Vascular Smooth Muscle Specific Deletion of the Leptin Receptor Attenuates Leptin-Induced Alterations in Vascular Relaxation.

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Running title: VSM Leptin and vascular function

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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. In order to test this, we used control and VSM-LepR deficient mice (VSM-LepR KO) created with a tamoxifen-inducible specific Cre recombinase to delete the LepR gene in VSMC. 10-12 week old control and VSM-LepR KO mice were fed a diet containing tamoxifen (50 mg/kg) for six weeks, 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 minutes. 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 leptin receptor-mediated mechanism, suggesting a potential pathogenic role for leptin to increase cardiovascular risk during obesity.
Key Words: Endothelial function, obesity, acetylcholine, hypertension, Cre recombinase
Introduction

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). While 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 which 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). While leptin receptors are highly expressed in the brain and nervous system, they are also present in the periphery including the vasculature. 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 synthase to increase nitric oxide (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 STAT3, Shp2, and 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 up-
regulation of SOCS3 expression (6).

While these diverse actions of leptin in the vasculature are established in the literature, the
specific effect of leptin’s action in VSM cells has yet to be elucidated. We utilized a Cre-loxP
based approach to effectively knockout leptin receptors from VSM cells in order 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 \textit{in vivo} (14; 27). We bred these floxed leptin
receptor mice to 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 weeks of age. Breeding pairs of floxed leptin receptor mice (LepRFlox) 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). Since this exon contains the signal sequence, Cre-mediated deletion of this exon results in the loss of all LR splice variants (14). Vascular smooth muscle-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, Inc., Madison, WI). Mice were fed the tamoxifen diet for a period of 6 weeks after which time they were switched back to the standard diet. Experiments were then performed 2 weeks 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 sec every 15 min for 7 consecutive days. Mice were then euthanized and tissues collected. Blood pressure data was analyzed using Dataquest ART version 3.1 software (Data Sciences International, Minneapolis, MN). Mean arterial blood pressure (MAP) was separated into daytime and nighttime values and
then two readings 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 as 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, NaCl 118.3, KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, and
180 glucose 11) saturated with 95\% O\(_2\) and 5\% CO\(_2\). Resting tension was adjusted step-wise to reach
a final tension of 0.25 grams. 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 pre-
contracted with the thromboxane A2 mimetic U46619 (0.4 \(\mu\)g/ml). A subset of vessels were
incubated with leptin (100 ng/ml) or vehicle (TrisHCl 0.1 mM) for 30 minutes prior to, 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 (Viagen Biotech Inc., Los Angeles, CA) according to manufacturers
guidelines. Vessels from the brain, heart, and kidney were prepared by passing the tissue
through a 75 \(\mu\)m 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’-
GTCACCTAGGTTAATGTATTC-3’ and LepRprimer3-5’-GCAATTCATATCAAAACGCC-3’.
PCR was performed using the following protocol: 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec 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 knockout mice. RNA was isolated from aortas using Nucleo Spin RNA kit (Macherey-Nagel, Durew, Germany) according to manufacturers guidelines. cDNA was derived from 500 ng of the purified total RNA using the iScript RT kit (BioRad, CA). Real-time PCR was performed on 1 ul of the cDNA using iTaq University SYBR-Green Kit (BioRad, CA). PCR was performed using the following protocol: 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec for 40 cycles. The difference in the threshold cycle (Ct) for the target gene of each sample was compared to the Ct for 18s rRNA to yield the delta Ct. The results are expressed as the fold difference for each target gene compared to the level in wild-type mice. The PCR primers used for real-time PCR were as follows:

- LEPR+5’CTATGTGGTTTTTGTACACTGG; LEPR5’AGGTGAGAGAAAGGAGTCC
- Nox1F+5’AATGCCAGGATCGAGGA; Nox1R+5’GATGGAAGCAAAGGGAGTCA
- Nox2F+5’CCCTTTGTACACAGCAGAGTT; Nox2R
- CAATCCCGCTCCACTACATCA; Nox4F5’GGATCACAGAAGGTCCCTAGCAG;
- Nox4R5’GCCGCTACATGCACACCTGAGAA; SOD1F5’CGGCTTTCTCGTCTTGTCTCTC;
- SOD1R5’CGAAGTGGATGCTTCCCTGC; SOD2F5’TAAAAGCCAGATCATGCA;
- SOD2R5’GGTGGCGTGTAGTGTTT; SOD3F5’CAGAACCAGCAATGCAAGTG;
- SOD3R5’CTGAGTGGTGGTAAAGTTGCTTGG; 18s+5’TAGTCCCTGTCCCTTGTA;
- 18s- 5’-GATCCGGGGCCTCAG

8
Western Blot. Total abundance of NOS3 and phosphorylation of NOS3 at serine1177 was 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 minutes. Rat kidney inner medulla was used as a positive control. 22.5 μL of homogenized sample were separated by electrophoresis (200 V, 40 min, 4–15% TGX Stain-Free Precast gel, Bio-Rad Laboratories) and transferred onto nitrocellulose membranes (Bio-Rad Trans-Blot Turbo, High MW program). The membrane was then blocked for 1 hour and cut at the 75 kDa marker. The top half was incubated with the rabbit polyclonal phosphorylated NOS3-Ser1177 antibody (Cell Signaling) overnight at 4°C, and the lower half was incubated with β-actin (Sigma). Membranes were then incubated with the secondary antibodies (rabbit 1:1,000 for p-NOS3 and mouse 1:30,000 for actin, Bio-Rad Laboratories) for 1h at room temperature and then developed with enhanced chemiluminescent reagents (Thermo Scientific). The top half of the membrane was stripped (ReBlot Plus Mild, Millipore), and incubated overnight with a primary antibody against NOS3 (BD Transduction, 1:250) prior to incubation with the mouse secondary antibody (mouse, 1:1000). Bands were then quantified using the VersaDoc Imaging System and Image Lab 3.0 Software (Bio-Rad Laboratories). 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 mean ± S.E.M. Differences between treatment groups were determined using an unpaired t-test or a one-way analysis of variance with a post hoc test.
A $P<0.05$ was considered to be significant. All analyzes were performed with SigmaStat (Systat software, Inc., Richmond, CA, USA).

**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 weeks in their chow. After a two week 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 (Figure 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 (Figure 1A). Next, levels of leptin receptor mRNA were determined from aorta by real-time PCR. Leptin receptor mRNA levels were decreased by 80% in the aorta of knockout as compared to wild-type mice (Figure 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 (Figure 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 knockout of the LepR did not
change ACh mediated relaxation in the carotid artery (Figure 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 (Figure 3A) or U46619 (Figure 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 Ser1177 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 (Figure 4A & B). However, the levels of
phospho Ser 1177 NOS3 were significantly diminished in the aorta VSM LepR KO as compared
to control mice (Figure 4A & 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 as compared to control mice (Figure 5A). This significant decrease in vascular NOX4 expression was associated with a 2-fold induction of NOX1 expression in the aorta of VSM LepR KO as compared to control mice (Figure 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 as compared to control mice (Figure 5B).

VSM deletion of leptin receptor has no effect on blood pressure or heart rate.

In order to determine the effect of loss of VSM leptin receptors on basal blood pressure, we measured conscious 24-hour blood pressure in tamoxifen treated control and KO mice for 7 days by radiotelemetry. Knockout of VSM leptin receptors did not have any effect on 24 hour mean arterial pressure (MAP) or heart rate (Figure 6A & B). Additionally, no effect of VSM leptin receptor knockout was observed on either daytime or nighttime MAP (Figure 6C & 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 anti-oxidant 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 in VSM LepR KO mice 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 maybe 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 Lep R KO mice exhibited a greater than 90% decrease in
vascular expression of NOX4 as compared to 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-mediated superoxide production (43; 60; 63). It is not known if 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. Superoxide dismutases (SODs) are a family of proteins which 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 of VSM LepR KO as compared to 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 while VSM specific deletion of SOD3 is associated with enhanced superoxide production and attenuation of bioavailable NO, it does not have 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 which 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 versus 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 (Figure 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). Leptin’s ability 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 serine 1177 results in increased activity and is one mechanism by which the activity of the protein is regulated (16; 38). VSM cell deletion of leptin receptors was associated with decreased levels of phospho Ser 1177 NOS3. Since 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 down-regulation of NOS3 phosphorylation in endothelial cells. While several studies have demonstrated that leptin can
promote vascular relaxation via enhancement of NO production, other studies have demonstrated that high doses of leptin promotes 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 current 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 knockout mice. Both of these questions need to be addressed in future experiments.

Perspectives and Significance

We developed a novel model of VSM cell specific knockout 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 of vascular segments 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 knockout 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.
Acknowledgements

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


Table 1- Body Weight, Heart Weight and Indicies of Cardiac Hypertrophy in Wild-type and VSM LepR KO mice

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<th>VSM LepR KO</th>
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All values are given as Mean + SE. N=5.
**Figure Legends**

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

**Figure 2**- A) Acute incubation with leptin inhibits acetylcholine (ACh) mediated relaxation and is restored with the superoxide scavenger tempol (1mM). Leptin was added to the bath for acute exposure. *=P<0.05 vs. vehicle treated. # = $P<0.05$ vs. leptin treated, n=6/group. B) Leptin impairment of acetylcholine (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 + Leptin, n=6/group.

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

**Figure 4**- Western blot analysis of total and Ser 1177 phosphorylated NOS3 from the aorta of VSM LepR KO and control mice. A) Representative Western blot. B) Ratio of total NOS3 to actin. B) Ratio of phospho NOS3 to total NOS3. * = $P<0.05$ vs. Con.

**Figure 5**- Expression levels of A) NADPH oxidase isoforms and B) Superoxide Dismutase (SOD) isoforms from the aorta of VSM LepR KO and control mice. * = $P<0.05$ vs. Con.
Figure 6- Blood Pressure and heart rate in VSM LepR KO and Con mice. Panel A- 24 hour mean arterial pressure (MAP). Panel B- 24 hour heart rate. Panel C- circadian blood pressure. Panel D- circadian heart rate. n=5.
**Figure 1**

A) Gel electrophoresis showing deletion products in Con and KO samples from different tissues:
- MWM
- Aorta
- Brain
- Carotid
- Coronary
- Kidney
- Mesenteric

B) Bar graph showing fold change in leptin receptor expression:
- Con: 1.2
- KO: 0.2

* indicates a significant difference.
Figure 2

**A**

% Relaxation vs. ACh (LogM)

- Leptin
- Leptin + Tempol
- Vehicle

**B**

% Relaxation vs. ACh (LogM)

- Con + Leptin
- KO + Leptin
- KO + Vehicle
- Con + Vehicle
Figure 3

A

% Relaxation vs SNP (LogM)

B

Tension (g) vs U46619 (μg/ml)
Figure 4

A

Phospho-NOS3(ser1177)  
NOS3  
actin

Kidney Papilla

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Aorta

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B

Total NOS3/Actin

C

Phospho NOS3/Total NOS3

* Con, n=4  
* KO, n=4
Fold Induction vs. WT

Con, n=4
KO, n=4

Nox1
Nox2
Nox4

* Figure 5
Figure 6

Mean Arterial Pressure (mmHg)

A

B

Con, n=5
KO, n=5

Day Night Day Night

C

D

Heart Rate (BPM)

Figure 6