Leptin Regulates Neointima Formation After Arterial Injury Through Mechanisms Independent of Blood Pressure and the Leptin Receptor/STAT3 Signaling Pathways Involved in Energy Balance

Peter F. Bodary, Yuechun Shen, Miina Öhman, Kristina L. Bahrou, Fernando B. Vargas, Sarah S. Cudney, Kevin J. Wickenheiser, Martin G. Myers Jr, Daniel T. Eitzman

**Background**—Leptin is an adipocyte-derived hormone critical for energy homeostasis and implicated in vascular disease processes. The relevant cellular leptin receptor pools and signaling pathways involved in leptin-related vascular phenotypes in vivo are unclear.

**Methods and Results**—Arterial injury was induced in wild-type (wt), leptin-deficient (lepb/ob), and leptin receptor–deficient (leprdb/db) mice. Compared with wt mice, lepb/ob and leprdb/db mice were protected from the development of neointima. Bone marrow transplantation experiments between wt and leprdb/db mice indicated that the vascular protection in leprdb/db mice was not attributable to lack of leptin receptor expression on bone marrow–derived elements. To investigate the role of the lepr-mediated signal transducer and activator of transcription 3 (STAT3) signaling pathway in the response to vascular injury, leprs/s mice homozygous for a leptin receptor defective in STAT3 signaling underwent femoral arterial injury. Despite similar obesity and blood pressure levels, the neointimal area in leprs/s mice was significantly increased compared with leprdb/db mice.

**Conclusions**—The molecular mechanism by which the leptin receptor mediates neointima formation and vascular smooth muscle cell proliferation is largely independent of the STAT3-dependent signaling pathways involved in energy balance.

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**Key Words:** atherosclerosis ■ obesity ■ remodeling ■ restenosis

Leptin is an adipocyte-derived hormone initially described for its profound effects on energy balance,1 which are largely mediated via leptin receptors expressed on cells in the hypothalamus.2 Leptin receptors have also been shown to be expressed on other cell types that may affect vascular disease processes including white blood cells,3 platelets,4 endothelial cells,5 and vascular smooth muscle cells (VSMCs).6 These receptor pools are likely to be biologically relevant as leptin has been demonstrated to promote angiogenesis,7 thrombosis,8–10 atherosclerosis,9 and neointimal hyperplasia11,12 in animal models. However, the relevant leptin receptor pools responsible for these vascular effects are unclear.

The interaction of leptin at the leptin receptor results in the phosphorylation of critical tyrosine residues13 and subsequent activation of additional intracellular signaling. Several pathways have been implicated in post-leptin receptor signaling events toward appetite and metabolism including PI3 kinase,14 AMP kinase,15 and signal transducer and activator of transcription 3 (STAT3).16 Tyr1138 of the leptin receptor mediates activation of STAT3. Substitution of Tyr1138 with serine blocks STAT3 activation in cultured cells and in mice in which the leptin receptor is replaced by this mutant (leprs/s).16 The obesity of these leprs/s mice closely resembles that of leptin receptor–deficient (leprdb/db) mice.16 However, the role of this pathway in mediating leptin receptor responses on vascular phenotypes in vivo is unknown.

This report examines the effects of leptin and leptin deficiency on neointimal hyperplasia after vascular injury. We demonstrate that the in vivo effect of leptin is not mediated by bone marrow–derived elements, is at least partially independent of leptin receptor-mediated STAT3 signaling, and is associated with the proliferation of vascular smooth muscle cells.

**Methods**

**Mice**

Wild-type (wt), leptin-deficient (lepb/ob), and leptin receptor–deficient (leprdb/db) mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Targeted leptin receptor mutant mice with failure to activate STAT3 signaling pathways (leprs/s) were generated as
previously described.\textsuperscript{16} Lepr\textsuperscript{ob} mice had been backcrossed to the C57BL/6J strain for 7 generations. Male animals, between 8 and 11 weeks of age, were fed a standard laboratory rodent diet (#5001, TestDiet, Richmond, Ind) in specific pathogen-free facilities. 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.

Femoral Artery Injury

Mouse femoral artery injury was performed using a model of wire-induced vascular injury as described previously.\textsuperscript{17} This model causes endothelial damage at the site of injury followed by reproducible intimal hyperplasia. Mice were anesthetized with intraperitoneal sodium pentobarbital (120 mg/kg; Butler Co), except for mice that received a BMT, which were anesthetized with inhaled isoflurane (Baxter Healthcare Corporation) using a Veterinary Anesthesia and Monitoring device (SurgiVet Inc). Mice were then secured in the supine position and placed under a dissecting microscope (Nikon SMZ-2T, Mager Scientific Inc). After a midline incision was made on the ventral left thigh region, the femoral nerve was isolated and pulled aside. The distal and proximal ends of the femoral artery were held with surgical suture for temporary control of blood flow. A small arterial branch superior to the muscular branch of the femoral artery was cauterized (DELC Cauterizer, Aaron Medical Industries Inc). One drop of 1% lidocaine was used to prevent arterial spasm. An arteriotomy of the femoral artery muscular branch was then performed. A strong guide wire (0.38 mm diameter, No. C-SF-15-15, Cook) was introduced 1 cm into the femoral artery through this arteriotomy. Blood flow through the femoral artery was restored by 15, Cook) was introduced 1 cm into the femoral artery through this arteriotomy. Blood flow through the femoral artery was restored by a radiotelemetry system.\textsuperscript{20} Mice of each genotype were instrumented with radiotelemetry devices, which allowed continuous nonrestrained monitoring of blood pressure. A transmitter catheter (Data Sciences) was inserted into the left carotid artery when the mouse was under isoflurane anesthesia, and the blood pressure transmitter body (Data Science, PA-C20) was routed to the abdominal cavity. After recovery, the mouse was housed in an individual cage and provided with regular mouse chow and tap water ad libitum. Mean arterial pressure was recorded at 5-minute intervals for at least 2 weeks duration. After data collection, the 5 minute sampling periods were averaged over 5 consecutive days (once diurnal rhythm was established). The 5 minute averages were then furthered condensed into 24 separate 1 hour values for each hour during the entire light-dark cycle for the purpose of graphical presentation and statistical analysis.

Cell Culture

VSMC were isolated from 7 to 14 week-old wt, lep\textsuperscript{ob/ob}, and lep\textsuperscript{ob} mice (n=3 each group) as previously described.\textsuperscript{21} Cells were grown in Dulbecco modified Eagle medium (DMEM, Gibco Inc) containing 10% fetal bovine serum (FBS, Gibco Inc) and 1% penicillin/streptomycin, and passaged as described previously.\textsuperscript{22} For experiments, cells between passages 4 and 9 were made quiescent by incubation with serum-free DMEM for 30 hours before use. The VSMC proliferation assay\textsuperscript{23} and quantification of cell number by crystal violet staining and optical density reading\textsuperscript{24} were performed as previously described. Briefly, cells were seeded in 96-well plate in DMEM containing 0.5% FBS at a density of 4×10\textsuperscript{4} well and allowed to attach overnight. Medium was then replaced with DMEM containing 2% FBS and Insulin-Transferrin-Selenium premix (Gibco Inc) in the presence of either leptin (100 ng/mL; R&D Systems) or vehicle. After 3 days of incubation, cells were fixed with 10% glutaraldehyde, stained with 0.1% crystal violet solution, and dissolved with 10% acetic acid. The wells were read at 590 nm in a microplate reader (VersaMax, Molecular Devices Corp). All assays were performed in triplicate wells and 2 to 3 replications of the experiment were performed.

Statistical Analysis

Values are expressed as mean±SEM. The statistical significance of differences between groups was determined by 1-way ANOVA followed by Dunn post-hoc analysis when more than 2 experimental groups were included. The student 2-tailed t test was performed when only 2 groups were being compared. For BMT and blood pressure measurements, a 2-way analysis of variance was performed followed by the Tukey HSD test to examine significant main effects. P<0.05 was considered significant.

Results

Effect of Leptin and Leptin Receptor on Neointimal Formation

To determine the influence of leptin and the leptin receptor on neointimal formation, femoral injury was induced in wt, lep\textsuperscript{ob/ob}, and lep\textsuperscript{ob} mice. Four weeks after injury, the average femoral artery neointimal area and ratio of intima/media in lep\textsuperscript{ob} and lep\textsuperscript{ob} mice was significantly reduced compared with wt mice (Figure 1 and Table 1). There were no differences in the medial area between wt, lep\textsuperscript{ob/ob}, and lep\textsuperscript{ob}. The neointimal area was comprised predominantly of
virus vector. Because leptin may affect thrombosis after vascular injury, which could in turn affect neointimal formation, ad-leptin was injected 1 day after injury to reduce potential effects on acute thrombosis. Leptin levels of \textit{lep}^{ob/ob} mice treated with ad-leptin reached a peak of 230.3±58.9 ng/mL at one week after ad-leptin injection and gradually declined to 84.0±33.2 ng/mL at 2 weeks, 8.8±1.6 ng/mL at 3 weeks and 2.1±0.6 ng/mL at 4 weeks. The neointima of \textit{lep}^{ob/ob} mice receiving ad-leptin was significantly higher compared with that of \textit{lep}^{ob/ob} mice receiving no treatment (Figure 1). The neointima of \textit{lep}^{ob/ob} mice receiving a control adenovirus was not different from that of \textit{lep}^{ob/ob} mice receiving no treatment (data not shown). This data supports the effect of leptin on neointimal formation and also demonstrates that leptin is not required at the time of injury to promote neointimal formation. The neointima of \textit{lep}^{ob/ob} mice receiving ad-leptin was also examined to confirm the effects of the leptin receptor on neointimal formation and to serve as a control for nonspecific inflammatory effects of the adenovirus. The neointima of \textit{lep}^{ob/ob} mice treated with ad-leptin was not different from that of \textit{lep}^{ob/ob} mice receiving no treatment (Figure 1).

**Effect of Bone Marrow Transplantation on Neointima Formation**

To examine whether the leptin receptor–deficient phenotype was attributable to the lack of leptin receptor on circulating blood elements, BMT was performed from \textit{lep}^{ob/ob} to \textit{wt}, \textit{wt} to \textit{lep}^{ob/ob}, and \textit{lep}^{ob/ob} to \textit{lep}^{ob/ob} mice. Control mice not receiving bone marrow after irradiation died 7 days later, demonstrating effective bone marrow ablation after the irradiation. Six weeks after BMT, femoral arterial injury was performed to induce intimal hyperplasia. The neointima of \textit{wt} mice receiving bone marrow from \textit{lep}^{ob/ob} mice was significantly greater than that of \textit{lep}^{ob/ob} mice receiving bone marrow from \textit{lep}^{ob/ob} mice (Figure 2). Similarly, the neointimal formation of \textit{lep}^{ob/ob} mice receiving ad-leptin was not different from that of \textit{lep}^{ob/ob} mice receiving no treatment (data not shown). This data supports the effect of leptin on neointimal formation and also demonstrates that leptin is not required at the time of injury to promote neointimal formation. The neointima of \textit{lep}^{ob/ob} mice receiving ad-leptin was also examined to confirm the effects of the leptin receptor on neointimal formation and to serve as a control for nonspecific inflammatory effects of the adenovirus.
timia of leprdb/db mice receiving wt bone marrow was significantly less than the neointima observed in wt mice receiving bone marrow from wt mice (Figure 2).

Effect of LepR-Mediated STAT3 Signaling on Neointima Formation
To determine the effect of leptin receptor-STAT3 signaling on neointimal formation, leprs/s mice (n/H11005), underwent femoral arterial injury. Despite development of an obese phenotype similar to that observed in leprdb/db mice (Table 2),16 leprs/s mice developed greater intimal hyperplasia than leprdb/db mice (P/H11021 0.02; Figure 3a through 3c).

Effect of LepR-Mediated STAT3 Signaling on Arterial Blood Pressure
To examine whether the neointimal formation of the leprs/s was related to arterial blood pressure we measured the mean arterial pressure of wt, leprdb/db, and leprs/s mice. Consistent with previous studies using radiotelemetry, we detected a reduced arterial pressure in leprdb/db compared with wild-type mice.24 Similarly, the leprs/s were observed to have reduced blood pressure compared with wild-type (Figure 4). No difference in blood pressure was observed between leprs/s and wt, leprdb/db, and letrs/s mice were significantly heavier than wt mice. Leptin treatment reduced body weight and insulin in lepob/ob, but not in leprs/s. Lepr/s mice developed greater body wt than wt mice with an obese phenotype similar to leprdb/db mice. *compared to wt; †compared to lepob/ob; ‡compared to leprdb/db. * ˆ† P/H11021 0.05.

Table 2. Effect of Leptin on Body Weight and Metabolic Profile

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body weight (g)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/ml)</th>
<th>Glucose (mg/dl)</th>
<th>Insulin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>28.3±0.4</td>
<td>50.0±4.9</td>
<td>31.9±5.3</td>
<td>204.0±13.5</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Lepob/ob</td>
<td>50.5±3.6**</td>
<td>81.6±8.1**</td>
<td>38.1±2.7</td>
<td>282.8±66.2</td>
<td>8.5±2.5*</td>
</tr>
<tr>
<td>Lepdb/db</td>
<td>54.8±2.0**</td>
<td>110.0±6.3**</td>
<td>56.9±13.0</td>
<td>340.4±50.0*</td>
<td>7.6±3.3*</td>
</tr>
<tr>
<td>Lepob/ob + lep</td>
<td>28.6±1.6</td>
<td>60.0±6.9</td>
<td>29.9±2.5</td>
<td>212.3±25.4</td>
<td>1.39±0.3*</td>
</tr>
<tr>
<td>Lepdb/db + ad-lep</td>
<td>29.8±1.6</td>
<td>68.3±7.9</td>
<td>38.6±3.6</td>
<td>114.3±11.4*</td>
<td>1.79±0.2*</td>
</tr>
<tr>
<td>Lep/s</td>
<td>47.5±1.6**</td>
<td>80.0±11.8</td>
<td>27.1±3.0</td>
<td>178.7±30.3</td>
<td>10.6±0.6**</td>
</tr>
</tbody>
</table>

Lepob/ob and leprdb/db mice were significantly heavier than wt mice. Leptin treatment reduced body weight and insulin in lepob/ob, but not in leprdb/db. Lepr/s mice developed greater body wt than wt mice with an obese phenotype similar to leprdb/db mice. *compared to wt; †compared to lepob/ob; ‡compared to leprdb/db. * ˆ† P/H11021 0.05.
lepr<sup>db/db</sup> mice suggesting that the leptin-mediated effects on blood pressure are mediated by the Lepr-STAT3 pathway.

**Effect of Leptin on SMC Proliferation**

To further elucidate relevant signaling pathways, VSMCs were isolated from wt, lepr<sup>ob/ob</sup>, and lepr<sup>db/db</sup> mice. Similar to previous studies, leptin induced a significant increase in cellular proliferation in wt cells compared with vehicle (leptin=0.40±0.006 versus vehicle=0.37±0.005; P<0.03). As previously shown, this leptin effect was completely blocked with the phosphoinositide 3 kinase inhibitor, wortmannin (leptin + wort=0.37±0.006; P<0.02 compared with leptin + vehicle). VSMCs from lepr<sup>ob/ob</sup> mice also responded to leptin with an increase in proliferation compared with vehicle control (leptin=0.67±0.01 versus vehicle=0.49±0.02; P<0.00001), and this effect was blocked with wortmannin (leptin + wort=0.53±0.001, P<0.00003). VSMCs from lepr<sup>db/db</sup> mice did not respond to leptin treatment.

**Discussion**

Leptin is a 16-kDa peptide hormone with structural features similar to those of the long-chain helical cytokine family. The expression of receptors for leptin are ubiquitous, indicating that leptin may affect multiple physiological processes. To determine the importance of leptin and the leptin receptor on neointimal hyperplasia, we performed femoral wire injury on wt, lepr<sup>s/s</sup> and lepr<sup>db/db</sup> mice. Consistent with previous studies, lepr<sup>ob/ob</sup> and lepr<sup>db/db</sup> mice were protected from neointimal formation. Similar to the findings of Schafer et al, who used an iron chloride model of carotid injury, we have shown that leptin treatment (recombinant murine leptin or ad-leptin) increases neointima in the leptin-deficient mice but not in mice lacking the leptin receptor, confirming a causal role for leptin and the leptin receptor in neointimal formation. In addition, cell proliferation analyses revealed that lepr<sup>ob/ob</sup> and lepr<sup>db/db</sup> mice had significantly less cellular proliferation in the femoral arterial wall after injury compared with wt mice. This suggests that leptin may augment VSMC proliferation after arterial injury. Previous studies have demonstrated leptin receptor expression by VSMCs after arterial injury, supporting a direct effect of leptin on vascular smooth muscle cells.

In addition to a potential role of the VSMCs, we sought to directly examine the importance of bone marrow–derived leptin receptor in leptin-mediated effects on neointimal hyperplasia. For these studies, we performed femoral wire injury after BMT from lepr<sup>db/db</sup> to wt and from wt to lepr<sup>db/db</sup> mice. Interestingly, the recipient phenotype was unchanged in both of these experiments compared with control groups receiving marrow from genetically identical donors. This suggests that the in vivo effect of the platelet and leukocyte leptin receptor is negligible toward the response to vascular injury.

Similar to other cytokine receptors, the leptin receptor signals by activating a Jak family tyrosine kinase (Jak2) that autophosphorylates numerous tyrosine residues and phosphorylates tyrosine residues (Tyr985 and Tyr1138) on the leptin receptor gene has been substituted by a mutant leptin receptor (lepr<sup>S1138</sup>) containing a Tyr1138-Ser substitution that is defective in STAT3 binding. These mice develop obesity similar to that observed in lepr<sup>db/db</sup> mice, although other phenotypes associated with leptin receptor deficiency are less affected indicating the relevance of other signaling pathways such as the IRS-protein-PI3 kinase pathway. The pathways responsible for the vascular effects of leptin are unknown, although both STAT3 and PI3K lepr signaling pathways have been implicated in VSMC proliferation in vitro. To dissect these pathways in vivo, lepr<sup>ob/ob</sup> mice with defective STAT3 signaling were studied in the femoral arterial injury model. These mice developed an obese

**Figure 4.** Comparison of mean arterial pressure between wt, lepr<sup>ob/ob</sup>, and lepr<sup>db/db</sup> mice. Note the diurnal variation of the mean arterial pressure during the light/dark cycle. Values represent mean of 4 to 5 mice for each genotype (wt: n=5; lepr<sup>db/db</sup>: n=4; lepr<sup>ob/ob</sup>: n=4). Two-way analysis of variance results in significant effects of time (diurnal variation) and genotype. Post-hoc analysis reveals that wild-type mice have significantly elevated mean arterial pressure compared with both lepr<sup>ob/ob</sup> and lepr<sup>db/db</sup> mice (P<0.05).
phenotype similar to that observed in the \textit{leprd/db} group, as previously described,\textsuperscript{16} but they exhibited significantly greater intimal hyperplasia after arterial injury, suggesting that leptin receptor–mediated STAT3 signaling is not responsible for the leptin effect on neointimal formation after injury. The robust effects of exogenous leptin in these experiments could be partially mediated by effects on arterial blood pressure. Leptin has been shown to influence sympathetic activation with elevations in blood pressure.\textsuperscript{25-28,29} Furthermore, this effect on sympathetic activity appears to be centrally mediated and may not be subject to the same degree of leptin resistance as observed with leptin-receptor related feeding behavior.\textsuperscript{30} To examine whether the in vivo effects observed in the \textit{lepr}\textsuperscript{ob} mice were mediated by elevations in blood pressure, we performed radiotelemetry arterial blood pressure recordings. Consistent with previous publications we observed reduced blood pressure in \textit{lepr}\textsuperscript{ob} mice.\textsuperscript{24} In addition, the blood pressure of the \textit{lepr}\textsuperscript{ob} mice was also lower than wt and was not different than \textit{lepr}\textsuperscript{db/db} mice. Therefore, although high doses of exogenous leptin probably elevated blood pressure at some time points in our exogenous leptin experiments, we have demonstrated that elevations in blood pressure are not mediating the increased neointimal response of the \textit{lepr}\textsuperscript{ob} mice as compared with the \textit{lepr}\textsuperscript{db/db} mice.

To further examine the potential underlying mechanism of the increased neointimal response of the \textit{lepr}\textsuperscript{ob} compared with the \textit{lepr}\textsuperscript{db/db}, we used a cell culture system to examine VSMC proliferation. Leptin has previously been shown to stimulate proliferation of VSMCs in culture\textsuperscript{6} and our in vivo studies demonstrated reduced intimal VSMC proliferation in leptin deficient and leptin receptor deficient mice. Therefore, we examined whether the \textit{lepr}\textsuperscript{db/db} and \textit{lepr}\textsuperscript{ob} mice responded differently to recombinant leptin in vitro. VSMCs isolated and cultured from \textit{lepr}\textsuperscript{db/db} did not respond to leptin treatment whereas leptin significantly stimulated the proliferation of VSMCs isolated from \textit{lepr}\textsuperscript{ob} mice, consistent with the in vivo phenotype. Furthermore, this leptin effect on \textit{lepr}\textsuperscript{ob} VSMC’s was completely blocked after incubation of the VSMCs with a PI3 kinase inhibitor, implicating this pathway in leptin receptor-mediated VSMC proliferation.

Limitations of this study include the supraphysiologic plasma concentrations of leptin obtained in the leptin replacement experiments. These experiments serve as proof of principle that leptin affects neointimal hyperplasia. The increased neointimal hyperplasia observed in the \textit{lepr}\textsuperscript{ob} mice compared with the \textit{lepr}\textsuperscript{db/db} mice, where no exogenous leptin was added, demonstrates the potential of physiological elevations of leptin, as observed in obese states, to affect this vascular end point. An additional limitation of this study is lack of definitive proof of the relevant leptin receptor cellular pool. Although we have demonstrated that leptin effects on neointimal hyperplasia are at least partially independent of Lepr-STAT3 signaling, not dependent on blood pressure elevation, and independent of bone marrow–derived elements, it remains possible that other Lepr-mediated central nervous system pathways could influence vascular disease.

In conclusion, these data suggest that leptin enhances neointimal formation through an interaction with the leptin receptor resulting in smooth muscle cell proliferation. These effects are mediated by a leptin receptor signaling pathway that is distinct from the major pathway involved in energy balance. These findings suggest that it may be possible to design therapeutic agents capable of promoting the beneficial metabolic activities of leptin while avoiding the potential hazard of vascular complications.

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Disclosures
None.

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