Interestingly, UCP-1 mRNA abun-

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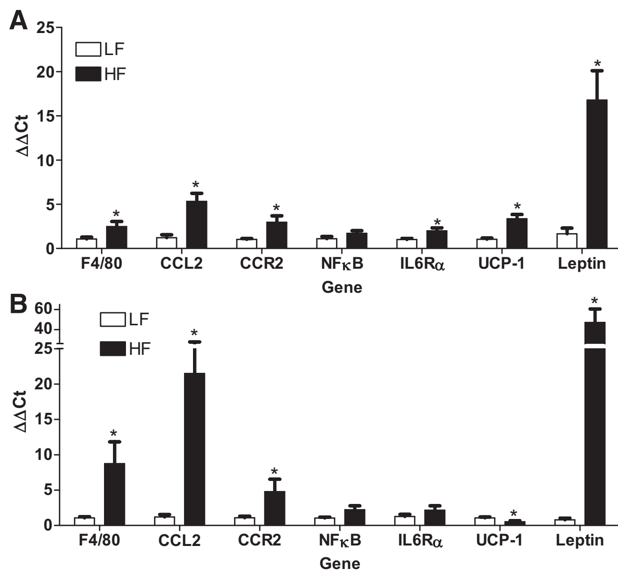

Figure 2. Effects of obesity on mRNA abundance of macrophage markers and proinflammatory chemokines in periaortic adipose tissue surrounding thoracic vs abdominal aortas. Periaortic adipose tissue surrounding thoracic vs abdominal aortas of LF- and HF-fed mice (4 months) was analyzed for mRNA abundance by real-time PCR. A, mRNA abundance in periaortic adipose tissue surrounding thoracic aortas from LF and HF-fed mice. B, mRNA abundance in periaortic adipose tissue surrounding abdominal aortas of LF- and HF-fed mice. F4/80 mRNA abundance increased to a greater extent in periaortic adipose tissue surrounding abdominal than thoracic aortas. Similarly, obesity-induced elevations in mRNA abundance of CCL2, CCR2, and leptin were greater in adipose tissue surrounding abdominal than thoracic aortas. mRNA abundance of UCP-1 was increased by obesity in thoracic periaortic adipose tissue, but decreased in abdominal. Data are mean±SEM from n=5 mice per group. *$P<0.05$ compared to LF.

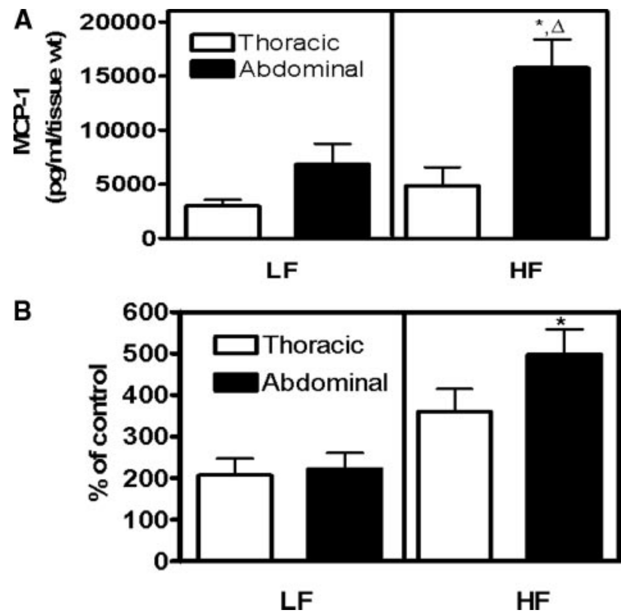

Figure 3. MCP-1 release (A) and macrophage migration (B) in periaortic adipose tissue explants from abdominal aortas are increased by obesity. Periaortic adipose tissue explants were prepared from thoracic or abdominal aortas of LF- and HF-fed mice (4 months) and incubated as described. A, MCP-1 release into the media was increased in periaortic explants of abdominal aortas from HF- compared to LF-fed mice. There was no effect of HF-feeding on MCP-1 release from thoracic periaortic adipose tissue explants. B, Mouse peritoneal macrophage migration was increased by conditioned media from abdominal periaortic adipose explants of HF compared to LF-fed mice.*Significantly different from LF abdominal. Δ, significantly different from HF-thoracic. Data are mean±SEM from n=5 mice/group.

dance increased in periaortic adipose tissue surrounding thoracic aortas of obese mice (3-fold), but was reduced by obesity in abdominal periaortic adipose tissue (2-fold).

Periaortic adipose tissue surrounding abdominal aortas of obese mice exhibited a greater fold increase in mRNA abundance of F4/80 compared to thoracic periaortic adipose tissue (9-versus 2-fold, respectively; Figure 2). Moreover, mRNA abundance of MCP1 and CCR2, cytokines or receptors characteristic of M1 classically activated macrophages,[22] were increased to a greater extent in abdominal versus thoracic periaortic adipose tissue from obese mice (Figure 2). However, other proinflammatory factors, including NFκB and IL6 receptor α, were not altered in adipose explants from either region with HF feeding.

To determine whether these differences in periaortic adipose tissue surrounding thoracic versus abdominal aortas influence chemokine expression and macrophage infiltration, periaortic adipose tissue from 4-month LF and HF-fed mice was separated into thoracic and abdominal, and adipose tissue explants from each of these regions cultured to obtain conditioned media. Periaortic adipose explants from abdominal aortas of HF-fed mice released greater concentrations of MCP-1 compared to explants from thoracic aortas of HF-fed mice (Figure 3A). In addition, periaortic adipose explants from abdominal aortas of HF-fed mice released greater MCP-1 concentrations compared to abdominal adipose explants from LF-fed mice. To define whether elevations in MCP-1 release from periaortic adipose explants of abdominal aortas influenced macrophage migration, conditioned media from explants was used as a stimulus for migration of mouse peritoneal macrophages (MPMs) in transwell assays. In LF-fed mice, media from adipose tissue explants surrounding thoracic and abdominal aortas increased MPM migration by the same percent (Figure 3B). With HF feeding, periaortic adipose explants surrounding abdominal aortas increased MPM migration more than abdominal adipose explants from LF-fed mice.

Diet-Induced and Genetic Obesity Promotes AngII-Induced AAA Formation

Because obesity increased chemokine release and macrophage infiltration in periaortic adipose tissue surrounding abdominal aortas, the aortic region where AAAs form when mice are infused with AngII, we examined effects of diet and genetic obesity on AngII-induced AAAs. Use of mice with diet- or genetic-obesity enabled definition of contributions of the HF diet to AAA risk. Also, in studies with diet-induced obesity we included $LDLr^{-/-}$ mice fed the HF diet for 4 months before infusion of AngII, because this hypercholesterolemic model has been demonstrated previously to exhibit AngII-induced AAAs,[17] and controlled for effects of hypercholesterolemia on AAA risk.

Male C57BL/6 mice were fed a LF (4 months) or HF diet for 1, 2, or 4 months to produce differing durations and

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Table. Characteristics of AngII-Infused HF-Fed C57BL/6 and LDLr$^{-/-}$ Mice

	Body Weight Gain (g)	Retroperitoneal Adipose (%)	Blood Glucose (mg/dl)	Plasma Renin (ng/ml)	Systolic BP (mm Hg)	
					Pre-AngII	Post-AngII
Control (n=10)	13.2±0.9	1.1±0.1	107±7	1.20±0.4	122±5	157±12‡
1 month high fat (n=11)	9.8±0.6	1.5±0.1	104±6	3.54±0.9	111±6	121±7
2 months high fat (n=11)	18.8±1.5	1.6±0.2	101±6	3.02±1.4	104±5*	128±5‡
4 months high fat (n=25)	23.8±1.1*	1.9±0.2*	109±6	1.14±0.4	109±1*	148±4‡
LDLr$^{-/-}$, 4 months high fat (n=7)	18.3±1.9	1.5±0.3	161±15*†	1.27±0.4	121±5	174±7‡

BP indicates systolic blood pressure. Data are mean±SEM.
*$P<0.001$ compared to Control; †$P<0.05$ compared to other HF groups; ‡$P<0.05$ compared to baseline values.

magnitudes of obesity before the initiation of AngII infusion. Body weight gain increased with duration of HF-feeding in AngII-infused C57BL/6 mice and was significantly increased compared to control at 4 months (Table). Increases in body weight in AngII-infused HF-fed C57BL/6 mice were paralleled by increases in retroperitoneal adipose tissue mass (Table) and elevations in plasma concentrations of leptin and resistin (supplemental Table I). The duration of HF-feeding (1, 2, or 4 months) of AngII-infused C57BL/6 mice did not significantly alter serum cholesterol concentrations compared to control (Supplemental Figure IA). However, AngII-infused LDLr$^{-/-}$ mice fed the HF diet exhibited marked elevations in serum cholesterol concentrations compared to control- and HF-fed C57BL/6 mice. In HF-fed C57BL/6 mice (4 month), plasma HDL-cholesterol concentrations were the predominant serum lipoprotein (Supplemental Figure IB); in contrast, HF-fed LDLr$^{-/-}$ mice had a predominance of VLDL- and LDL-cholesterol.

Fasting blood glucose concentrations were not influenced by HF feeding in AngII-infused C57BL/6 mice, but were increased in 4-month HF-fed LDLr$^{-/-}$ mice compared to control (Table). Plasma renin concentrations were not significantly influenced by HF feeding in any group (Table). Systolic blood pressures before AngII infusion were decreased in mice fed the HF-diet for 2 or 4 months compared to control (Table). Infusion of AngII resulted in an increase in systolic blood pressure in control mice and C57BL/6 and LDLr$^{-/-}$ mice fed HF diets for 4 months compared to baseline (Table). In contrast, AngII infusion did not significantly increase systolic blood pressures in 1-month HF-fed C57BL/6 mice.

AAA incidence increased progressively as a function of the duration of HF feeding in C57BL/6 mice (18, 36, and 60% incidence for 1, 2, and 4 months of HF-feeding, respectively; Figure 4A, supplemental Table II). AAA incidence was significantly increased in 4-month HF-fed C57BL/6 mice ($P<0.05$) compared to control (22%) and 1-month HF-fed groups. Moreover, AAA incidence was similar in 4-month HF-fed C57BL/6 and LDLr$^{-/-}$ mice (60 and 57%, respectively).

To define effects of genetic obesity on AAAs, independent of HF-feeding, we infused either saline or AngII into *ob/+* and leptin-deficient (*ob/ob*) mice fed normal diet. Systolic blood pressure increased equally in *ob/+* and *ob/ob* mice infused with AngII compared to saline (20 and 19% increase, respectively; Supplemental Table III). Leptin-deficient *ob/ob* mice exhibited characteristics of the metabolic syndrome, including increased body weight, visceral adiposity, and elevated systemic concentrations of glucose, cholesterol (supplemental Table III), insulin and resistin, compared to lean *ob/+* controls (supplemental Table I). Plasma HDL-cholesterol concentrations predominated in sera from *ob/+* mice (saline- and AngII-infused), whereas *ob/ob* mice had both HDL and LDL- cholesterol (supplemental Figure IC). Plasma renin concentrations were increased with obesity, but were decreased by AngII infusion in both *ob/ob* and *ob/+* mice (Supplemental Table III). Infusion of AngII decreased body weight in *ob/ob*, but not in *ob/+* mice. Interestingly, AngII infusion decreased fasting blood glucose concentrations in both strains (Supplemental Table III) and improved glucose tolerance in *ob/ob* mice, quantified by area under the curve (supplemental Figure II).

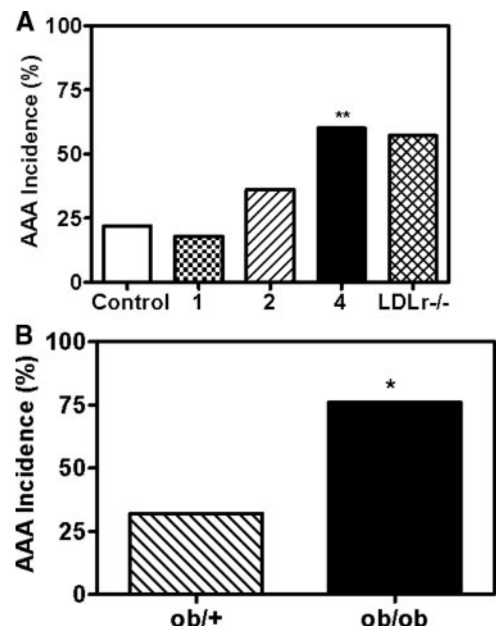

Figure 4. Diet-induced and genetic obesity increase AngII-induced AAAs. A, C57BL/6 mice were fed control or HF diets for 1, 2, or 4 months. Age-matched LDLr$^{-/-}$ mice were fed the HF diet for 4 months. AAA incidence increased progressively with longer durations of HF-feeding in C57BL/6 mice (18%, 36%, and 60% AAA incidence, respectively). AAA incidence was similar in both C57BL/6 and LDLr$^{-/-}$ mice fed a HF diet for 4 months (57% and 60%, respectively). B, AAA incidence was increased in *ob/ob* (76%) compared to *ob/+* mice (32%). Data are mean±SEM from n=10 per group. *$P<0.05$ vs *ob/+*. **$P<0.05$ compared to control, 1-month HF.

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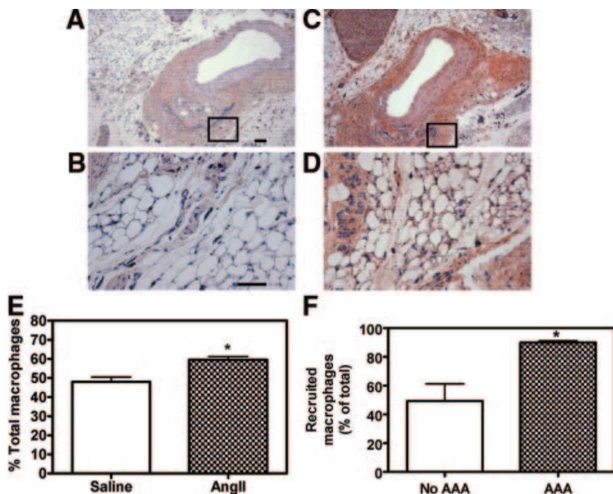

Figure 5. Infusion of AngII promotes inflammation in aneurysms and adipose tissue from obese mice. A through D, Aneurysmal sections with isotype-matched IgG control (A and B) and CD68-positive immunostaining in an aneurysmal tissue section from an AngII-infused *ob/ob* mice (C and D). Macrophages are present in adventitia and periaortic adipose tissue. Boxes represent regions illustrated at higher magnification in B and D. **E**, AngII infusion promotes macrophage infiltration into the stromal vascular fraction of retroperitoneal white adipose tissue from *ob/ob* mice (n=5/group). **F**, Macrophage infiltration into the stromal vascular fraction of white adipose tissue from mice who developed AAAs compared to mice that did not develop AAAs (n=4 no AAA; n=3 AAA). Data are mean±SEM. *Significantly different from saline or no AAA, $P<0.05$.

Similar to mice with diet-induced obesity, *ob/ob* mice exhibited a significant increase in AAA incidence compared to lean *ob*/+ controls (76% versus 32%, respectively, $P<0.05$; Figure 4B, supplemental Table II). In addition, mice with genetic obesity had a greater incidence of aortic rupture (32% of obese *ob/ob* mice compared to 4% of lean *ob*/+ mice). To determine whether improved glucose tolerance or increased body weight predisposed to enhanced AAA formation in *ob/ob* mice, we calculated the HOMA-IR (an index of insulin resistance[23,24]) for *ob*/+ and *ob/o*b AngII-infused mice and fit a logistic regression model with AAA as the response variable and HOMA-IR values or body weight as explanatory variables. Mean HOMA-IR values for AngII-infused *ob/ob* and *ob*/+ mice were 36±9 and 3±1, respectively ($P=0.003$). Logistic regression analysis demonstrated that body weight (odds ratio [OR], 1.29; 95% confidence interval [CI], 1.07 to 1.57; $P=0.008$), but not the HOMA-IR value (OR, 0.94; 95% CI, 0.86 to 1.02; $P=0.144$), emerged as a significant predictor of AAA formation.

Infusion of AngII Promotes Inflammation in Aneurysmal and Adipose Tissue of Obese Mice

In AAA tissue sections from AngII-infused *ob/ob* mice, macrophage immunostaining was present in the adventitia and surrounding adipose tissue (Figure 5C and 5D). To determine whether infusion of AngII promoted further inflammation in adipose tissue, we isolated the stromal vascular fraction (SVF) from retroperitoneal visceral adipose tissue of saline and AngII-infused HF-fed (4 month) C57BL/6 mice, labeled macrophages using a CD11b antibody conjugated to Alexa488, and assessed fluorescence with flow cytometry. We used retroperitoneal adipose as a visceral depot in close proximity to abdominal aortas. Total macrophage content of the SVF (CD11b+ cells) increased in AngII compared to saline-infused HF-fed mice (60±1 compared to 48±2% of cells, respectively; $P<0.05$; Figure 5E). Moreover, mice exhibiting AAAs in response to AngII infusion (AAA) had a greater percentage of newly recruited macrophages (CD11b+/total macrophages) in SVF (no AAA; 90±1 compared to 49±12%, respectively; $P<0.05$; Figure 5F).

Discussion

The major findings of the present study are that obesity increases macrophage infiltration and cytokine expression in periaortic adipose tissue surrounding abdominal aortas and markedly enhances AngII-induced AAA formation. Regional differences in adipocytes, with hypertrophied white adipocytes surrounding abdominal aortas of obese mice, were associated with increased abundance of macrophages, proinflammatory chemokines, and their receptors. Moreover, MCP-1 release and macrophage migration were increased in periaortic adipose explants from abdominal compared to thoracic aortas of obese mice. Mice with either diet-induced or genetic obesity exhibited markedly enhanced AAA formation, demonstrating that HF feeding is not required to increase AAA risk from obesity. AAA incidence was similar in HF-fed C57BL/6 and $LDLr^{-/-}$ mice, despite a 10-fold difference in serum cholesterol concentrations, demonstrating that hypercholesterolemia is not a major contributor to enhanced AAA risk from obesity. Moreover, AAA incidence correlated to body weight, but not to measures of insulin resistance, minimizing contributions of insulin sensitivity to enhanced AAA risk. AAA tissue sections from obese mice exhibited macrophage immunostaining in adventitial and periaortic adipose tissue. Finally, obese mice with AAAs had a greater percentage of newly recruited macrophages in visceral adipose tissue. These results demonstrate that obesity or AngII promote inflammation in periaortic adipose tissue surrounding abdominal aortas and increase susceptibility to AngII-induced AAAs.

Previous results demonstrated that AngII-induced AAA formation exhibits adventitial and medial macrophage infiltration early in aneurysm development.[18] Moreover, deficiency of CCR2 in bone marrow–derived stem cells reduced AngII-induced AAAs,[25,26] demonstrating the pivotal role of leukocytes in this AngII-induced vascular pathology. However, it is unclear whether leukocytes enter the vascular wall from the intimal or periadventitial spaces on infusion of AngII. Obesity generates a state of low grade inflammation characterized by increases in plasma concentrations of inflammatory factors and infiltration of macrophages into white adipose tissue.[27–30] Previous studies demonstrated an increase in macrophage infiltration into periaortic adipose tissue surrounding human coronary arteries from patients with atherosclerosis.[6] Moreover, HF-feeding in rats resulted in increased mass of white adipose tissue surrounding abdominal aortas.[7] Recent studies demonstrated that perivascular adipocytes surrounding human coronary arteries are an integral part of the blood vessel wall because adipocytes invaded the adven-

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titia.[3] In addition, perivascular adipocytes surrounding human coronary arteries had increased expression of inflammatory cytokines compared to other adipose depots. Our results extend these findings by demonstrating that diet-induced obesity in mice results in an increase in chemokine release, macrophage infiltration, and proinflammatory cytokine expression in periaortic adipose tissue. Moreover, effects of obesity on periaortic adipose tissue differed regionally, potentially related to the type of adipocytes surrounding different regions of the aorta. Collectively, these findings demonstrate that perivascular adipose tissue exhibits inflammation similar to other white adipose depots with obesity and support a role for this localized inflammation in enhanced AAA risk from obesity.

To our knowledge, this is the first study to demonstrate that obesity promotes mRNA abundance and release of chemokines and infiltration of macrophages more readily in adipose tissue surrounding abdominal compared to thoracic aortas. The type of adipocytes surrounding different aortic regions may mediate effects of obesity. Recent studies using transgenic aP2-Cre recombinase over expression of MCP-1 in mouse adipose tissue demonstrated that although both brown and white adipose tissue exhibited robust increases in MCP-1 expression, macrophage markers were increased to a greater extent in white than brown adipose tissue.[30] Collectively, these results suggest that the type of adipocytes surrounding aortic regions and the differential ability of brown versus white adipocytes to recruit macrophages may localize AAAs to abdominal aortas of AngII-infused mice.

We used a HF diet as one mode of inducing obesity and established that durations of HF feeding that increase adiposity are associated with enhanced susceptibility to AngII-induced AAAs. The use of C57BL/6 mice, with a predominance of HDL-cholesterol,[31] demonstrates that changes in lipoprotein distributions with obesity do not mediate increased AAA risk. Moreover, because obese C57BL/6 mice exhibited an AAA incidence similar to $LDLr^{-/-}$ mice, despite $\approx$10-fold lower serum cholesterol concentrations, dyslipidemia was not a major contributor to enhanced AAA risk in obese mice.

Obesity develops in *ob/ob* mice from hyperphagia on normal mouse diet without enrichment with either dietary fat or cholesterol. Similar to previous reports in $LDLr^{-/-}$ (HF-fed) or apolipoprotein E–deficient mice,[17,21] *ob/ob* mice exhibited a high incidence of AAA formation. These results demonstrate that elevated dietary fat is not a requirement for increasing AAA risk. Moreover, because hyperleptinemic HF-fed mice as well as leptin-deficient mice both exhibited enhanced AAA risk, then effects of obesity to promote AAA risk are independent of plasma leptin concentrations. Finally, because all mice infused with AngII exhibited similar increases in blood pressure, but disparate AAA incidences, then hypertension is not a mechanism for enhanced AAA risk from obesity.

An unexpected result of the present study was the effect of AngII infusion to decrease fasting blood glucose and improve glucose tolerance in *ob/ob* mice. The reduction in body weight in *ob/ob* mice infused with AngII may have contributed to improved insulin sensitivity. Diabetes has been reported as a negative risk factor for human AAA formation.[10,32] However, most studies examining diabetes and AAA risk have focused on hyperglycemia and have not segregated out influences from type 1 versus type 2 diabetes. If diabetes is protective against AAA formation, then it is plausible that enhanced AAA formation in *ob/ob* mice in this study resulted from improved insulin sensitivity on infusion of AngII. However, a contribution of changes in insulin sensitivity to the observed increase in AAA formation in obese mice is unlikely, because body weight and not insulin sensitivity was a significant predictor of AAA formation. These results demonstrate that body weight, but not insulin sensitivity, is associated with enhanced AAA risk from obesity.

An important finding of this study was the localization of macrophages to periaortic adipose tissue surrounding aneurysmal tissue from AngII-infused obese mice. Moreover, although a proinflammatory effect of AngII to promote macrophage infiltration has been demonstrated to contribute to atherosclerosis and AAA formation,[18,19] this is the first report demonstrating that infusion of AngII promotes macrophage infiltration into adipose tissue. Interestingly, abdominal adipose tissue macrophage content was increased in mice which developed AAAs, suggesting a pivotal role for adipose inflammation in aneurysm development.

In summary, results from this study demonstrate that obesity promotes AngII-induced AAAs. Enhanced AAA risk from obesity was not influenced by the mode of obesity induction, manipulation of serum cholesterol concentrations or lipoprotein distributions, changes in insulin sensitivity, blood pressure responses to AngII, or by leptin. In contrast, regional differences in periaortic adipocytes and their differential ability to promote chemokine release, macrophage infiltration, and proinflammatory cytokine expression related to enhanced AAA risk from obesity. These results suggest that localized inflammation in periaortic or visceral abdominal adipose tissue with obesity, or in response to infusion of AngII, may provide a macrophage rich milieu favoring entry into the aortic media to promote AAA formation. Future studies should explore weight loss by dietary manipulation as a nonpharmacologic lifestyle approach to blunt AAA progression. Moreover, because AngII infusion increased macrophage infiltration into adipose tissue, future studies should define whether these effects are attributed to effects of AngII at adipocyte angiotensin receptors.

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Disclosures

None.

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Supplement Material

Supplemental Table I. Plasma concentrations of insulin and adipokines from diet-induced and *ob/ob* mice.

	Insulin (pg/ml)	Leptin (pg/ml)	tPAI-1 (pg/ml)	Resistin (pg/ml)
Control	383 ± 70	7,768 ± 613	1,778 ± 310	2,069 ± 570
1 month high fat	738 ± 79	12,249 ± 925	1,275 ± 68	5,476 ± 725*
2 month high fat	535 ± 117	9,156 ± 1,113	1,802 ± 456	3,301 ± 231
4 month high fat	927 ± 254	17,043 ± 2,191*	1,730 ± 289	5,397 ± 697*
LDLr-/-, 4 month high fat	592 ± 135	4,731 ± 1,328	2,448 ± 483	3,620 ± 571
ob/+, AngII	433 ± 60	914 ± 84	1,815 ± 434	3,943 ± 484
ob/ob, AngII	5,475 ± 1,694†	99 ± 16†	2,044 ± 335	8,280 ± 1, 520**

*, P<0.05 compared to Control; **, P<0.05 compared to *ob*/+; †, P<0.01 compared to *ob*/+

Supplemental Table II. AAA Incidence Summary Table

Strain	n	Diet / Duration	AAA Incidence (%)
C57BL/6	10	Control / 4 months	22
C57BL/6	11	HF / 1 month	18
C57BL/6	11	HF / 2 months	36
C57BL/6	25	HF / 4 months	60
LDLR-/-	7	HF / 4 months	57
ob/+	25	Control / 1 month	32
ob/ob	25	Control / 1 month	76

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Supplemental Table III. Characteristics of *ob/ob* and *ob/+* mice infused with either saline or AngII.

	Body weight (g)	Retroperitoneal adipose (%)	Blood glucose (mg/dl)	Serum cholesterol (mg/dl)	Plasma renin (ng/ml)	Systolic BP (mmHg)
ob/+, saline (n =10)	32.4 ± 0.3	0.5 ± 0.1	145 ± 11	180 ± 21	3.8 ± 0.6	131 ± 4
ob/+, AngII (n =25)	31.1 ± 0.5	0.3 ± 0.0	109 ± 5 *	193 ± 29	1.0 ± 0.1 *	165 ± 3 *
ob/ob, saline (n = 10)	58.6 ± 1.1 †	4.1 ± 0.3 †	153 ± 15	274 ± 33 †	6.9 ± 1.2 †	136 ± 4
ob/ob, AngII (n = 25)	50.9 ± 1.2 *†	4.0 ± 0.3 †	116 ± 9 *	277 ± 61 †	1.5 ± 0.1 *†	169 ± 9 *

Data are mean ± SEM. *, P < 0.05 compared to saline-infused, within genotype;

†, P < 0.01 compared to *ob*/+, within treatment

Supplemental Table IV. Primer sequences.

Gene	Forward and Reverse Primers	Product Size
IL-6 receptor alpha	5’-GCCCAAACACCAAGTCAAGT-3’ 5’-TATAGGAAACAGCGGGTTGG-3’	187
UCP-1	5'-GGGCCCTTGTAAACAACAAA-3' 5'-GTCGGTCCTTCCTTGGTGTA-3'	196
NF kappa B (p105)	5’-CTGACCTGAGCCTTCTGGAC-3’ 5’-GCAGGCTATTGCTCATCACA-3’	177
CCL2	5’-CCTGCTGCTACTCATTCACC-3’ 5’-TGTCTGGACCCATTCCTTCT-3’	158
CCR2	5’-AGAGAGCTGCAGCAAAAAGG-3’ 5’-GGAAAGAGGCAGTTGCAAAG-3’	185
Leptin	5'-AGACAGTGAGCCCCAAGAAA-3' 5'-GGAACAAAACTCCCCACAGA-3'	178
F4/80	5’-CTTTGGCTATGGGCTTCCAGTC-3’ 5’-GCAAGGAGGACAGAGTTTATCGTG-3’	165

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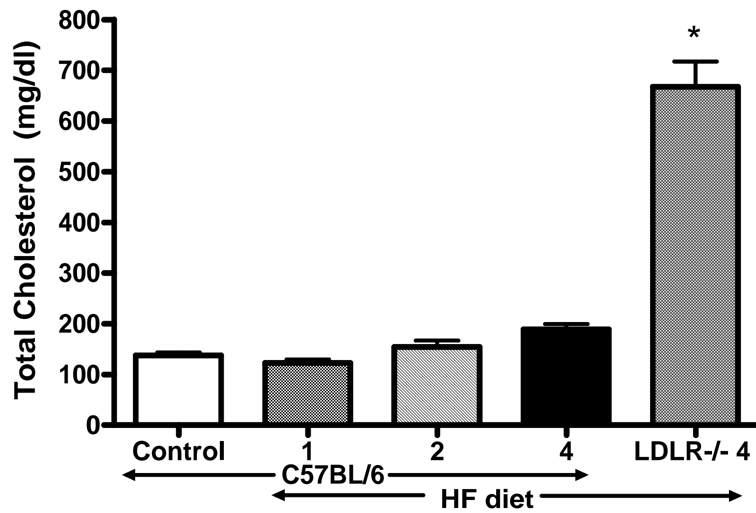

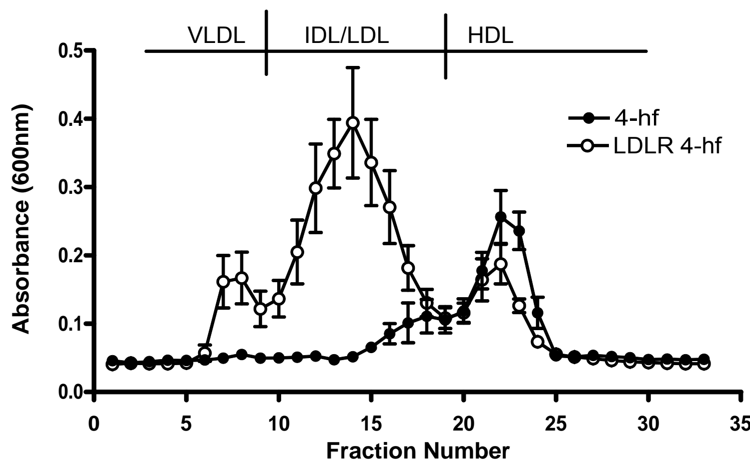

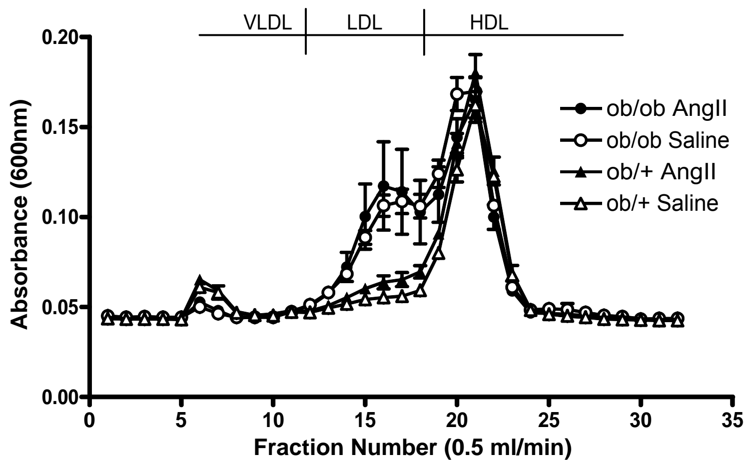

Supplemental Figure I.

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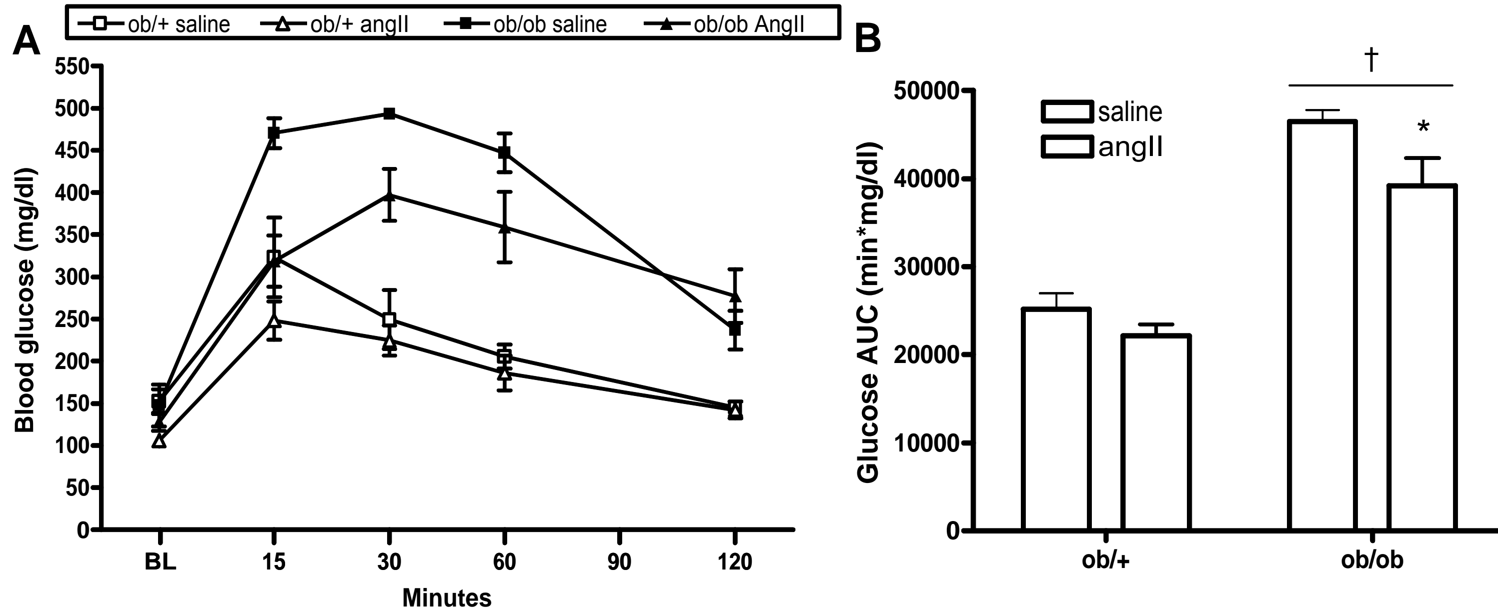

Supplemental Figure II.

Supplemental Figure Legends

Supplemental Figure I. Serum cholesterol concentrations and lipoprotein cholesterol distributions in mice with diet or genetic obesity. **A**, Total serum cholesterol concentrations in C57BL/6 mice fed a LF (4 month) or HF diet for 1, 2 or 4 months prior to AngII infusion (28 days). Serum cholesterol concentrations were not different in LF versus HF-fed mice at any time point. LDLr-/- mice fed the HF diet for 4 months had increased serum cholesterol concentrations compared to LF or HF-fed mice. Data are mean + SEM from n = 10 mice/group. *, significantly different from 4 month HF-fed mice. **B,C**, Lipoprotein-cholesterol distributions of 4 month HF-fed C57BL/6 or LDLr-/- mice (B) or genetically obese (*ob/ob*) versus lean (*ob/+*) mice (C) indicates the increase in total cholesterol of obese mice is due to an elevation of LDL-cholesterol. Values were obtained from n = 5 mice/group run individually. Data are mean ± SEM.

Supplemental Figure II. Effect of obesity and AngII infusion on glucose tolerance. Glucose regulation in *ob*/+ and *ob/ob* saline-(n = 5 per strain) and AngII- (n = 10 per strain) infused mice. (A) Intraperitoneal glucose tolerance test (IPGTT): Blood glucose was measured at baseline. After an i.p. injection of 20% glucose (2mg/g), blood glucose values were monitored for 120 minutes. (B) Results quantified by area under the curve; *ob/ob* mice exhibit reductions in glucose tolerance that is improved by infusion of AngII. Data are mean ± SEM; †, $P < 0.05$ compared to ob/+; *, $P < 0.05$ compared to Saline.

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Supplemental Methods

Measurement of serum and plasma components. Serum cholesterol and triglyceride concentrations were determined in all mice using enzymatic kits (Wako Pure Chemical, Richmond, VA). Lipoprotein cholesterol distribution was performed by size exclusion chromatography as described previously.[1] Plasma renin concentration was measured in all mice by generation of angiotensin I in the presence of excess angiotensinogen substrate as described previously.[2,3] Plasma insulin and adipokine concentrations were determined in AngII-infused mice (diet-induced and genetic obesity) using a mouse adipokine lincoPlex multiplex immunoassay kit. The homeostasis model assessment index (HOMA-IR; an index of insulin resistance) value was calculated in AngII-infused *ob*/+ and *ob/ob* mice as = glucose (in mg/dl) * insulin (in uIU/ml)/405.[4,5]

Glucose tolerance test. During week 4 of saline or AngII infusion, *ob*/+ and *ob/ob* mice were fasted in clean cages for 6 hours of the light cycle. Subsequently, mice were injected i.p. with a 20% glucose solution (2 mg/g) and blood glucose levels were measured over 120 minutes (15, 30, 60, and 120 minutes following injection) (Freestyle Flash glucometer; Abbott Diabetes Care Inc., Alameda, CA).

Quantification of AAAs. For diet-induced obesity studies using C57BL/6 and LDLr-/- mice, aneurysm incidence was quantified by grading of excised, cleaned tissue by 3 independent observers blinded to the experimental design as described previously.[6] For studies using *ob*/+ and *ob/ob* mice, since periaortic adipose tissue was not cleaned from excised aortas, aneurysm incidence was quantified by an increase (>50%) in lumen

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diameter of the suprarenal region of the abdominal aorta as defined by ultrasound (RMV704 scanhead, 40 MHz transducer, 6 mm focal length; Visualsonics, Canada).[7] For AAA detection by ultrasound, mice were anesthetized (ketamine/xylazine, 100/10 mg/kg, i.p.) and restrained in a supine position for ultrasonography. Short axis scans of aortas were performed on abdominal aortas from the level of the left renal arterial branch moving vertically to the suprarenal region. Cine loops of 300 frames were acquired throughout the renal region of abdominal aortas and used to determine the maximal diameters in the suprarenal region of abdominal aortas. Severity of AAAs was defined by grading of cleaned, excised aortas by 3 observers blinded to the experimental design according to methods described previously.[1,8-10]

RNA isolation and extraction. Aortas (from arch to ileal bifurcation) from 4 month LF or HF-fed C57BL/6 mice were removed and placed in RNALater (Ambion, Austin, TX) at 4C. The following day, periaortic adipose tissue (approx. 80-100 mg/mouse) was removed from aortas and RNA extracted using the SV Total RNA Isolation System (Promega, Madison, WI). RNA was quantified using UV spectroscopy (260 nm) and reverse transcribed using a RETROSCRIPT kit (Ambion, Austin, TX). cDNA was amplified using a SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA). mRNA was quantified using the $\Delta\Delta C_T$ method. Estimation of amplified gene products was normalized to 18S RNA to compensate for variations in loading. Primer sequences are illustrated in Supplemental Table IV.

Periaortic adipose tissue explants. Periaortic adipose tissue from thoracic (aortic arch to diaphragm) and abdominal (diaphragm to infrarenal branch) aortas was dissected in

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DMEM with 1% pencillin-streptomyosin G, 0.2% BSA. Tissue pieces from each aortic region of individual mice were weighed, minced, and placed in 6-well culture plates (Costar, Corning, NY) containing the culture media (1 ml) and incubated at 37C for 5 hours. Conditioned media was used for measurement of MCP-1 release (0.5 ml) or for macrophage migration assays (0.5 mls).

Macrophage migration assays. Macrophage migration was performed using a 24-well Transwell plate (8 µm pore size; Costar, Corning, NY). Peritoneal macrophages (1 x 10^6 cells) from male C57BL/6 mice were loaded into the upper chambers, and the lower chamber was filled with either control (non-conditioned media) or conditioned media from periaortic adipose tissue explants. Transwell plates were then incubated at 37C for 5 hours. Media was removed from the upper chamber. Cells in the bottom chamber were then fixed in methanol and stained with Giemsa solution (Dade Behring, Marburg, Germany). Cell counts were performed by two different observers who were blinded to the study design. Migration was expressed as a percentage of basal cell migration (control).

MCP-1 Elisa. MCP-1 was measured in conditioned media from periaortic adipose tissue explants of abdominal and thoracic aortas from LF or HF-fed mice using a commercial kit (Thermo Fischer, Chicago, IL). MCP-1 concentrations were expressed as pg/ml of media and were normalized to explant tissue wet weight.

Periaortic adipose tissue: morphology and immunohistochemistry. Aortas with surrounding periaortic adipose intact from saline or AngII-infused *ob*/+ and *ob/ob* mice

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were fixed overnight in 4% paraformaldehyde and embedded in paraffin for subsequent analyses. The diaphragm was used as a landmark to denote thoracic and abdominal segments of the aorta. For the thoracic region (illustrated in Figure 1), we measured 3 mm above the diaphragm, and then removed 4-6 mm of thoracic aortas with periaortic adipose intact for paraffin embedding. Similarly, for abdominal regions (illustrated in Figure 1), we measured 4 mm below the diaphragm, and then removed 4-6 mm of abdominal aorta with periaortic adipose intact for histologic characterization.
Five micron tissue sections of thoracic and abdominal aortas were deparaffinized in xylene and histologically stained using hematoxylin and eosin (H&E) or for expression of CD68 (Abcam, clone FA-11, Cambridge, MA) with visualization using a rat IgG secondary biotinylated antibody (Vector Labs, BA-4001, Burlingame, CA). A peroxidase-based ABC system and red chromagen AEC (both from Vector Laboratories) were used to identify the antigen-antibody reaction. An isotype-matched rat IgG (Vector Labs, I-4000) was used as control, as was omission of primary antibody. Nuclei were visualized by staining with hematoxylin. Scale bars in A) and C) of Figure 5 represents 200 microns.

Quantification of macrophage infiltration into abdominal adipose tissue. On day 28 of saline or AngII infusion (1 month after injection of PKH26), the stromal vascular fraction containing macrophages was isolated from retroperitoneal adipose tissue adjacent to the abdominal aorta. Briefly, retroperitoneal adipose tissue was excised and minced in Kreb's bicarbonate buffer containing 1% bovine serum albumin (BSA). Minced adipose tissue was digested with collagenase (1 mg/ml; Worthington, Freehold, NJ) at 37C for 1 hour with shaking. The digested cell suspension was passed through a sterile 100 μm

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filter and centrifuged (500g, 10 min) to separate floating adipocytes from the stromal vascular fraction. Cell pellets were resuspended in 0.5 mls of red blood cell (RBC) lysis buffer (eBioscience, SanDiego, CA) and allowed to incubate for 5 minutes before addition of FACS sorting buffer (PBS with 1% FBS, 2 mM EDTA, and 25 mM HEPES) and centrifugation (500g, 1 min). Pellets were resuspended in FACS sorting buffer and stromal vascular cells (10^7 cells) incubated with anti-mouse CD11b antibody conjugated to Alexa Flour 488. After washing, cells were resuspended in 100 µl of 2% paraformaldehyde for fixation and subsequently analyzed for PKH26 (excitation 551 nm; emission 567 nm) and/or CD11b (Alexa Fluor 488, excitation 488 nm; emission 519 nm) fluorescence using FACS (fluorescence activated cell sorting; FACSCalibur flow cytometer, BD Biosciences, San Jose, CA). We used PKH26, a red, inert fluorescent \pard softlinedye (Sigma Aldrich, St. Louis, MI) that is taken up by phagocytic cells to label newly recruited adipose tissue macrophages, as described previously[11]. Prior to infusion of saline or AngII, HF-fed C57BL/6 mice (4 months, a time point demonstrated to exhibit AngII-induced AAAs, see Figure 3) were injected (i.p.) with PKH26 dye (0.1 mmol/l in Diluent B) per the manufacturer's instructions. The percentage of newly recruited macrophages was calculated as cells CD11b+ divided by total macrophages (CD11b+ and CD11b+PKH26+).

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