Vascular smooth muscle-specific deletion of the leptin receptor attenuates leptin-induced alterations in vascular relaxation

Michael J. Ryan, T. Taylor Coleman, Jennifer M. Sasser, Katarina M. Pittman, Michael W. Hankins, and David E. Stec

1Department of Physiology and Biophysics, Mississippi Center for Obesity Research, Cardiovascular-Renal Research Center, Jackson, Mississippi; and 2Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi

Submitted 20 July 2015; accepted in final form 23 February 2016

Ryan MJ, Coleman TT, Sasser JM, Pittman KM, Hankins MW, Stec DE. Vascular smooth muscle-specific deletion of the leptin receptor attenuates leptin-induced alterations in vascular relaxation. Am J Physiol Regul Integr Comp Physiol 310: R960–R967, 2016. First published March 2, 2016; doi:10.1152/ajpregu.00336.2015.—Obesity is a risk factor for cardiovascular disease and is associated with increased plasma levels of the adipose-derived hormone leptin. Vascular smooth muscle cells (VSMC) express leptin receptors (LepR); however, their physiological role is unclear. We hypothesized that leptin, at levels to mimic morbid obesity, impairs vascular relaxation. To test this, we used control and VSM-LepR knockout mice (VSM-LepR KO) created with a tamoxifen-inducible specific Cre recombinase to delete the LepR gene in VSMC. Control (10–12 wk old) and VSM-LepR KO (10–12 wk old) mice were fed a diet containing tamoxifen (50 mg/kg) for 6 wk, after which vascular reactivity was studied in isolated carotid arteries using an organ chamber bath. Vessels were incubated with leptin (100 ng/ml) or vehicle (0.1 mM Tris·HCl) for 30 min. Leptin treatment resulted in significant impairment of vessel relaxation to the endothelial-specific agonist acetylcholine (ACh). When these experiments were repeated in the presence of the superoxide scavenger tempol, relaxation responses to ACh were restored. VSM-LepR deletion resulted in a significant attenuation of leptin-mediated impaired ACh-induced relaxation. These data show that leptin directly impairs vascular relaxation via a VSM-LepR-mediated mechanism, suggesting a potential pathogenic role for leptin to increase cardiovascular risk during obesity.

Obesity is a significant risk factor for the development of cardiovascular disease, as it often appears clinically with other risk factors such as diabetes, hypertension, and hyperlipidemia, giving rise to the metabolic syndrome. Another common cardiovascular risk factor associated with obesity is impaired vascular relaxation. Studies in animal models of obesity have documented that obesity is associated with impaired vascular relaxation (13, 24, 29, 39, 42). Similar observations have also been made in human patient populations (5, 58). Although impaired vascular relaxation has been widely documented in obesity, the underlying cause responsible for this phenotype has yet to be established.

One potential candidate for mediating the altered vascular relaxation in obesity is leptin. Leptin is a hormone produced from white adipocytes that acts on its receptors in the hypothalamus to control food intake and energy expenditure. Mutations in leptin and its receptor cause genetic obesity in rodents and humans (30, 40, 46, 62, 64). Although leptin receptors are highly expressed in the brain and nervous system, they are also present in the periphery including the vasculature. Leptin receptors are expressed in both endothelial and vascular smooth muscle (VSM) cells (54). Interestingly, in obesity, plasma leptin levels are very high, suggesting a central resistance to the effects of leptin on appetite and energy expenditure; however, the peripheral actions are believed to remain intact (15, 35).

Leptin has been reported to have several conflicting effects on the vasculature. Leptin has been found to both promote and inhibit VSM cell growth and proliferation (8, 22, 32, 44, 51, 56, 63). Studies in both animal models as well as human patient populations have linked leptin to the development and progression of atherosclerosis (7, 36, 50, 57). Leptin also has confounding effects on vascular resistance. It can promote the release of the potent vasoconstrictor, endothelin, and increase the expression of its receptors in both endothelial and VSM cells (12, 23, 48). Leptin also increases the expression of renin-angiotensin system components (angiotensinogen, angiotensin receptors), and it is well known that renin-angiotensin activation has profound vascular effects (63). However, leptin also increases the levels and activity of both endothelial and neuronal nitric oxide (NO) synthase (NOS) to increase NO levels, which is a major vasodilator (4, 31, 51, 53, 59). Leptin signals via the long form of the leptin receptor through activation of signal transducer and activator of transcription 3, Shp2, and phosphatidylinositol 3-kinase 3-kinase (PI3K)/Akt pathways and acutely increases NO levels through activation of the PI3K/Akt pathway (20, 59). However, prolonged exposure to leptin has been reported to attenuate NO production and phosphorylation of NOS3 through upregulation of suppressor of cytokine signaling-3 expression (6).

Although these diverse actions of leptin in the vasculature are established in the literature, the specific effect of leptin in VSM cells has yet to be elucidated. We utilized a Cre-loxP-based approach to effectively knock out leptin receptors from VSM cells to determine the effect of VSM cell leptin receptors on vascular reactivity. We utilized a mouse model in which exon 1 of the leptin receptor gene was flanked by loxP sites to create a model with loss of all leptin receptor splice variants in VSM cells in vivo (14, 27). We bred these floxed leptin receptor mice with a tamoxifen-inducible Cre recombinase expressed specifically in VSM cells to test the hypothesis that VSM cell leptin receptors mediate the alterations in vascular...
relaxation in response to high leptin levels typically observed in obesity.

METHODS

*Animals.* All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Experiments were performed on male and female mice between 10 and 15 wk of age. Breeding pairs of floxed leptin receptor mice (LepRflx) mice were obtained from Dr. Jeffrey M. Friedman, Rockefeller University, and offspring were maintained on a C57BL/6 background as previously described (21). These mice contain loxP sites, which flank exon 1 of the leptin receptor gene (14). Because this exon contains the signal sequence, Cre-mediated deletion of this exon results in the loss of all leptin receptor splice variants (14). VSM-specific inducible Cre mice (SM22-MerCreMer) were as originally described (27). All mice were fed a standard diet containing 0.29% NaCl and provided water ad libitum. Tamoxifen was administered via a specially prepared diet, which was identical in composition to the standard diet but contained tamoxifen at a concentration of 50 mg/kg (Harlan Laboratories, Madison, WI). Mice were fed the tamoxifen diet for a period of 6 wk, after which time they were switched back to the standard diet. Experiments were then performed 2 wk after switching back to the standard diet.

**Blood pressure.** Blood pressure was measured in mice by radiotelemetry. Mouse radiotelemetry transmitters (TA11PA-C10; Data Sciences International, Minneapolis, MN) were implanted into the carotid artery of mice as previously detailed (10). Mice were allowed 10 days to fully recover from surgery. After this time, blood pressures were measured for 10 s every 15 min for 7 days. Mice were then euthanized and tissues collected. Blood pressure data were analyzed using Dataquest ART version 3.1 software (Data Sciences International). Mean arterial blood pressure (MAP) was separated into daytime and nighttime values, and then two readings were averaged to get a daily MAP. MAP over each of the 7 days of recording was then averaged to get an individual measurement for each mouse as well as reported daytime and nighttime averages.

**Vascular ring preparation.** Mouse carotid arteries were removed and prepared for vessel reactivity studies as previously published (52). Carotid arteries were incubated in Krebs buffer (in mmol/l: pH 7.4, 118.3 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25.0 NaHCO\(_3\), and 11.0 glucose) saturated with 95% O\(_2\)-5% CO\(_2\). Resting tension was adjusted stepwise to reach a final tension of 0.25 g. For each animal, at least two vessel segments were studied with the averaged response equal to an \(n\) of 1. Concentration-dependent relaxation (10\(^{-8}\)-10\(^{-4}\) mol/l) to acetylcholine (ACH) and sodium nitroprusside (SNP) were assessed in vessel segments precontracted with the thromboxane A2 mimetic U46619 (0.4 µg/ml). A subset of vessels was incubated with leptin (100 ng/ml) or vehicle (Tris·HCl 0.1 mM) for 30 min before and during the concentration response curve to ACh. These studies were conducted in the presence or absence of Tempol (1 mM) to test the role of reactive oxygen species.

**PCR of leptin receptor deletion product.** PCR was performed on genomic DNA isolated from the aorta, brain vessels, carotid artery, coronary arteries, kidney arteries, and mesenteric arteries using the Direct PCR kit (Vigten Biotech, Los Angeles, CA) according to manufacturer’s guidelines. Vessels from the brain, heart, and kidney were prepared by passing the tissue through a 75-µm sieve and collecting the vascular tissue retained. Individual mesenteric arteries were cleared of all surrounding adipose tissue and collected by microdissection. PCR for the leptin deletion product was performed using the following primers: LepRprimer1-5’-GTCACCTAGGTATTATGTATTC-3’ and LepRprimer3-5’-GCAATTCATATCAAAACGCC-3’. PCR was performed using the following protocol: 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 35 cycles. PCR products were visualized on 2% agarose gels.

**Real-time PCR.** Real-time PCR was performed on RNA isolated from aortas of wild-type and KO mice. RNA was isolated from aortas using Nucleo Spin RNA kit (Macherey-Nagel, Durew, Germany) according to manufacturer’s guidelines. cDNA was derived from 500 ng of the purified total RNA using the iScript RT kit (Bio-Rad, Hercules, CA). Real-time PCR was performed on 1 µl of the cDNA using iTaq University SYBR-Green Kit (Bio-Rad). PCR was performed using the following protocol: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles. The difference in the threshold cycle (Ct) for each sample was compared with the Ct for 18S rRNA to yield the delta Ct. The results are expressed as the fold difference for each target gene compared with the level in wild-type mice. The PCR primers used for real-time PCR were as follows: LEPRF+, 5’-CTATGTGTGGTTTGTATA-CCTG; LEPR, 5’-AGTGAGAGAGAAGGAGTGAT; Nox1 forward+, 5’-ATGGCCAGAGAGTACGATGAGAT; Nox1 reverse+, 5’-GATGGAAGCCAAAGGGAGTG; Nox2 forward+, 5’-CCCTTTGTTGTA-CGAGGATTAAGACGT; Nox2 reverse, 5’-CGGCTGCATAGTCACCTGAGAG; SOD1 forward, 5’-CCGTTCTCTGTGTCTGCTTCTC; SOD1 reverse, 5’-CGAAAGTGATGGTTCTCCTG; SOD2 forward, 5’-TAAACGGC-AGCATGAGCA; SOD2 reverse, 5’-GGTGGGCGGTGGAGATT-GTTC; SOD3 forward, 5’-CAGAAACGCAATGCGCTTG; SOD3 reverse, 5’-CTGAGTGCTGGTTAAGTGCTTGTG; 18S forward, 5’-TTAGTCTTGTCCCTTGTGACAC; 18S-5’-GATCCAGGAGGCGTCTCATTACAC.

**Western blot.** Total abundance of NOS3 and phosphorylation of NOS3 at Ser 1177 were determined by Western blot. The thoracic aorta was homogenized in 250 µl of lysis buffer, centrifuged at 10,000 g for 10 min at 4°C, and the homogenate was then mixed with loading buffer and heated to 85°C for 5 min. Rat kidney inner medulla was used as a positive control. Homogenized sample (22.5 µl) was separated by electrophoresis (200 V, 40 min, 4–15% TGX Stain-Free Precast gel, Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad Trans-Blot Turbo, High MW program). The membrane was then blocked for 1 h and cut at the 75-kDa marker. The top half was incubated with the rabbit polyclonal phosphorylated NOS3-Ser1177 antibody (Cell Signaling Technology, Beverly, MA) overnight at 4°C, and the lower half was incubated with β-actin (Sigma, St. Louis, MO). Membranes were then incubated with the secondary antibodies (rabbit 1:1,000 for p-NOS3 and mouse 1:30,000 for actin, Bio-Rad) for 1 h at room temperature and then developed with enhanced chemiluminescent reagents (Thermo Scientific, Rockford, IL). The top half of the membrane was stripped (ReBlot Plus Mild; Millipore, Billerica, MA) and incubated overnight with a primary antibody against NOS3 (1:250; BD Transduction, San Jose, CA) before incubation with the mouse secondary antibody (mouse, 1:1,000). Bands were then quantified using the VersaDoc Imaging System and Image Lab 3.0 Software (Bio-Rad). NOS3 phosphorylation was calculated as the integrated optical density (IOD) of the phospho-NOS3 band to the IOD of the NOS3 band, and NOS3 abundance was calculated as the ratio of the IOD of NOS3 to the IOD of β-actin.

**Statistics.** All data are presented as means ± SE. Differences between treatment groups were determined using an unpaired t-test or a one-way ANOVA with a post hoc test (Dunnett’s). \(P < 0.05\) was considered to be significant. All analyzes were performed with SigmaStat (Systat Software, Richmond, CA).

**RESULTS**

Tamoxifen-mediated deletion of leptin receptors in vascular smooth muscle cells. Detection of the deleted leptin receptor was performed using a specific PCR-based assay, which specifically amplifies the deletion product of the leptin receptor gene. Mice were administered tamoxifen for 6 wk in their chow. After a 2-wk recovery period, mice were euthanized,
and blood vessels from different vascular beds were collected. PCR of several different vascular segments was then performed from control flox and flox mice expressing the inducible VSM cell-specific Cre recombinase (Fig. 1A). The 400-bp PCR deletion product was only observed in vascular segments from the tamoxifen-treated KO mice and not from control (Con) mice (Fig. 1A). Levels of leptin receptor mRNA were next determined from aorta by real-time PCR. Leptin receptor mRNA levels were decreased by 80% in the aorta of KO compared with wild-type mice (Fig. 1B).

**Acute incubation with leptin inhibits ACh but not SNP-mediated relaxation, and it is restored with the superoxide scavenger tempol.** Acute incubation of isolated carotid arteries from control mice with a concentration of leptin that mimics levels achieved in morbid obesity caused significantly impaired vessel relaxation to the endothelial-specific agonist ACh (Fig. 2A) but not to smooth muscle-mediated relaxation caused by SNP (data not shown). When these experiments were repeated in the presence of the superoxide scavenger tempol, relaxation responses to ACh were restored, suggesting that leptin-mediated oxidative stress contributed to the impairment.

**Leptin-mediated impairment of ACh relaxation is blunted in VSM LepR KO mice.** To specifically test the role of VSM LepR in mediating vascular responses to leptin, we utilized VSM LepR KO mice and littermate controls (Con). Conditional KO of the LepR did not change ACh-mediated relaxation in the carotid artery (Fig. 2B). Consistent with our data above, carotid arteries from control littermates incubated with leptin exhibited significant impairment to ACh-mediated relaxation. However, this impairment in response to acute incubation with leptin was significantly attenuated in carotid arteries from VSM LepR KO mice. No differences in the concentration response curves to SNP (Fig. 3A) or U46619 (Fig. 3B) were observed in carotid arteries from control and KO mice.

**VSM deletion of leptin receptor decreases vascular phospho-NOS3 without affecting total NOS3 levels.** The levels of both total NOS3 and phospho Ser 1177 NOS3 were determined by Western blot using aortic lysates from control and VSM LepR KO mice. Total NOS3 levels did not differ in the aorta between control and VSM LepR KO mice (Fig. 4, A and B). However, the levels of phospho Ser 1177 NOS3 were significantly diminished in the aorta of VSM LepR KO mice compared with control mice (Fig. 4, A and C).

**VSM deletion of leptin receptor decreases vascular NOX4 and SOD3 levels.** The effect of VSM deletion of the leptin receptor on enzymes involved in regulating the production of vascular superoxide was determined by real-time PCR using RNA derived from the aorta of control and VSM LepR KO mice. NADPH oxidases are a family of proteins responsible for the generation of superoxide in the vasculature. NOX4, which is the major isoform of NADPH oxidase present in the vasculature, was decreased by 90% in the aortas of VSM LepR KO mice compared with control mice (Fig. 5A). This significant decrease in vascular NOX4 expression was associated with a twofold induction of NOX1 expression in the aorta of VSM

---

**Fig. 1.** Molecular characterization of the vascular smooth muscle (VSM) leptin receptor (LepR) knockout (KO) mice. A: PCR validation of LepR KO in vessels isolated from various tissues in control (Con) and KO mice. B: expression of LepR mRNA levels in the aorta of Con and KO mice via real-time PCR. MWM, molecular weight marker. *P < 0.05 vs. Con, n = 4/group.

**Fig. 2.** A: acute incubation with leptin inhibits acetylcholine (ACh)-mediated relaxation and is restored with the superoxide scavenger tempol. Leptin was added to the bath for acute exposure. *P < 0.05 vs. vehicle-treated mice. †P < 0.05 vs. leptin-treated mice, n = 6/group. B: leptin impairment of ACh-mediated relaxation of the carotid artery is blunted in VSM LepR KO mice. Leptin was added to the bath for acute exposure, and the ACh response was paired from the same artery. *P < 0.05 vs. Con + vehicle and KO + vehicle. #P < 0.05 vs. Con + vehicle and KO + vehicle. †P < 0.05 vs. Con + vehicle, n = 6/group.
LepR KO compared with control mice (Fig. 5A). VSM LepR KO mice also exhibit a 90% decrease superoxide dismutase (SOD) 3 expression and a 20% increase in SOD2 expression in the aorta compared with control mice (Fig. 5B).

VSM deletion of leptin receptor has no effect on blood pressure or heart rate. To determine the effect of loss of VSM leptin receptors on basal blood pressure, we measured conscious 24-h blood pressure in tamoxifen-treated control and KO mice for 7 days by radiotelemetry. KO of VSM leptin receptors did not have any effect on 24-h MAP or heart rate (Fig. 6, A and B). Additionally, no effect of VSM leptin

![Image of graph showing % Relaxation vs SNP (LogM) for Con and KO mice](image1)

Fig. 3. Responses of the carotid artery of VSM LepR KO and Con mice to sodium nitroprusside (SNP) (A) and the thromboxane receptor agonist, U46619 (B); n = 6/group.

![Image of graph showing Tension (g) vs U46619 (ug/ml) for Con and KO mice](image2)

![Image of graph showing Fold Induction vs WT for NOX and SOD isoforms](image3)

Fig. 5. Expression levels of NOX isoforms (A) and superoxide dismutase (SOD) isoforms (B) from the aorta of VSM LepR KO and control mice. WT, wild-type. *P < 0.05 vs. Con.

![Image of Western blot analysis of total and Ser 1177 phosphorylated NADPH oxidase 3 (NOS3) from the aorta of VSM LepR KO and Con mice.](image4)

Fig. 4. Western blot analysis of total and Ser 1177 phosphorylated NADPH oxidase 3 (NOS3) from the aorta of VSM LepR KO and Con mice. A: representative Western blot. B: ratio of total NOS3 to actin. C: ratio of phospho NOS3 to total NOS3. *P < 0.05 vs. Con.
receptor KO was observed on either daytime or nighttime MAP (Fig. 6, C and D). In addition, no differences in body weight, heart weight, heart weight-to-body weight ratio, or heart weight-to-body length ratio were found between control and KO mice (Table 1).

**DISCUSSION**

Obesity is associated with an increased incidence of cardiovascular diseases. As it often appears with other metabolic abnormalities such as diabetes, hyperlipidemia, hypercholesterolemia, and inflammation, it is difficult to specifically determine the importance of obesity alone in the development of cardiovascular disease such as hypertension and heart disease. One of the potential links between obesity and the development of hypertension is the hormone leptin, which is released primarily from white adipose tissue and is elevated in obesity. Elevated leptin levels as observed in obesity can directly cause hypertension through a central mechanism resulting in increased sympathetic outflow to peripheral organs like the kidney (11, 41, 49, 55). High plasma levels may also promote hypertension and cardiovascular disease through impairment of vascular relaxation. In our study, we observed a significant effect of acute high leptin levels similar to those observed in obese individuals to attenuate ACh-mediated vascular relaxation. This response was likely due in part to leptin-mediated increases in VSM cell oxidative stress, as treatment with the antioxidant compound tempol significantly attenuated the effect of high leptin levels on ACh-mediated vascular relaxation. The effect of leptin to attenuate ACh-mediated relaxation was also a VSM cell-dependent effect, as deletion of leptin receptors also prevented this response. The alterations in the ability of leptin to attenuate ACh-mediated relaxation was not due to alterations in vascular reactivity in these mice, as they exhibited normal responses to sodium nitroprusside and U46619. Previous studies have reported that leptin can cause increases in the levels of oxidative stress in endothelial cells and cardiomyocytes (9, 17). The mechanism by which leptin increases vascular oxidative stress is not known but may be mediated via stimulation of NAD(P)H oxidases (18). NOX4 and NOX1 are thought to be the major isoforms of NAD(P)H oxidase in the vasculature responsible for superoxide production (3, 33). VSM LepR KO mice exhibited a >90% decrease in vascular expression of NOX4 compared with control mice and an increase in vascular NOX1 expression. It is possible that the leptin receptor acting either directly or through increases in angiotensin II or endothelin is responsible for maintaining NOX4 expression levels in the vasculature to increase NAD(P)H oxidase in the vasculature responsible for superoxide production (43, 60, 63). It is not known whether the observed increase in vascular NOX1 levels in the VSM LepR KO mice is a compensatory response to loss of vascular NOX4; however, it is clear that the enhanced NOX1 expression is not sufficient to mediate the effect of leptin to attenuate ACh-mediated relaxation in the VSM LepR KO mice. SODs are a family of proteins that are responsible for protecting the vasculature from reactive oxygen species (ROS), such as superoxide (37). We observed significant decreases in the expression of the extracellular isoform of SOD, SOD3, in the vasculature.

Table 1. **Body weight, heart weight, and indices of cardiac hypertrophy in wild-type and VSM LepR KO mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>VSM LepR KO</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>28.2 + 1.2</td>
<td>27.6 + 1.2</td>
<td>0.69</td>
</tr>
<tr>
<td>Heart Weight, mg</td>
<td>113 + 7.2</td>
<td>115 + 7.8</td>
<td>0.85</td>
</tr>
<tr>
<td>HW:BW, mg:cm</td>
<td>4 + 0.2</td>
<td>4.2 + 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>HW:BL, mg:cm</td>
<td>11.6 + 0.6</td>
<td>11.8 + 0.8</td>
<td>0.83</td>
</tr>
</tbody>
</table>

All values are given as means + SE; \( n = 5 \). VSM, vascular smooth muscle; LepR, leptin receptor; KO, knockout; HW, heart weight; BW, body weight; BL, body length.
of VSM LepR KO compared with control mice. SOD3 expression is believed to be regulated by factors such as angiotensin II and endothelin-1, which may be attenuated in VSM-lacking leptin receptors (45, 47). Recent studies have demonstrated that, although VSM-specific deletion of SOD3 is associated with enhanced superoxide production and attenuation of bioavailable NO, it does not have a significant effect on blood pressure either under basal conditions or in response to angiotensin II infusion (34). It is possible that the decrease in SOD3 expression in the vasculature of VSM LepR KO mice is reflective of the NOX4-mediated decrease in vascular superoxide production. However, additional studies are required to fully elucidate this relationship.

Our findings of attenuation of vascular relaxation by high leptin levels, which are mediated by increased oxidative stress, are in agreement with a recent study that demonstrated that acute leptin treatment impairs vascular relaxation in second-order branches of mesenteric arteries (61). This study demonstrated that the effect of leptin to impair vascular relaxation was also mediated by a central mechanism, as celiac ganglionectomy blocked the effect of leptin on endothelial function (61). These results, when combined with the results of the present study, suggest that leptin may impair vascular relaxation by two mechanisms, a centrally mediated mechanism and a direct leptin receptor-mediated effect on VSM cells. It is possible that each of these mechanisms plays a different role in the regulation of vascular function in smaller resistance arteries vs. larger arterioles with the direct effect of leptin being more pronounced in the larger arteries and the central sympathetic mechanism regulating smaller resistance arterioles. It should be noted that VSM cell leptin receptors in our model were knocked out in both large arterioles such as the carotid artery and aorta as well as small mesenteric resistance arteries (Fig. 1A). However, we did not address the role of leptin in mediating vascular function in small mesenteric arterioles in the present study.

Leptin has been previously described to promote vascular relaxation by enhancement of NO production from endothelial cells (2, 25, 31). The ability of leptin to increase NO production is thought to be an important counterbalance to its central effects to raise blood pressure (1, 19, 28). Acute leptin treatment (hours) has been demonstrated to increase NOS3 phosphorylation through a PI3K-Akt mediated pathway (59). Enhancement of NOS3 phosphorylation at Ser 1177 results in attenuation of vascular relaxation by induction of endothelial expression of NOS3. Because NOS3 is expressed in endothelial cells of the vasculature, it is unclear how VSM-specific deletion of leptin receptors could influence the regulation of endothelial NOS3 activity. One possibility is that decreased leptin-mediated VSM superoxide production increases the bioavailability of NO, which results in a downregulation of NOS3 phosphorylation in endothelial cells. Although several studies have demonstrated that leptin can promote vascular relaxation via enhancement of NO production, other studies have demonstrated that high doses of leptin promote vascular dysfunction via uncoupling of NOS3 and increased peroxynitrite formation (26). Our results would suggest that the VSM leptin receptor plays an important role in leptin-induced vascular dysfunction through a mechanism dependent on superoxide generation.

We did not find any effect of VSM cell deletion of leptin receptors on basal blood pressure or heart rate in the present study. Thus it is apparent that VSM cell leptin receptors do not contribute to the regulation of blood pressure under basal conditions. However, we did not determine the effect of loss of VSM cell leptin receptors on leptin-induced hypertension, as it was not the focus of the present study. It is possible that loss of VSM cell leptin receptors could attenuate leptin-induced hypertension by reducing leptin-mediated oxidative stress in the vasculature and enhancing leptin-mediated NO production from endothelial cells. It would also be interesting to determine the effect of chronic leptin treatment on relaxation in the VSM cell leptin receptor KO mice. Both of these questions need to be addressed in future experiments.

**Perspectives and Significance**

We developed a novel model of VSM cell-specific KO of leptin receptors to assess the impact of high circulating levels of leptin on vascular function. We found that high leptin levels attenuate ACh-mediated vasorelaxation, and this effect was reversed by pretreatment with the antioxidant compound tempol. We further demonstrate that VSM cell-specific loss of leptin receptors attenuated the effect of leptin to inhibit ACh-mediated vascular relaxation. VSM deletion of the leptin receptor was associated with decreased aortic expression of NOX4 and SOD3 and phosphorylation of NOS3 at Ser 1177. VSM cell-specific KO of leptin receptors had no effect on body weight, basal blood pressure, heart rate, and cardiac hypertrophy. This model could be beneficial for determining the role of VSM cell leptin receptors in other vascular pathologies, such as atherosclerosis, and in understanding the role of leptin receptors in vascular stiffness.

**GRANTS**

This work was supported by grants from the National Heart, Lung and Blood Institute (PO1HL-051971) and the National Institute of General Medical Sciences (P20GM-104357).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


