Leptin-enhanced neointimal hyperplasia is reduced by mTOR and PI3K inhibitors

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Despite the use of the sirolimus (rapamycin) drug-eluting coronary stent, diabetics are at increased risk of developing in-stent restenosis for unclear reasons. Hyperleptinemia, which often coexists with diabetes and metabolic syndrome, is an independent risk factor for progression of coronary artery disease. It has not been determined whether elevated circulating leptin decreases the efficacy of the sirolimus drug-eluting stent in inhibiting neointimal hyperplasia, the process underlying restenosis after stenting. Here we show that leptin activates the mammalian target of rapamycin (mTOR) signaling pathway in primary murine vascular smooth muscle cells (VSMC) and stimulates VSMC proliferation in a PI3K-dependent fashion. Exogenous leptin, administered at levels comparable to those found in obese humans, promotes neointimal VSMC hyperplasia in a murine femoral artery wire injury model. Leptin significantly increases the dose of the mTOR inhibitor sirolimus that is required for effective inhibition of neointimal formation. Combination therapy with LY294002, a PI3K inhibitor, and sirolimus effectively inhibits leptin-enhanced neointimal hyperplasia. These data show that, in the setting of hyperleptinemia, higher doses of an mTOR inhibitor, or combination therapy with mTOR and PI3K inhibitors, inhibits neointimal hyperplasia after arterial injury. These studies may explain the higher rates of restenosis observed in diabetics treated with a sirolimus-eluting coronary stent and suggest a potential novel therapeutic approach for inhibiting in-stent restenosis in such patients.

Coronary stenting is a widely used treatment for coronary artery disease. Restenosis after coronary stenting occurs primarily through a process of neointimal hyperplasia involving vascular smooth muscle cells (VSMC) (1). By inhibiting the proliferation and migration of VSMC (2), the sirolimus (rapamycin)-eluting coronary stent has markedly decreased the rates of restenosis and repeat revascularizations in randomized clinical trials (3). Nevertheless, despite the use of the sirolimus-eluting stent, diabetic patients remain at increased risk of developing in-stent restenosis (4, 5).

Elevated circulating leptin is a risk factor for stroke and progression of coronary artery disease, independent of body mass index, lipids, glucose, and inflammatory markers (6–9). Leptin can promote processes involved in atherogenesis, including platelet aggregation, inflammation, endothelial dysfunction, and VSMC proliferation and migration (10).

In mammals, the hormone leptin is physiologically secreted in response to increased body fat mass and acts at the hypothalamus to decrease appetite and increase energy expenditure (11). Absence of leptin in ob mutant mice or resistance to leptin’s effects in db mutant mice (by loss of the receptor isoforms that have intact cytoplasmic signaling domains) leads to obesity and hyperglycemia (11). Cases of leptin or leptin receptor deficiency are rare in humans (12). In humans, obesity, particularly abdominal obesity, is associated with high circulating leptin levels, which may be explained by hypothalamic leptin resistance (13, 14). Exogenous leptin or a high-fat diet, which up-regulates leptin, promotes neointimal formation in a murine arterial injury model (15, 16), whereas leptin signaling-defective db mice are resistant to neointimal formation (15–17). Thus, we hypothesized that hyperleptinemia contributes to the increased in-stent restenosis and reduced efficacy of the sirolimus-eluting stent observed in diabetic patients (18, 19).

Leptin activates multiple signaling molecules including mammalian target of rapamycin (mTOR), PI3K, and MAPK. Activation of the MAPK pathway by stimulation of cytokine or leptin receptors has been observed in the hypothalamus and peripheral tissues (20). Activation of PI3K is necessary for serum- and leptin-mediated migration of VSMC in vitro (21, 22). The PI3K family is an emerging drug target in cancer, where mutations in the PI3K family is an emerging drug target in cancer, where mutations in the PI3K, the phosphatase and tensin homologue (PTEN) tumor suppressor, or Akt lead to gain-of-PI3K function (23). Combined mTOR and PI3K inhibition have been shown to be synergistic against some cancers in in vitro and in vivo models (24, 25). On this basis, we tested the hypothesis that up-regulation of leptin, as occurs in diabetes and metabolic syndrome, could antagonize sirolimus-dependent inhibition of VSMC proliferation and migration by activating PI3K pathways.

Results

Leptin Stimulates VSMC Proliferation and Migration. We first assessed the effect of leptin on the proliferation and migration of early-passage murine aortic primary VSMC. C57BL/6J VSMC were serum-starved and subsequently treated with leptin at increasing concentrations 0.06, 0.6, or 6 ng/mL (1, 10, and 100 ng/mL) for 72 h (Fig. 1A). As observed previously for human VSMC (15, 16, 26), leptin increased murine VSMC proliferation in WT and leptin-deficient (ob/or) cells but not in leptin-receptor-defective cells (db/db) in a dose-dependent fashion (Fig. 1 A and B). Serum stimulation, in contrast, increased VSMC proliferation equivalently in ob/or- and db/db-derived cells (Fig. 1B). Leptin [6 ng/mL, a level found in obese humans (13)] enhanced PDGF-induced migration of WT but not db/db VSMC (Fig. 1C). Thus, leptin stimulates murine VSMC proliferation and migration in vitro via the leptin receptor.

Leptin Activates the mTOR, MAPK and PI3K Pathways in VSMC. Leptin signaling in the mammalian hypothalamus occurs through activation of the mTOR pathway (27), but it has not been determined whether leptin regulates mTOR in VSMC. In primary murine VSMC, leptin (6 ng/mL) stimulated the phosphorylation of
S6K (Fig. 2A) and Ser-473 of Akt (Fig. 2B). Leptin (0.6 nM), a physiological level in lean humans (13), stimulated S6K phosphorylation, albeit less robustly (data not shown). Leptin stimulated the Thr-308 phosphorylation of Akt (23) (Fig. 2A) and the phosphorylation of MAPK (20) (Fig. 2B) and the phosphorylation of MAPK (20) (Fig. 2C). Protein levels of S6K, Akt, and MAPK did not change significantly (Fig. 2). Thus, in vitro leptin can activate the mTOR, MAPK, and PI3K signaling pathways in murine VSMCs.

Leptin Stimulates PI3K-Dependent VSMC Proliferation. To determine whether MAPK and PI3K activity is important for leptin-induced proliferation of murine VSMC, we stimulated serum-starved primary VSMC with 6 nM leptin in the absence or presence of pharmacological inhibitors of MAPK kinases (U0126) (28) and PI3K (LY294002) (29) or overexpression of PTEN. Addition of 10 μM U0126 inhibits VSMC proliferation induced by the adipokine resistin (30). U0126 (10 μM) prevented leptin-stimulated phosphorylation of MAPK (Fig. 3A Inset) but did not significantly alter leptin-induced proliferation (Fig. 3A). However, LY294002 (10 μM), which prevents neointimal hyperplasia in cultured porcine coronary arteries (22), inhibited leptin-stimulated VSMC proliferation (Fig. 3A). To further explore the role of the PI3K pathway in leptin-induced proliferation, primary VSMCs were infected with adenoviral recombinant adenovirus expressing human PTEN (31). Overexpression of PTEN (Fig. 3B Inset) inhibited leptin-induced proliferation significantly at the higher MOI compared with the control virus (Fig. 3B). These results suggest that leptin-induced proliferation of murine VSMC requires PI3K activation.

Leptin Enhances Neointimal Hyperplasia After Arterial Injury and Reduces the Efficacy of Sirolimus-Dependent Growth Inhibition. We confirmed that leptin increases neointimal hyperplasia in a murine arterial injury model (15, 16). In the femoral artery wire injury model, neointimal hyperplasia is normally observed within 1 week after injury and peaks at 2 weeks (32). In our experiments, WT C57BL/6J mice on a normal diet were randomized to receive a sham operation (n = 8), wire injury followed by vehicle treatment for 14 days (n = 9), or wire injury followed by treatment with murine recombinant leptin (0.4

Fig. 1. Leptin stimulates murine VSMC proliferation and migration. VSMC proliferation was assessed by using an MTS-based assay in serum-starved primary aortic VSMC from WT (A), ob/ob (B), or db/db (B) mice, which were stimulated with leptin or serum as indicated. (C) VSMC migration was assessed by using a modified Boyden’s chamber assay. Primary WT and db/db VSMC were stimulated with vehicle or 6 nM leptin, and migration to PDGF-BB was determined by subtraction of migration in the absence of PDGF-BB; data not shown. Triplicate experiments were quantitated, and control is vehicle-treated. *, P < 0.05; #, P < 0.001 compared with control (Dunnett’s test in A and B and Student’s t test in C). Bar shading indicates the genotypes.

Fig. 2. Leptin activates mTOR and MAPK in murine VSMC. Serum-starved WT murine VSMCs were stimulated with 6 nM leptin and subjected to immunoblotting. (A) Phosphorylated S6K (S6K-P, Top) and total S6K (Middle). (B) 473-phosphorylated Akt (Akt-P, Top), T308-phosphorylated Akt (Akt-P, Upper Middle), and total Akt (Lower Middle). (C) Phosphorylated MAPK (p44-P and p42-P, Top) and total MAPK (p44 and p42, Middle). Bar graphs represent the protein levels determined by densitometry of phosphorylation (normalized to total protein and untreated control) from 3 independent experiments. *, P < 0.05 compared with control.

Fig. 3. Leptin stimulates VSMC proliferation in a PI3K-dependent fashion. VSMC proliferation was assessed by using an MTS-based assay in cells treated with LY294002 or U0126 (A) or infected with adenoviruses (B). Adenoviruses (Ad or Ad-PTEN) were infected at an MOI of 30 or 100 as indicated. Triplicate experiments were quantitated; PBS-treated cells were used as control. *, P < 0.001 compared with the control; #, P < 0.001 compared with leptin alone; #, P < 0.001 compared with leptin plus Ad. Representative Western blots show phosphorylated MAPK (p44-P and p42-P, A Inset Upper) and total MAPK (p44 and p42, A Inset Lower) after treatment with U0126 or vehicle for 10 min and PTEN (B Inset Upper) and cdk2 (B Inset Lower) expression after infection with the indicated adenoviruses.
To investigate the possibility that leptin may oppose the inhibitory effect of sirolimus on neointimal hyperplasia, we tested the combined effect of leptin and increasing concentrations of sirolimus on neointimal formation in the murine femoral artery injury model. Mice were randomized to treatment with 1 mg/kg sirolimus (n = 8); 4 mg/kg sirolimus (n = 8); 9 mg/kg sirolimus (n = 7); 0.4 mg/kg leptin and 1 mg/kg sirolimus (n = 8); 0.4 mg/kg leptin and 4 mg/kg sirolimus (n = 8); or 0.4 mg/kg leptin and 9 mg/kg sirolimus (n = 7), each daily for 14 days. Sirolimus administered i.p. at doses of 1, 4, and 9 mg/kg per day has been shown to inhibit VSMC migration from murine aortic explants (34). In the absence of leptin, sirolimus significantly decreased neointimal formation (Fig. 4B and Table 1) by 2-fold at the lowest dose (1 mg/kg per day) compared with vehicle, 5-fold at the intermediate dose (4 mg/kg per day), and 6-fold at the highest dose (9 mg/kg per day). Leptin significantly increased neointimal formation despite 1 or 4 mg/kg sirolimus per day (Fig. 4B). At the highest dose (9 mg/kg per day) of sirolimus complete inhibition of leptin-enhanced neointimal formation was observed (Fig. 4B and Table 1). Our results suggest that leptin increases the sirolimus dose necessary to inhibit neointimal hyperplasia, both by increasing the degree of hyperplasia caused by arterial injury and by reducing the inhibitory effects of sirolimus on neointimal formation.

Cooperative Inhibition of Leptin-Induced Neointimal Hyperplasia by an mTOR and PI3K Inhibitor. Next, we examined the ability of the PI3K inhibitor LY294002 alone or in combination with 4 mg/kg sirolimus (a dose that is partially effective in the presence of leptin) to inhibit leptin-enhanced neointimal formation after femoral artery injury. LY294002 (1.2 mg/kg per day) reached an effective circulating level by 7 days of daily administration, as indicated by the inhibition of >80% of the basal Thr-308 phosphorylation of Akt (23) in descending aortas (Fig. 4C). Mice were randomized to treatment with leptin (0.4 mg/kg) and vehicle (n = 7); leptin (0.4 mg/kg), LY294002 (1.2 mg/kg), and vehicle (n = 7); or leptin (0.4 mg/kg), LY294002 (1.2 mg/kg), and sirolimus (4 mg/kg, n = 8) daily for 14 days. Leptin-enhanced neointimal formation was partially inhibited by either LY294002 (Fig. 4D and Table 1) or sirolimus (Fig. 4B and Table 1). However, a combination of LY294002 (1.2 mg/kg per day) and sirolimus (4 mg/kg per day) achieved nearly complete inhibition of neointimal formation (Fig. 4D and Table 1). These results suggest that combined therapy with PI3K and mTOR inhibitors suppresses leptin-enhanced neointimal hyperplasia after arterial injury in this murine model. LY294002 and sirolimus can cause decreased white blood cell counts. Peripheral blood cell counts showed that treatment with a combination of LY294002 (1.2 mg/kg) and sirolimus (4 mg/kg) decreased the absolute neutrophil count without significantly affecting the total white blood cell, lymphocyte, or platelet counts [supporting information (SI) Fig. S1].

Discussion

The present study demonstrates that leptin stimulates neointimal hyperplasia after vascular injury in mice and decreases the efficacy of sirolimus-dependent inhibition of neointimal hyperplasia. These findings may explain why some diabetic patients are relatively resistant to sirolimus as they exhibit higher in-stent restenosis rates than nondiabetic patients (4, 35). Leptin levels are elevated in the presence of obesity, and circulating levels are proportional to adiposity, consistent with the premise that obesity causes leptin resistance in peripheral tissues and the brain (36). This may be due, in part, to altered signaling within the leptin signaling pathways, such that downstream targets are already activated. Although leptin increases neointimal hyperplasia in nonobese mice, it is not clear whether our findings can
be generalized to obese animals, in which peripheral tissues may be partially resistant to leptin.

Leptin stimulated both murine VSMC migration and proliferation, although to a lesser extent than serum. Reduction of neointimal hyperplasia by sirolimus and LY294002 suggested that they inhibited VSMC migration (from the media to the intima) and/or proliferation. A contributing factor could be the antiinflammatory activity of combined therapy with sirolimus and LY294002 because the number of neutrophils in peripheral blood was decreased.

Leptin can activate multiple receptor-mediated signaling pathways, some of which regulate energy balance (20), but the pathways responsible for leptin’s vascular effects are relatively unknown. STAT3 plays a role in diurnal blood pressure regulation in mice, possibly via leptin-mediated sympathetic stimulation (37), but is not required for neointimal hyperplasia after arterial injury (16). PI3K has been implicated in VSMC migration and proliferation in vitro (21, 38), and overexpression of PTEN inhibits neointimal hyperplasia and macrophage accumulation after arterial injury (39), suggesting that PI3K activity plays a role in neointimal hyperplasia after vascular injury. In the present study we administered leptin to achieve levels that have been observed in obese individuals. In the presence of elevated leptin, the sirolimus dose required to effectively inhibit neointimal hyperplasia was increased likely because leptin activates a sirolimus-insensitive growth pathway mediated by PI3K that promotes neointimal hyperplasia.

Our data suggest that, as is the case in the treatment of cancer (40, 41), in-stent restenosis may be more effectively treated in individuals with high circulating leptin levels by therapy with higher doses of sirolimus or combinatorial therapy targeting both mTOR and PI3K. Of the 3 classes of mammalian PI3K, the class 1A PI3K has been identified as the most important for cellular growth and survival (23). Growth factors including insulin activate mTOR through class 1A PI3K; however, nutrients lead to mTOR activation through signals from a class 3 PI3K (hVPS34) (42, 43). PI3K inhibition may have been synergistic with sirolimus in terms of mTOR inhibition, but neither may be mTOR-independent pathway(s) inhibited by PI3K inhibition that are responsible, at least in part, for the leptin-induced neointimal hyperplasia.

Our data suggest that high leptin levels such as those observed in obese individuals and some diabetic patients may be deleterious by promoting enhanced neointimal hyperplasia after vascular injury. Higher sirolimus concentrations or combination therapy that includes an mTOR and PI3K inhibitor on a drug-eluting stent might be more efficacious in preventing in-stent restenosis in diabetic patients than the current sirolimus-eluting stent.

**Limitations**

Type 2 diabetes, often part of the metabolic syndrome, is commonly associated with vascular complications, including atherosclerosis and postangioplasty restenosis. The murine model of exogenous leptin administration used in the present study creates a hyperleptinemic state that recapitulates the increased neointimal hyperplasia and much of the altered metabolism of mice on a high-fat diet without the increase in body fat mass (15). A caveat is that this animal model does not cause diabetes (15); instead leptin promotes improved glucose metabolism in mice, rescuing the hyperglycemia and insulin resistance in leptin-deficient ob mice (16).

Circulating levels of sirolimus after similar i.p. administration are not known (44). The sirolimus doses that we used were based on previous studies showing that they were effective doses to inhibit neointimal hyperplasia (34, 45). The fact that these doses were less effective in conditions of hyperleptinemia showed that relative resistance to rapamycin could occur. However, local sirolimus concentrations achieved by stent-based delivery must be considerably higher than those achieved by systemic administration. The present study, having examined the effect of LY294002 only at a single dose, was not intended to characterize the dose response of neointimal hyperplasia to LY294002 but rather to explore the interaction between the PI3K and mTOR pathways. Indeed, higher doses of LY294002 may inhibit neointimal hyperplasia more effectively. As a cautionary note, the long-term effects of using high-dose LY294002 or another PI3K inhibitor in stent-based therapy are not known.

**Materials and Methods**

**Reagents.** Recombinant human leptin was purchased from R & D Systems. Recombinant PDGF-BB was purchased from Sigma–Aldrich. Rapamycin, U0126, and LY294002 were purchased from Calbiochem. Rabbit polyclonal anti-phospho-(Thr202/Tyr204)-p42/p44MAPK, anti-p42/p44MAPK, anti-phospho-(Ser473)-Akt, anti-phospho-(Ser 308)-Akt, anti-Akt, anti-Akt-pSubs, anti-phospho(Thr563)p70S6K, and anti-p70S6K antibodies were purchased from Cell Signaling Technology. Mouse anti-smooth muscle α-actin antibody was purchased from Sigma, PTEN antibody was from Santa Cruz Biotechnology, and biotinylated Mac-2 and CLB993B antibodies were from Cedarlane.

**VSMC Proliferation and Migration Assays.** Primary VSMC were isolated from aortic explants of WT, ob/ob, or db/db mice (46). VSMC proliferation was determined by using the Cell Titer Aqueous One Solution Proliferation Assay (Promega). Migration to PDGF-BB was assessed in a modified Boyden chamber assay (47).

**Western Blotting.** VSMC were plated on 100-mm dishes, serum-starved for 72 h in DMEM containing 0.1% FBS, and stimulated with 0.6 or 10 nM (10 or 100 ng/mL) leptin. Extracts were prepared by brief sonication in HKM buffer (40 mM Hepes-KOH, pH 7.6/7.5 mM MgCl2/0.5 mM DTT) or, in the case of Akt phosphorylation studies, by lysing cells on ice for 30 min in RIPA buffer. Seventy micrograms of protein per sample were subjected to immunoblotting.

**Table 1. Morphometric measurements of cross sections of injured femoral arteries from indicated treatment groups**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Lumen</th>
<th>Media</th>
<th>Intima</th>
<th>TVA</th>
<th>I/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8</td>
<td>0.013 ± 0.006</td>
<td>0.015 ± 0.002</td>
<td>0.001 ± 0.001</td>
<td>0.029 ± 0.006</td>
<td>0.057 ± 0.047</td>
</tr>
<tr>
<td>Injury</td>
<td>9</td>
<td>0.012 ± 0.003</td>
<td>0.016 ± 0.003</td>
<td>0.008 ± 0.001</td>
<td>0.036 ± 0.006</td>
<td>0.608 ± 0.126</td>
</tr>
<tr>
<td>Injury + SRL, 1 mg/kg per day</td>
<td>8</td>
<td>0.012 ± 0.003</td>
<td>0.014 ± 0.001</td>
<td>0.005 ± 0.001</td>
<td>0.029 ± 0.003</td>
<td>0.334 ± 0.043</td>
</tr>
<tr>
<td>Injury + SRL, 4 mg/kg per day</td>
<td>8</td>
<td>0.012 ± 0.005</td>
<td>0.015 ± 0.003</td>
<td>0.001 ± 0.000</td>
<td>0.028 ± 0.006</td>
<td>0.121 ± 0.027</td>
</tr>
<tr>
<td>Injury + SRL, 9 mg/kg per day</td>
<td>8</td>
<td>0.015 ± 0.002</td>
<td>0.015 ± 0.002</td>
<td>0.001 ± 0.000</td>
<td>0.030 ± 0.004</td>
<td>0.094 ± 0.037</td>
</tr>
<tr>
<td>Injury + leptin</td>
<td>10</td>
<td>0.010 ± 0.004</td>
<td>0.015 ± 0.001</td>
<td>0.024 ± 0.004</td>
<td>0.053 ± 0.007</td>
<td>1.883 ± 0.140</td>
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<tr>
<td>Injury + leptin + SRL, 1 mg/kg per day</td>
<td>8</td>
<td>0.010 ± 0.002</td>
<td>0.019 ± 0.001</td>
<td>0.024 ± 0.005</td>
<td>0.056 ± 0.007</td>
<td>1.269 ± 0.254</td>
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<tr>
<td>Injury + leptin + SRL, 4 mg/kg per day</td>
<td>8</td>
<td>0.012 ± 0.004</td>
<td>0.017 ± 0.002</td>
<td>0.009 ± 0.001</td>
<td>0.035 ± 0.006</td>
<td>0.654 ± 0.172</td>
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<tr>
<td>Injury + leptin + SRL, 9 mg/kg per day</td>
<td>7</td>
<td>0.014 ± 0.002</td>
<td>0.017 ± 0.003</td>
<td>0.001 ± 0.000</td>
<td>0.031 ± 0.004</td>
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<tr>
<td>Injury + leptin + LY</td>
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<td>0.013 ± 0.002</td>
<td>0.020 ± 0.003</td>
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<td>0.044 ± 0.006</td>
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<tr>
<td>Injury + leptin + LY + SRL, 4 mg/kg per day</td>
<td>8</td>
<td>0.015 ± 0.002</td>
<td>0.018 ± 0.002</td>
<td>0.001 ± 0.000</td>
<td>0.034 ± 0.003</td>
<td>0.055 ± 0.025</td>
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Data are presented as mean ± SD. SRL, sirolimus; LY, LY 294002; TVA, total vessel area.
by standards protocols. Imaging and densitometry were performed on an Odyssey infrared system (LI-COR).

Adenovirus Infection. Purified recombinant adenovirus AdPTEN and control empty adenovirus were gifts from Chris Kontos (Duke University Medical Center, Durham, NC) (31). Titters were assessed by using the Adeno-X kit (Clontech), and viruses were added to serum-starved VSMC at the indicated MOI 18 h before the addition of leptin or vehicle.

Animal Models and Drug Treatments. All experiments were performed on female mice. WT C57BL6/J-background mice and mice deficient for leptin (C57BL6/J-Lepob/-ob/ob) or defective for the leptin receptor (C57BL6/J-m +/- Lepob/-ob/db/db) were purchased from Jackson Laboratories. Mice were obtained at 6–8 weeks of age and at an average weight of 0.015 kg. Animals were fed standard rodent chow and tap water ad libitum.

All surgical and injection procedures were approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center. Mice were subjected to femoral artery wire injury or sham injury 24 h before starting pharmacological treatments, which consisted of a single daily i.p. injection for 14 days according to 1 of 2 experimental protocols. Protocol I groups, up to 400–µl total volume: sham, injury (plus vehicle for leptin and sirolimus), injury plus leptin (plus vehicle for sirolimus), injury plus sirolimus (1 mg/kg, plus PBS), injury plus sirolimus (4 mg/kg, plus PBS), injury plus sirolimus (9 mg/kg, plus PBS), injury plus leptin plus sirolimus (1 mg/kg), injury plus leptin plus sirolimus (4 mg/kg), injury plus leptin plus sirolimus (9 mg/kg). Protocol II groups, up to 350–µl total volume: sham, injury plus leptin (plus vehicle for sirolimus and LY), injury plus leptin plus LY294002 (plus vehicle for sirolimus), injury plus leptin plus LY294002 plus sirolimus (4 mg/kg). Leptin stock solution was prepared in PBS. Sirolimus (rapamycin) stock solution was prepared in 0.33% DMSO/PBS. Leptin and sirolimus stock solutions were sonicated before use and diluted to their final concentrations in 0.9% saline. For these analyses, 250–µl samples of intracardiac blood were obtained by insertion of a 0.5 M EDTA-rinsed 22-gauge needle through the thoracic wall of mice under isofluorane anesthesia. Samples were stored in K+ -EDTA-treated microtubes (Fisher Scientific).

Femoral Artery Injury and Morphometry. Endoluminal injury of bilateral femoral arteries was performed under general anesthesia as previously described (32). Computer-aided quantitative morphometry was performed (46) with minor modification. Briefly, mice were killed with CO2 asphyxiation and perfused with PBS before en bloc excision of hind limbs. Common femoral arteries at the level of injury were fixed in 10% zinc formalin and embedded in paraffin, equally divided into 4 segments lengthwise, sectioned on 5-µm thickness, and stained with Elastic-Van Gieson (Columbia University Core Histology facility). Photomicrographs were analyzed by using Photoshop (Adobe Systems) and Scion Image software. Sections (3 per mouse) from comparable arterial segments were analyzed by 2 experienced researchers blinded to the treatment groups. The I/M ratio for each section was obtained by dividing the area of the (neo)intima by the area of the media, then I/M ratios for a given treatment group were averaged. In this way, for example, I/M ratio data for injury plus leptin (from Protocol I) represented the analysis of 3 sections per mouse × 7 mice = 21 sections.

Immunohistochernistry. For antigen retrieval, deparaffinized formalin-fixed sections were boiled for 10 min in 10 mM sodium citrate (pH 6). Primary antibody (α-actin at 1:200, Mac-2 at 1:100, or CL8993B at 1:500 dilution) was incubated overnight at 4°C in 1% normal goat serum-PBS. For actin staining, after washes of primary antibody, secondary antibody conjugated with an Alexa Fluor 680 fluorophore was incubated for 1 h at room temperature in the dark as 1/250 dilution in 1% normal goat serum-PBS. For Mac-2 and CL8993B staining, a tertiary system was used in which the primary antibodies were obtained as biotin conjugates, the secondary reagent was a prediluted high-affinity streptavidin conjugated to horse-radish peroxidase (R & D Systems), and the tertiary detection reagent was tyramide conjugated to Alexa Fluor 680 (PerkinElmer). Sections were counterstained with DAPI. Images were acquired on a Carl Zeiss fluorescent microscope, which was equipped with an Apotome module and Axiosview software, under similar settings to ensure equal background signals between slides in adjacent skeletal muscle.

Complete Blood Counts. For these analyses, 250–µl samples of intracardiac blood were obtained by insertion of a 0.5 M EDTA-rinsed 22-gauge needle through the thoracic wall of mice under isofluorane anesthesia. Samples were stored in K+ -EDTA-treated microtubes (Fisher Scientific).

Statistical Methods. Bar graphs with error bar data show mean ± standard deviations. Multiple comparisons were made on GraphPad Prism software by 1-way analysis of variance followed by post hoc analysis of means with Tukey’s HSD test or, in the case of comparison to a control, with Dunnett’s test. P < 0.05 was considered significant.

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References:


Supporting Information

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Fig. S1. Complete blood counts with WBC differential. Mice were subjected to 14 days of i.p. injections with the indicated therapies (leptin and sirolimus at 4 mg/kg per day, LY294002 at 1.2 mg/kg per day), and intracardiac blood samples were prepared in K⁺-EDTA for analysis. Triplicate experiments were quantified. Negative controls are vehicle-treated. *, P < 0.05 compared with vehicle; #, P < 0.05 compared with leptin. (A) WBC and absolute lymphocyte (ALC) counts. (B) Absolute neutrophil counts (ANC). (C) Platelet counts.