Leptin Promotes Vascular Remodeling and Neointimal Growth in Mice

Katrin Schäfer, Martin Halle, Colin Goeschen, Claudia Della, Marianne Pynn, David J. Loskutoff, Stavros Konstantinides

Objectives—Human obesity is associated with elevated leptin levels and a high risk of death from cardiovascular disease. In the present study, we investigated the effects of leptin on vascular wound healing and arterial lesion growth in mice.

Methods and Results—Wild-type mice placed on an atherogenic, high-fat diet had elevated (9-fold) leptin levels compared with their counterparts maintained on normal chow, and the former demonstrated significantly enhanced neointimal thickening after carotid artery injury with ferric chloride. The lesions forming in response to injury strongly expressed leptin receptor mRNA and protein. Unexpectedly, the atherogenic diet had no effect on injured vessels from leptin-deficient ob/ob mice despite aggravating obesity, diabetes, and hyperlipidemia in these animals. Daily administration of leptin to ob/ob mice during the 3-week period after injury reversed this phenotype, dramatically increasing neointimal thickness and the severity of luminal stenosis. Exogenous leptin also enhanced lesion growth and increased cellular proliferation in injured arteries from wild-type mice but had no effect on vessels from leptin receptor–deficient db/db mice.

Conclusions—Our results raise the possibility that there might be a direct, leptin receptor-mediated link between the hyperleptinemia in human obesity and the increased risk for cardiovascular complications associated with this condition. (Arterioscler Thromb Vasc Biol. 2004;24:112-117.)

Key Words: leptin • obesity • neointima

Health problems related to excess body weight have reached the dimensions of an epidemic in Western societies. In particular, obesity has led to an increase in morbidity and mortality due to cardiovascular disease. It has been suggested that obesity indirectly promotes the progression of atherosclerotic lesions by inducing a multitude of metabolic and hemodynamic derangements that have a high atherogenic potential. However, adipocytes also synthesize and secrete a number of hormones, growth factors, cytokines, proteases, and protease inhibitors, and some of these proteins might directly contribute to the atherosclerotic and atherothrombotic risk associated with obesity. In particular, the adipocyte-derived hormone leptin, a major regulator of food intake and body weight, was recently shown to enhance platelet aggregation and arterial thrombosis. Moreover, leptin was reported to promote angiogenesis, modulate inflammatory and immune responses, impair arterial distensibility, and induce proliferation and migration of vascular smooth muscle cells. These processes are known to be involved in the pathophysiology of atherosclerosis, raising the possibility that the elevated levels of leptin in human obesity might promote the growth of vascular lesions. However, the effects of leptin on vascular remodeling after endothelial injury in vivo have not yet been systematically studied.

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lesion size in ob/ob and WT mice but had no effect on injured vessels of db/db mice. These results indicate that the receptor-mediated effects of leptin on the vessel wall might represent a novel, direct link between obesity and cardiovascular disease.

Methods

Experimental Animals and Carotid Artery Injury
C57BL/6J WT mice and mice deficient for leptin (C57BL/6-Lep-/-; ob/ob) or the leptin-receptor (C57BL/6-m +/- Lep-/-; db/db) were from Jackson Laboratories, Bar Harbor, Me. Mice were fed normal rodent chow until the age of 8 weeks and then either switched to an HFD containing 21% wt/wt fat and 0.15% wt/wt cholesterol (No. 88137, Harlan Winkelmann) or maintained on normal chow. At the age of 4 months, mice were anesthetized by inhalation of methoxyflurane (Metofane, Janssen) and subjected to carotid artery injury by intraperitoneal injection. Leptin injections were started 24 hours after injury and continued for 3 weeks until carotid artery harvest. Leptin was injected once daily between 7AM and 9 AM, started 24 hours after injury and continued for 3 weeks until carotid artery injury. Leptin was injected once daily between 7AM and 9 AM, with the animals having free access to food and water. Control mice received 100 μL of the vehicle (normal saline) instead. All animal care and experimental procedures were approved by the Animal Research Committee of the University of Goettingen and complied with national guidelines.

Metabolic Parameters and Leptin Levels in Plasma
Plasma glucose, cholesterol, and triglyceride levels were determined enzymatically (Sigma). Plasma leptin levels were measured with a specific enzyme-linked immunoassay for detecting murine leptin (Quantikine, R&D Systems). All measurements were performed in duplicate for each sample.

Histochemistry
For morphometric analysis, paraffin-embedded sections were stained with Verhoeff’s elastic stain and analyzed (ImagePro Plus; Media Cybernetics). Five sections equally spaced throughout the injured segment (at 200-μm intervals) were evaluated, and the results were averaged for each animal. Groups of 5 to 20 animals were compared. Immunohistochemistry was performed as described.15 Smooth muscle cells (SMCs) were detected by a monoclonal anti-mouse α-actin antibody (horseradish peroxidase labeled; Dako) followed by incubation with aminoethyl-carbazole (AEC) reagent (Zytomed), and macrophages were identified with a rat anti-mouse Mac-3 antibody (PharMingen). Oxidized LDL was detected with an antibody against murine malondialdehyde (MDA)-conjugated LDL (a kind gift of Dr W. Palinski, University of California, San Diego; dilution, 1:50) and an avidin-biotin phosphatase detection system (Universal APAAP kit; Dako). Plasminogen activator inhibitor-1 was localized with a polyclonal rabbit anti-mouse PAI-1 antibody (Santa Cruz Biotechnology; dilution, 1:20), and tissue factor was observed with a goat anti-human TF antibody (American Diagnostica; dilution, 1:100).

To quantitatively assess the presence of proliferating cells within the lesions developing after injury, the number of PCNA-positive cells (Zytomed) was counted manually and expressed as a percentage of the total number of cells (within the external elastic lamina) per cross section. Three sections equally spaced throughout the injured segment were evaluated and the results averaged for each animal. Groups of 8 (leptin-treated vs vehicle treated) WT animals were compared.

Isolation and Culture of Vascular SMCs
Murine aortal SMCs were obtained from the thoracic aorta of WT mice and were maintained in SM culture medium (PromoCell) supplemented with 10% (vol/vol) fetal calf serum, 0.5 ng/mL EGF, 2.0 ng/mL bFGF, 5 μg/mL insulin, and gentamicin/amphotericin B at 37°C in a humidified 95% air–5% CO2 atmosphere. Subcultured SMCs (third or fourth passage) were used in the experiments.

SMC Proliferation Assay
Cells (4×103/well) were added to a 96-well tissue culture plate and allowed to attach for 1 hour. Recombinant murine leptin (1, 10, 100, and 1000 ng/mL in PBS), PDGF (40 ng/mL in PBS), or an equal volume of PBS was added. After incubation for 72 hours, cells were washed once with PBS and stained with 0.5% crystal violet solution in methanol. Plates were gently washed in water to remove unbound stain and allowed to dry overnight. Crystal violet stain bound to the total protein of attached cells was redissolved in 100% ethanol/0.1 mol/L sodium citrate (1:1) and absorbance determined at 540 nm. Proliferation of leptin- or PDGF-treated SMCs was expressed as percentage of PBS-treated cells (100%). Three sets of experiments were performed.

Gene Expression Studies
Total RNA from cultured vascular SMCs was extracted with Ultraspex RNA (Biotecx) and chloroform and analyzed for mRNA expression of the common (Ob-RC) and the long (Ob-RL) isoform of the leptin receptor by RT-PCR.15 In situ hybridization was used to study the expression of Ob-R in sections from mouse carotid arteries.15,19 The pcRII vector (Invitrogen) carrying the 474-bp Sac1/Ala fragment of the common form of the mouse Ob-R cDNA was linearized and used as a template for the in vitro transcription of digoxigenin-labeled antisense or sense riboprobes with SP6 or T7 RNA polymerases, respectively, in the presence of digoxigenin-UTP (Roche). For the detection of RNA, sections were incubated with alkaline phosphatase–conjugated anti-digoxigenin antibody (Roche; 1:500).20 Bromochloroindoxyl phosphate and nitro blue tetrazolium were used as color reagents, and sections were counterstained with Nuclear Fast Red. Parallel (control) sections were hybridized with Ob-R sense riboprobe.

Statistical Analysis
Comparisons between WT, ob/ob, and db/db mice or between leptin-treated and vehicle-treated mice were performed with Student’s t test or 1-way ANOVA followed by the Bonferroni test. All statistical tests were 2-sided with a probability value of <0.05 indicating statistical significance.

Results

HFD Enhances Neointima Formation in WT But Not in ob/ob Mice
The ferric chloride model of carotid artery injury was used to study the vascular wound healing response in WT, ob/ob, and db/db mice. To enhance the expected differences between the mouse genotypes, some of the mice were switched to an atherogenic HFD 2 months before injury. As shown in the Table, ob/ob mice fed normal chow exhibited a marked increase in body weight and plasma glucose levels compared with their age-matched WT counterparts. Moreover, placing ob/ob mice on the HFD severely aggravated the metabolic syndrome in these animals. The HFD also aggravated the metabolic syndrome of obese, leptin receptor–deficient db/db mice (n=5; not shown), resulting in plasma glucose levels of 408±35 mg/dL, plasma cholesterol levels of 208±17 mg/dL, and plasma triglyceride levels of 84±3.8 mg/dL (compare to results in the Table).
Histologic and morphometric analyses were performed on cross sections from arterial vessels harvested 3 weeks after injury. Representative sections are shown in Figure 1, and the quantitative morphometric results are summarized in Figure I (available online at http://atvb.ahajournals.org). As expected, WT mice fed normal chow developed small neointimal lesions in response to injury, resulting in minimal luminal narrowing (Figure 1A). Neointimal growth appeared to be slightly more pronounced in ob/ob mice fed normal chow, but the difference did not reach statistical significance (Figure 1, panel E vs A and Figure IA; compare appropriate open bars). When WT mice were placed on HFD, the development of vascular lesions after injury was significantly enhanced (Figure 1, panel C vs A). The medial and particularly the intimal layer were increased in thickness and size (area) in these animals, thus leading to a higher intima-media ratio (0.30±0.06 under HFD compared with 0.14±0.03 under normal chow; \( P=0.03 \)) and more severe luminal stenosis (21±4.9% vs 7.6±1.4%; \( P=0.0017 \); Figure IB, open bars).

Immunohistochemical analysis of the vascular lesions developing in WT mice fed the HFD (Figure 2) demonstrated \( \alpha \)-actin–positive SMCs (predominantly in the neointima) and Mac-3–positive macrophages (predominantly in the media) 3

| Metabolic Parameters of Wild-Type and ob/ob Mice at the Time of Vascular Injury |
|--------------------------------|----------------|---------------|----------------|----------------|
|                                | WT             | WT+HFD        | ob/ob          | ob/ob+HFD      |
|                                | n=29           | n=20          | n=28           | n=14           |
| Body weight (g)                | 34±0.7         | 41±1.5        | 54±1.5         | 68±1.0         |
| Leptin (ng/mL)                 | 2.2±0.4        | 20±4.0        | ...            | ...            |
| Glucose (mg/dL)                | 153±8.6        | 182±9.8       | 356±32         | 544±38         |
| Cholesterol (mg/dL)            | 75±4.5         | 154±9.0       | 93±9.6         | 210±7.9        |
| Triglycerides (mg/dL)          | 66±4.5         | 104±5.4       | 72±2.1         | 109±2.3        |

Data represent the mean values±SEM in mouse plasma. WT, wild-type mice; HFD, high-fat diet.

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Figure 1. Leptin enhances neointimal growth and luminal stenosis after arterial injury: histologic analysis. Representative histologic sections (Verhoeff’s elastic stain; magnification, ×600) of carotid arteries from mice 3 weeks after injury. Mice were treated with saline (A, C, E, G, I) or leptin (B, D, F, H, J) as described in Methods. Arrows denote the internal elastic lamina with the neointima above it and the media below. As indicated, mice were maintained on normal chow or an HFD.

Figure 2. Immunohistochemical analysis of lesions developing in mice after experimental arterial injury. Representative sections of lesions from WT mice fed HFD, 3 weeks after injury. Arrows indicate \( \alpha \)-actin–positive SMCs (upper left) and Mac-3–positive macrophages (upper right). Macrophages also stained strongly for oxidized LDL (oxLDL; lower left) and tissue factor (lower right). Magnification, ×1000. IEL denotes the internal elastic lamina.
weeks after injury. The macrophages appeared to be foam cells because they stained strongly for oxidized LDL (ox-LDL; Figure 2), and they were shown to express tissue factor (Figure 2) and plasminogen activator inhibitor-1 (PAI-1; not shown). Unexpectedly, the atherogenic HFD did not increase the vascular lesions developing after injury in ob/ob mice (Figure 1, panel G vs E; and Figure 1A and B, open bars) despite further increases in body weight, glucose levels, and lipid levels in these animals (Table).

**Exogenous Leptin Dramatically Increases Neointima Formation in WT and ob/ob Mice**

The above observations suggested that additional factors might be required to mediate the effects of obesity on vascular wound healing and neointimal growth. In particular, we hypothesized that the absence of a pronounced neointima in ob/ob mice placed on an HFD might be due to the lack of (functional) leptin in these animals. The Table shows that WT mice placed on the HFD developed a 9-fold increase in their plasma leptin levels by the time they were subjected to vascular injury. Therefore, additional experiments were performed to determine whether leptin influenced the vascular response to injury. Recombinant murine leptin (0.6 μg/g body weight) was administered daily by intraperitoneal injection to the mice throughout the 3-week wound healing period after arterial injury. Control mice received an equal volume of vehicle (normal saline).

Although daily leptin injections did not significantly lower plasma glucose levels, they reduced the mean body weight of WT mice fed the HFD by 15% and that of ob/ob mice fed either normal chow or HFD by 24% and 26%, respectively (data not shown). As expected, vehicle-treated controls in all groups exhibited a slight increase in body weight over the 3-week interval. Leptin treatment significantly lowered plasma cholesterol levels in ob/ob mice fed the HFD (159±9.5 vs 210±7.9 mg/dL in vehicle-treated mice; P=0.002), and it also reduced plasma triglyceride levels in WT (86±2.9 vs 104±5.4 mg/dL; P=0.006) and ob/ob (80±3.8 vs 109±2.3 mg/dL; P<0.001) mice fed the HFD. Importantly, however, in spite of the partial correction of the obesity and hyperlipidemia, leptin treatment dramatically increased both lesion size and the severity of luminal stenosis after arterial injury (Figure 1, panel B vs A; D vs C; F vs E; and H vs G). In fact, the effects of leptin on neointimal growth and luminal narrowing (Figure IA and IB, respectively; compare open and solid bars) were profound in both WT and ob/ob mice regardless of the type of diet (normal chow or HFD). Immunohistochemical studies revealed that the neointimal lesions developing in WT and ob/ob mice after daily leptin treatment over the 3-week period consisted predominantly of α-actin–positive SMCs (not shown).

To begin to investigate how leptin might stimulate neointimal growth, we examined the effects of daily leptin treatment on cellular proliferation within the vascular lesions. The number of cells (within the external elastic lamina) per cross section was slightly increased in leptin-treated WT mice compared with their vehicle-treated counterparts (155±14 vs 126±6 cells, P=0.39). However, PCNA staining revealed that leptin administration over 3 weeks was associated with a significantly higher proportion of proliferating cells both in the neointima (43±9%, vs 20±5% in vehicle-treated mice P=0.041) and the media (77±3% vs 56±4%; P<0.001) of injured vessels. Because, as mentioned above, the majority of cells within the neointima were α-actin–positive SMCs, we examined the effects of increasing concentrations of leptin on the proliferation of vascular SMCs in vitro. Leptin was shown to stimulate the proliferation of cultured SMCs in a dose-dependent manner (Figure II, available online at http://atvb.ahajournals.org).

**Effects of Leptin on Vascular Remodeling Are Mediated by the Leptin Receptor**

RT-PCR of cultured murine SMCs isolated from the aorta of WT mice revealed that these cells expressed the leptin receptor (Figure IIIB). Moreover, in situ hybridization and immunohistochemical analysis of the lesions developing in the arterial wall after injury demonstrated that the cells in these lesions strongly expressed leptin receptor mRNA and antigen (Figure 3). Expression of the leptin receptor was observed both in the neointima and the media, and it was localized to endothelial cells, vascular SMCs, and macrophages. These findings suggested that the effects of leptin on vascular remodeling could be mediated by its receptor. To test this hypothesis, leptin was injected daily into leptin receptor-deficient db/db mice after arterial injury. As in the ob/ob mice, ferric chloride only induced a very small neointima in vehicle-treated db/db mice fed HFD (Figure II). However, in contrast to the results with ob/ob mice, exogenous leptin had no effect on neointimal or medial growth in db/db mouse vessels (Figure 1J and Figure 1).

**Discussion**

Existing evidence indicates that obesity-related insulin resistance results in a systemic prothrombotic state21 and might enhance the progression of atherosclerotic lesions.22 How-
ever, additional pathomechanisms might link excess body weight to the risk for cardiovascular disease. For example, although the ob/ob mouse is a powerful tool for studying human obesity and diabetes, these mice do not spontaneously develop advanced atherosclerotic lesions. The data in the present report suggest that the absence of such lesions in ob/ob mice might reflect the absence of (functional) leptin in these animals.

In these studies, we used the ferric WT and ob/ob mice. Unexpectedly, the vascular lesions developing after injury in the obese and diabetic ob/ob mice remained small, even when those animals were placed on an HFD that led to further increases in their body weight, glucose, and lipid levels. These observations suggest that additional factors apart from insulin resistance and the metabolic syndrome are required to mediate the effects of obesity on vascular remodeling.

The ob/ob mouse expresses a mutant, nonfunctional form of leptin, the hormone that reduces food intake and energy expenditure in periods of nutritional abundance. As a consequence of this defect, ob/ob mice develop uncontrolled hyperphagia and excessive obesity. In contrast to ob/ob mice, and paradoxically, obese humans, who are characterized by overexpression of leptin in the adipose tissue and elevated circulating levels of the hormone, they often develop resistance to the weight-reducing effects of leptin. Interestingly, leptin appears to have a much broader biologic role than simply regulating body weight. For example, it might affect platelet aggregation and arterial thrombosis. Moreover, there is evidence to suggest that leptin might be involved in the homeostasis of the vessel wall, possibly by acting on endothelial cells, macrophages, and/or SMCs.

Recently, the leptin receptor (Ob-R) was detected in human atherosclerotic lesions, and post-hoc analysis of the data from a large clinical study suggested that leptin levels might independently predict future coronary events in hypercholesterolemic men. However, the implications of these observations for the arterial remodeling process have not been tested in appropriate animal models of vascular injury.

To test the hypothesis that the lack of (functional) leptin in the ob/ob mice might have been responsible for the unexpectedly small lesions that developed in these mice after arterial injury, recombinant murine leptin was administered daily by intraperitoneal injection to the mice throughout the 3-week wound healing period after injury. Leptin (or vehicle) treatment was started 24 hours after induction of injury to minimize the possibility that exogenous leptin might indirectly enhance lesion growth by preventing early embolization of arterial thrombi and the provisional matrix necessary for cell migration and neointima formation. In these experiments, leptin treatment dramatically increased lesion size and the severity of luminal stenosis in both ob/ob and WT mice irrespective of the type of diet (HFD or normal chow). Importantly, exogenous leptin enhanced neointimal growth in these mice despite leading to significant reductions in body weight and plasma cholesterol levels. Further in vivo and in vitro studies showed that this effect was associated with an increase of cell proliferation within the vascular lesions.

The effects of leptin on vascular lesion growth appeared to be specifically mediated by the leptin receptor. For example, strong expression of this receptor was demonstrated in endothelial cells, SMCs, and macrophages within the lesions developing in WT mouse vessels after injury. Furthermore, daily administration of leptin over the 3-week period after injury failed to increase neointimal growth in leptin receptor-deficient db/db mice. In the db/db mouse, a point mutation inserts a premature stop codon into the transcript for the long isoform of the leptin receptor (Ob-R_L). Because only Ob-R_L contains all protein motifs required for signal transduction, the resulting defect in leptin signaling leads to severe obesity and metabolic abnormalities, which are indistinguishable from those developing in the ob/ob mouse, despite the presence of high leptin concentrations in the circulation. In contrast, leptin resistance in hyperleptinemic obese humans appears to involve a limitation of leptin transport across the blood-brain barrier and, possibly, impaired leptin signaling within the hypothalamus. Further studies are needed to clarify whether the hyperleptinemia associated with human obesity enhances the effects of the hormone on peripheral organs (including the vasculature) or whether leptin resistance is a generalized phenomenon not confined to the central nervous system.

Our (preliminary) experiments showed that SMCs isolated from the aorta of WT mice expressed the leptin receptor and that leptin stimulated murine SMC proliferation in vitro. Our results thus confirm and extend those of a previous in vitro study on rat aortic SMCs. However, because platelets, endothelial cells, and macrophages also express the leptin receptor, further studies are necessary to determine whether the mechanisms by which leptin promotes neointimal growth also involve its pleiotropic effects on thrombosis, angiogenesis, and inflammation/immune function.

Finally, it needs to be mentioned that experimental models of arterial injury, including the ferric chloride model used in the present study, cannot exactly reproduce the pathophysiology of human atherosclerosis. Notwithstanding this important limitation, lesions developing in mice after injury with ferric chloride exhibit several histologic characteristics of human atherosclerotic plaques, and their systematic study helps dissect basic pathomechanisms of the vascular wound healing (remodeling) process.

In conclusion, our results suggest that leptin modulates vascular remodeling in vivo and that elevated leptin levels dramatically promote lesion growth after experimental vascular injury in the mouse. These observations, together with previous studies showing that leptin also enhances platelet aggregation and stabilizes arterial thrombi, raise the possibility that the increased levels of leptin in obese humans might directly contribute to the elevated risk for cardiovascular disease associated with this condition. This emerging hypothesis has broad clinical implications, and it might help define the objectives of future therapeutic strategies aimed at reducing the disease burden resulting from excess body weight.

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