Recombinant Leptin Promotes Atherosclerosis and Thrombosis in Apolipoprotein E–Deficient Mice

Peter F. Bodary, Shufang Gu, Yuechun Shen, Alyssa H. Hasty, Joshua M. Buckler, Daniel T. Eitzman

Objective—The direct role of leptin in vascular disease remains controversial. The objective of this study was to examine the effects of leptin treatment on atherosclerosis and thrombosis in atherosclerotic-prone mice.

Methods and Results—Sixteen-week-old, male apolipoprotein E–deficient mice were treated with injections of recombinant leptin (125 μg per day IP; n=10) or vehicle (n=10) for 4 weeks. Leptin treatment resulted in reduced epididymal fat (352±30.7 versus 621±61.5 mg; P=0.005) and fasting insulin (0.57±0.25 versus 1.7±0.22 ng/mL; P=0.014). Despite these metabolic benefits, leptin treatment resulted in an increase in atherosclerosis (8.0±0.95% versus 5.4±0.59% lesion surface coverage; P<0.05). Leptin treatment also resulted in a shortened time to occlusive thrombosis after vascular injury (21±2.1 versus 34.6±5.4 minutes; P=0.045).

Conclusions—These studies indicate that exogenous leptin promotes atherosclerosis and thrombosis and support the concept that elevations of leptin may increase the risk for cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words:

Obesity is a growing epidemic and will likely have a major impact on cardiovascular disease. The link between obesity and cardiovascular disease is unclear, but recent and emerging evidence indicates that factors produced by adipose tissue may directly influence vascular disease.1 leptin, a hormone produced primarily by adipocytes, has been clearly demonstrated to play an important role in body weight regulation through effects on feeding and energy expenditure.2 In addition to central effects of leptin on appetite and metabolism, numerous peripheral effects of leptin have been discovered recently. Leptin receptors have been identified on several cell types, including vascular endothelial cells,3 monocyte/macrophages,4 and platelets.5 Plasma leptin levels have also been correlated with cardiovascular complications in humans, an effect independent of body mass index and traditional risk factors.6–8

Nevertheless, the direct role of leptin in vascular disease remains controversial. Atherosclerotic mouse models with complete deficiency of leptin suggest that the absence of leptin might promote atherosclerosis.9,10 However, these studies have been confounded by the extreme obesity and dyslipidemia that result from the loss of leptin-mediated central effects.9,10 As a result, the direct effect of leptin on atherosclerosis has not yet been addressed. Therefore, to examine the direct role of leptin in atherosclerosis, we analyzed the effect of exogenous recombinant murine leptin on atherosclerosis using apolipoprotein E (apoE)–deficient mice.

In addition, we tested the effects of chronic leptin therapy on thrombosis in these atherosclerotic-prone mice to determine whether potential metabolic improvements achieved with leptin therapy would outweigh the acute prothrombotic effect of leptin described previously.11,12

Methods

Mice Male apoE-deficient mice were purchased from the Jackson Laboratory (stock No. 002052; Bar Harbor, Maine) and provided Western chow (TD88137; Harlan Teklad) from 14 to 20 weeks of age. All procedures complied with the principles of laboratory and animal care established by the National Society for Medical Research and were approved by the University of Michigan committee on use and care of animals.

Leptin Treatment Beginning at 16 weeks of age, mice received 200 μL intraperitoneal injections daily of either 125 μg of recombinant murine leptin (R & D Systems) or vehicle control (n=10 per group). The dose of leptin chosen was based on a protocol used to achieve weight loss and fertility in leptin-deficient mice.13 We determined that a 4-week duration of injections at this dose would be adequate to test the hypothesis that leptin promotes atherosclerosis in this particular model of hyperlipidemia.14

Analysis of Atherosclerosis At 20 weeks of age, all mice (n=10 per group) were euthanized via exsanguination while under intraperitoneal pentobarbital an-
esthesia (100 mg/kg). Mice were perfused with saline and fixed using formalin with a 25-gauge needle inserted into the left ventricle at a rate of 1 mL/min. After formalin fixation, the arterial tree was meticulously dissected from the carcass and placed in 70% ethanol for ≥72 hours. The surface area occupied by atherosclerosis was then quantitated at the thoracic aorta and major branches, including the brachiocephalic, carotid, and subclavian arteries, via Oil Red O staining and quantitative morphometry, as described previously.13 The lesion area was calculated for the control and treatment groups and expressed as a percentage of total surface area examined (n=10 per group). Additional analysis of lesion thickness was performed on hema-toxylin and eosin–stained cross-sections obtained from the ascending aorta and brachiocephalic and carotid arteries. For the aorta, sections were taken every 300 μm, beginning just distal to the aortic valve. For the brachiocephalic artery, sections were taken every 100 μm, beginning at the origin of the vessel. For the carotid arteries, sections were taken every 200 μm, beginning in the distal common carotid artery, just proximal to the bifurcating segment. Four consecutive sections were analyzed from each site per animal (n=10 per group). Images were analyzed using Image-Pro Plus software (Media Cybernetics). Intimal and medial areas were measured, and the ratio of intima/media area was calculated.

Cross-sections from the brachiocephalic artery were also examined for cellular composition and proliferation indices (n=3 per group). Smooth muscle cells were detected by smooth muscle α-actin staining using an α-actin monoclonal antibody (1:50; Cedarlane Laboratories Limited). Macrophages were identified with a rat anti-mouse Mac-3 monoclonal antibody (1:10; BD Biosciences), followed by detection with biotin-goat anti-rat IgG (Accurate Chemical & Scientific Corp.). To identify proliferating cells within the atherosclerotic lesions, mice were injected with 5-bromodeoxyuridine (BrdUrd) hours before death at the dose recommended by the manufacturer. BrdUrd incorporation was detected with BrdUrd labeling reagent and staining kit (Zymed) in 3 sections per mouse. Cells were counted manually and expressed as a percentage of total cells in the lesion.

**Plasma and Fat Analyses**

Serum insulin and adipokines were measured from all mice (n=10 per group) after a 5-hour fast using a Luminex100 multi-analyte kit (MadKine-71K; Linco Research Inc.). Total cholesterol was determined using plasma collected after cardiac puncture containing 0.15 mol/L NaCl, 0.01 mol/L Na2HPO4, and 1 mmol/L EDTA. Forty 0.5-mL fractions were collected, and cholesterol contents were assayed in fractions 11 through 40 over a Superose 6 column (Amersham Biosciences) in buffer containing 0.15 mol/L NaCl, 0.01 mol/L Na2HPO4, and 1 mmol/L EDTA. Forty 0.5-mL fractions were collected, and cholesterol contents were assayed in fractions 11 through 40 using cholesterol reagent from Raichem. Cholesterol reagent from Raichem, with glycerol as a standard according to manufacturer instructions. Total plasma triglyceride levels were measured using the GPO kit from Roche, with 0.03 mg/mL as a standard. Subcutaneous fat pads were removed and immediately weighed at the time of death in the 72 hours. The surface area occupied by atherosclerosis was then quantitated at the thoracic aorta and major branches, including the brachiocephalic, carotid, and subclavian arteries, via Oil Red O staining and quantitative morphometry, as described previously.13 The lesion area was calculated for the control and treatment groups and expressed as a percentage of total surface area examined (n=10 per group). Additional analysis of lesion thickness was performed on hema-toxylin and eosin–stained cross-sections obtained from the ascending aorta and brachiocephalic and carotid arteries. For the aorta, sections were taken every 300 μm, beginning just distal to the aortic valve. For the brachiocephalic artery, sections were taken every 100 μm, beginning at the origin of the vessel. For the carotid arteries, sections were taken every 200 μm, beginning in the distal common carotid artery, just proximal to the bifurcating segment. Four consecutive sections were analyzed from each site per animal (n=10 per group). Images were analyzed using Image-Pro Plus software (Media Cybernetics). Intimal and medial areas were measured, and the ratio of intima/media area was calculated.

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**Statistical Analysis**

All results are expressed as mean±SEM. Differences between leptin and vehicle groups were determined with a t test; P<0.05 was considered statistically significant.

**Results**

**Effect of Leptin Therapy on Body Weight, Adiposity, and Fasting Insulin**

Leptin-treated mice experienced significant and sustained weight loss during the leptin treatment period compared with control mice (Figure 1A). This weight difference was associated with a significantly smaller epididymal fat pad (leptin 352±30.7 mg; vehicle 621±61.5 mg; P=0.005) and lower fasting serum insulin levels (vehicle 1.7±0.22 ng/mL; leptin 0.57±0.25 ng/mL; P<0.014).

**Effect of Leptin Therapy on Atherosclerotic Burden**

Despite salutary effects of leptin on adiposity and insulin levels, the analysis of total lesion area revealed significantly greater atherosclerosis in the leptin-treated compared with vehicle-treated mice (Figure 1B; P=0.032). The effects of leptin were particularly apparent at bifurcation sites, including the carotid and brachiocephalic arteries. Lesion surface area in leptin-treated mice was 2-fold higher in the brachiocephalic artery (Figure 2E through 2G) and 1.5-fold higher in the carotid arteries (Figure 2A, 2B, and 2G). This proatherogenic effect of leptin was even more apparent when lesion thickness, expressed as intima-to-media ratio, was analyzed at the brachiocephalic (3.5-fold increase; Figure 2H) and carotid arteries (2.4-fold increase; Figure 2D). There were no significant differences in the medial area between the groups. Analysis of cellular composition revealed a trend toward a higher percentage of vascular smooth muscle cells (vehicle 18.4±6.6%; leptin 36.6±15.6%; P=0.34) and proliferation index (vehicle 3.1±0.6% BrdUrd positive; leptin 6.3±1.2% BrdUrd positive; P=0.08) in the leptin-treated group. No differences in macrophage content were observed between the groups. In contrast, no significant difference in atherosclerosis involving the ascending aorta was noted by surface lipid

**Figure 1.** Leptin effects on body weight and atherosclerotic lesion development. A, Mean body weight during the study for the leptin and vehicle groups. The arrow indicates the initiation of injections. The inset displays the mean weight change between groups from the initiation of injections until death; P<0.0001; n=10 per group. B, Atherosclerotic lesion development in vehicle- and leptin-treated mice for the thoracic aorta and major arteries expressed as the percentage of surface area covered with lesion; P=0.032; n=10 per group.
staining (vehicle 4.1±0.78%; leptin 4.6±0.54%; P=0.60) or cross-sectional lesion analysis (intima-to-media ratio: vehicle 0.15±0.012; leptin 0.17±0.02; P=0.29) between the 2 groups of mice. The state of vascular relaxation during perfusion fixation was similar between the 2 groups as judged by no differences in lumen area at nondiseased vascular segments (data not shown). In addition, similar carotid blood flow velocities were present between groups during the photochemical injury experiments using the same pentobarbital dosing regimen (vehicle 0.74±0.11 mL/min; leptin 0.56±0.07 mL/min; P=NS).

Effect of Leptin Treatment on Blood Lipids and Adipokines

Total cholesterol and triglyceride levels were not significantly different between groups (cholesterol–vehicle 1020±80 mg/dL; leptin 913±25 mg/dL; P=0.21; triglyceride–vehicle 94±11 mg/dL; leptin 75±7.6 mg/dL; P=0.20). Fractionation of pooled plasma samples into lipoprotein classes revealed elevations in very-low density lipoprotein levels in the leptin-treated mice (data not shown). No significant differences were evident between groups for plasma levels of tumor necrosis factor-α, interleukin-6, monocyte chemoattractant protein-1, or plasminogen activator inhibitor-1 after the 4 weeks of vehicle or leptin treatment (data not shown).

Effect of Leptin Therapy on the Response to Photochemical Vascular Injury

Mice receiving chronic leptin treatment formed an occlusive thrombus significantly faster than vehicle-treated mice (Figure 3).
lesion vascular smooth muscle cells observed in our study is consistent with the latter hypothesis.

Recent human population studies have indicated that elevated leptin may promote cardiovascular events.6–7 Because thrombosis is the cause of most clinical events, we examined the effect of leptin in an atherosclerotic mouse on the end point of occlusive vascular thrombosis. Interestingly, despite the beneficial effects of leptin therapy on adiposity, leptin treatment promoted thrombus formation, as evidenced by a shortened time to occlusive thrombosis. This finding extends previous studies with acute leptin administration by demonstrating that chronic leptin administration is prothrombotic even in the presence of reduced adiposity.

In conclusion, leptin treatment of apoE-deficient mice results in increased atherosclerosis and enhanced arterial thrombosis. These findings support the concept that elevations of leptin promote cardiovascular complications and indicate that exogenous leptin therapy might increase cardiovascular risk, especially in groups at risk for atherosclerosis.

Acknowledgments

This work was supported by grant funding from National Institutes of Health grant HL-073150 (D.T.E.) and the American Heart Association established investigator award (D.T.E.).

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Arterioscler Thromb Vasc Biol. published online June 9, 2005;
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

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