Cardiovascular and Sympathetic Effects of Disrupting Tyrosine 985 of the Leptin Receptor

Shannon M. Harlan, Donald A. Morgan, David J. Dellsperger, Martin G. Myers, Jr, Allyn L. Mark, Kamal Rahmouni

Abstract—Leptin acts in the brain to regulate food intake and energy expenditure. Leptin also increases renal sympathetic nerve activity and arterial pressure. The divergent signaling capacities of the leptin receptor (ObRb) mediate the stimulation of various intracellular pathways that are important for leptin control of physiological processes. We evaluated the cardiovascular and sympathetic consequences of disrupting the signal emanating from tyrosine985 of ObRb. For this, we used Lepr<sup>s/s<sup> (l/l), mice, which carry a loss of function mutation replacing tyrosine985 of ObRb with leucine. Body weight of l/l mice was not significantly different from wild-type controls. In contrast, radiotomeletry measurements revealed that the l/l mice had higher arterial pressure and heart rate as compared with controls. Ganglionic blockade caused a greater arterial pressure fall in the l/l mice relative to controls. In addition, leptin treatment induced a larger increase in arterial pressure and heart rate in the l/l versus wild-type mice. Finally, we compared the response of renal and brown adipose tissue sympathetic nerve activity to intracerebroventricular injection of leptin (2 μg) between l/l and control mice. Leptin-induced increase in renal sympathetic nerve activity was greater in l/l mice relative to controls. In contrast, the brown adipose tissue sympathetic nerve activity response to leptin was attenuated in the l/l mice relative to controls. These data indicate that selective loss of leptin receptor signaling emanating from tyrosine985 enhances the cardiovascular and renal sympathetic effects of leptin. These findings provide important insight into the molecular mechanisms underlying leptin’s effects on the sympathetic cardiovascular function and arterial pressure.

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Key Words: autonomic function • cardiovascular regulation • leptin

Leptin is an adipocyte hormone that acts in the central nervous system to modulate food intake and enhance energy expenditure through activation of sympathetic nerve traffic to thermogenic brown adipose tissue. 1,2 Leptin action in the central nervous system also increases blood pressure through activation of the sympathetic nervous system. 3,4 Indeed, in animal studies, elevating circulating leptin levels increase arterial pressure, which can be prevented by adrenal blockade. 5,6 In addition, leptin has emerged as an important culprit linking obesity, sympathetic overdrive, and hypertension based on epidemiological and experimental animal studies. 3,4,7 For instance, several animal models of obesity that have elevated arterial pressure were found to have preserved renal sympathetic and arterial pressure responses to leptin despite the resistance to the anorectic and weight-reducing actions of leptin. 8-11 Despite this, little is known about the molecular mechanisms underlying the sympathetic and cardiovascular responses triggered by leptin.

The long form of the leptin receptor (ObRb) is the major signaling isoform of the leptin receptor. 12 Leptin binding triggers the activation of the receptor-associated Janus kinase 2 tyrosine kinase. Once activated, Janus kinase 2 phosphorylates other tyrosine residues within the ObRb, including Tyr<sub>1138</sub>, Tyr<sub>1077</sub>, and Tyr<sub>985</sub>, each of which mediates the activation of distinct downstream signaling pathways. 13,14 ObRb activation also promotes the activation of phosphatidylinositol (PI) 3 kinase, although this appears to be cell type-specific and the mechanisms underlying this regulation remain unclear. 15 Whereas phosphorylated Tyr<sub>1138</sub> of ObRb recruits and activates signal transducer and activator of transcription (STAT) 3, the phosphorylation of Tyr<sub>1077</sub> promotes the tyrosine phosphorylation and activation of STAT5. 15 Activated STAT3 and STAT5 translocate to the nucleus to modulate gene transcription with important implications for the regulation of metabolism and body energy balance. 16 For instance, knock-in mice that have a mutation at Tyr<sub>1138</sub> of ObRb (Lepr<sup>s/s</sup>, s/s mice), which disrupts leptin-induced STAT3 signaling, are severely obese and hyperphagic, but in contrast to the mice lacking leptin or ObRb, these mice remain fertile and are less diabetic. 17

On the other hand, phosphorylation of Tyr<sub>985</sub> creates a binding site for the COOH-terminal SH2 domain of the...
tyrosine phosphatase, PTPN11 (also known as SHP2), leading to the activation of the extracellular signal-related kinase signaling pathway.\textsuperscript{13,14} Although Tyr\textsubscript{985} mediates most extracellular signal-related kinase stimulation during ObRb signaling, tyrosine phosphorylation sites on Janus kinase 2 appears to account for a fraction of extracellular signal-related kinase activation by leptin independently from ObRb phosphorylation.\textsuperscript{13} Tyr\textsubscript{985} also binds the suppressor of cytokine signaling-3, which acts as a negative regulator to inhibit STAT3 signaling.

A knock-in mouse model, Lepr\textsuperscript{L985} (l/l mice), carries a point mutation resulting in the substitution of the Tyr\textsubscript{985} with a Leu residue.\textsuperscript{18} This presumably blocks the recruitment of SHP2 and suppressor of cytokine signaling-3. The l/l mice tend to be lean and exhibit resistance to diet-induced obesity likely because of enhanced leptin sensitivity in the hypothalamus.\textsuperscript{18} In addition, the l/l mice exhibited a robust elevation in urinary excretion of norepinephrine, suggesting a higher sympathetic nerve discharge.\textsuperscript{19}

In the present study, we evaluated the consequences on the cardiovascular and sympathetic functions of disrupting Tyr\textsubscript{985} ObRb signaling in the l/l mice. We also examined the effects of leptin on arterial pressure, heart rate, and regional sympathetic nerve traffic in the l/l mice.

Methods

Animals
We used Lepr\textsuperscript{L985} (l/l) and Lepr\textsuperscript{R1131H}(s/s) mice on a C57BL/6 background generated previously.\textsuperscript{17,18} Heterozygous mice (obtained from Dr Myers, University of Michigan, Ann Arbor, MI) were bred to generate experimental mice (l/l and s/s) and wild-type (+/+ or +/l) controls needed for our study. Mice were genotyped using a custom-designed single nucleotide polymorphism assay that enabled accurate and reliable detection of the point mutation in these knock-in mouse models (Custom TaqMan SNP Genotype Assay; Applied Biosystems) as described previously.\textsuperscript{17,18}

We used adult mice (12 to 17 weeks of age), which were housed in a temperature-controlled room with a 12:12-light–dark cycle with free access to standard laboratory chow and tap water. All studies were approved by the University of Iowa Animal Research Committee.

Arterial Pressure and Heart Rate Measurement
Arterial pressure and heart rate were recorded in conscious state using continuous radiotelemetric measurement (PA-C10; Data Science Instruments) as described.\textsuperscript{9} Mice were anesthetized with intraperitoneal (IP) administration of a ketamine (91 mg/kg) and xylazine (9.1 mg/kg) cocktail. Under aseptic surgical conditions, the catheter of the telemeter was inserted in the left carotid artery and tied securely using 6-0 silk suture. The transmitter was tunneled subcutaneously from the neck area until the unit had reached the midabdominal region. The neck incision was saturated with 4-0 absorbable cat gut and then further sealed with tissue adhesive (Vet-Bond) along the incision line.

Animals were allowed to recover for 7 to 10 days before arterial pressure, heart rate, and locomotor activity were recorded continuously in the conscious unrestrained state for 7 days. The effect of ganglionic blockade on the hemodynamic parameters was tested using hexamethonium bromide (1 \( \mu \)g/g body weight, IP) during the daytime recording period (11 AM). The control period corresponded to the 15 minutes before injection, and the effect of hexamethonium bromide was considered the first recording acquired after injection with data collected every 5 minutes. The effect of leptin (60 \( \mu \)g, IP, twice daily) on arterial pressure and heart rate was measured for 6 days. Hemodynamic parameters were recorded for 10 seconds every 5 minutes and stored on a personal computer using Data Science Datquest software.

Sympathetic Nerve Recording
Mice were equipped with a lateral cerebroventricular (ICV) cannula as described elsewhere.\textsuperscript{9,10} One week after recovery, mice were anesthetized with the ketamine/xylazine cocktail, IP, and intubated (PE-50) to allow for spontaneous respiration of oxygen-enriched room air. Body temperature was kept constant at 37.5°C using a surgical heat lamp and a heat pad. The right jugular vein was cannulated to maintain anesthesia with \( \alpha \)-chloralose (initial dose: 25 mg/kg; sustaining dose of 6 mg/kg/h). Finally, the left carotid artery was cannulated for continuous measurement of arterial pressure and heart rate.

The nerves to the left kidney or brown adipose tissue (BAT) were identified using a dissecting scope and then mounted on a bipolar 36-gauge platinum–iridium electrode (Cooner Wire Co, Chaddsworth, CA). Once optimal recording parameters were established, the nerve fibers were fixed to the electrode with silicone gel (Kwik-Sil; World Precision Instruments Inc, Sarasota, FL). After completing the surgery, animals were allowed to stabilize before a 10-minute control period was obtained followed by ICV administration of leptin (2 \( \mu \)g) or vehicle (saline, 2 \( \mu \)L). In 1 cohort of l/l and control mice, we tested the role of P33 kinase in mediating leptin-induced increase in renal sympathetic nerve activity (SNA). For this, LY294002 (0.1 \( \mu \)g) was administered ICV. 10 minutes before ICV leptin (2 \( \mu \)g), SNA responses were followed for 4 hours.

Data on SNA were acquired and analyzed as previously described.\textsuperscript{9} The nerve electrodes were attached to a high-impedance probe (HIP-511; Grass Instruments Co, Quincy, MA). The signal was amplified 10\(^3\) times with a Grass P5 AC preamplifier and filtered at both low- (10 Hz) and high-frequency (1000 Hz) cutoff. This amplified, filtered signal was then sent to a speaker system and oscilloscope (Model 54501A; Hewlett-Packard Co, Palo Alto, CA). The signal was also routed to a MacLab analog–digital converter (Ad Instruments, Castle Hill, New South Wales, Australia) for recording and data analysis on a Macintosh computer. Background noise was excluded from the measurements of both renal and BAT SNA by correcting for postmortem activities.

Data Analysis
Sympathetic nerve responses are expressed as the percent change from the 10-minute baseline recording period. Results are shown as mean±SEM. Data were analyzed using Student t test, 1- or 2- way analysis of variance with or without repeated measures. When analysis of variance reached significance, a post hoc comparison was made using Fisher test. A \( P<0.05\) was considered to be statistically significant.

Results

Cardiovascular Effects of Disrupting Tyr\textsubscript{985} of ObRb in l/l Mice
Body weight of l/l mice (23.6±0.7 g) and wild-type controls (23.1±0.7 g) did not differ significantly (\( P=0.3\)), but visceral fat pads in the l/l mice (0.36±0.06 g) were significantly (\( P<0.02\)) less than wild-type controls (0.54±0.04 g; see Supplemental Figure I; available at http://hyper.ahajournals.org).

To evaluate the cardiovascular consequences of disrupting Tyr\textsubscript{985} of ObRb, we used radiotelemetry to assess arterial pressure, heart rate, and locomotor activity. Significant differences between l/l mice and wild-type controls were observed (Figure 1). Mean arterial pressure was significantly (\( P<0.05\)) elevated in the l/l mice during the 24-hour recording period (Figure 1A). The elevated mean arterial pressure in the l/l mice resulted from an increase in both sylvatic
(+20.0±3 mm Hg) and diastolic (+26±9 mm Hg) arterial pressure.

As shown in Figure 1B, the elevation in mean arterial pressure in l/l mice occurred during both the light phase (+26 mm Hg during the 12-hour day period, \(P=0.05\) versus controls) and the dark phase (+23 mm Hg during the 12-hour night period, \(P=0.04\) versus controls).

Relative to wild-type controls, the l/l mice had a significantly \((P=0.04)\) higher diurnal heart rate measured over the 24-hour period (Figure 1C). The elevation in heart rate in the l/l was present during both the light (+52 beats/min, \(P=0.01\)) and dark phases (+44 beats/min, \(P=0.02\); Figure 1D).

Locomotor activity tended to be higher in the l/l mice relative to controls during the active dark phase, but this was not statistically significant \((P=0.14, \text{Supplemental Figure II})\).

**Exaggerated Arterial Pressure Response to Ganglionic Blockade in l/l Mice**

To investigate the contribution of neurogenic mechanisms to the elevated arterial pressure in the l/l mice, we compared the arterial pressure response to ganglionic blockade with IP hexamethonium bromide (1 \(\mu g/g\) body weight) between l/l and control mice. As shown in Figure 2, in the l/l mice, the magnitude of the decrease in mean arterial pressure in response to ganglionic blockade was significantly greater than in wild-type controls \((-15.1±5.4 \text{ mm Hg in l/l mice versus } -0.1±4.6 \text{ mm Hg in controls}, P<0.05)\) indicating the importance of neurogenic mechanisms, presumably sympathetic tone, in maintaining arterial pressure elevation in the l/l mice.

**Enhanced Leptin-Induced Increases in Arterial Pressure in the l/l Mice**

To examine whether disruption of Tyr985 of ObRb affected the hemodynamic response to leptin, we evaluated the effect of 6 days of IP leptin treatment (60 \(\mu g\), twice daily) in l/l mice and control littermates that were instrumented for radiotelemetric recording of arterial pressure. The leptin-induced increase in arterial pressure was significantly \((P<0.05)\) greater in the l/l mice from Days 2 through 6 (+7.3±2.1 mm Hg at Day 6) as compared with the wild-type controls (+3.2±1.7 mm Hg at Day 6; Figure 3A). Similarly,
the sensitivity of renal SNA to leptin is augmented in l/l mice. We found that ICV leptin (2 μg) caused increases in renal SNA in both the wild-type and l/l mice. However, the response was significantly (P<0.05) greater in the l/l mice (Figure 4A–C). Indeed, ICV leptin increased renal SNA by 160%±27% in the l/l mice versus 108%±15% in the controls (P<0.02; Figure 4B). In the anesthetized state, the l/l mice tend to have higher baseline mean arterial pressure (85±4 mm Hg) and heart rate (335±17 beats/min) as compared with the controls (80±4 mm Hg and 325±25 beats/min, respectively). Consistent with our previous data in anesthetized mice,9,20,22 leptin caused no change in arterial pressure and heart rate relative to vehicle in either l/l or control mice (data not shown). In l/l and wild-type mice, ICV administration of vehicle caused no significant change in renal SNA during the 4-hour recording period (The effects of vehicle on renal SNA at 4 hour are shown in Figure 4C).

We previously demonstrated that leptin-induced sympathetic activation to kidney is PI3 kinase-dependent.22,23 Therefore, we tested whether inhibition of PI3 kinase with LY294002 attenuated renal sympathetic activation to leptin in l/l mice. We found that ICV pretreatment with LY294002 (0.1 μg) substantially attenuated the increase in renal SNA induced by ICV leptin (2 μg) in controls (18%±33%, n=4, P=0.005 versus leptin alone) as well as l/l mice (30%±39%, n=3, P=0.02 versus leptin alone).

Next, we tested whether the enhanced renal sympathetic activation to leptin is specific to disruption of Tyr985. For this, we examined the effect of leptin on renal SNA in the s/s mice. These mice carry a point mutation resulting in the replacement of the Tyr1138 in ObRb with a serine residue that selectively abolishes the activation of STAT3 by leptin.17 We found that despite severe obesity (Supplemental Figure III), in the s/s mice, ICV administration of leptin (2 μg) increased renal SNA by 97%±28%, which was not different (P=0.3) from the response obtained in the wild-type mice (Figure 4B–C).

**Attenuated BAT SNA Response to Leptin in l/l Mice**

Finally, we asked if the enhanced leptin-induced sympathetic nerve activation in the l/l mice is uniform or is specific to renal SNA. For this, we compared the effect of ICV administration of leptin (2 μg) on SNA with BAT between l/l and
wild-type mice (Figure 5). As expected, in wild-type mice, ICV leptin caused a gradual but robust increase in BAT SNA (Figure 5A). ICV leptin also increased BAT SNA in the l/l mice, but this increase was significantly ($P<0.05$) less in the l/l mice ($159\% \pm 27\%$) compared with control mice ($363\% \pm 72\%$; Figure 5B). Vehicle had no effect on BAT SNA in either control or l/l mice (Figure 5B).

**Discussion**

This study demonstrates that loss of function of Tyr985 of the leptin receptor in l/l mice increases arterial pressure and sympathetic cardiovascular responses to leptin. First, despite normal body weight, the l/l mice have elevated arterial pressure and heart rate. Second, the elevation in arterial pressure is neurogenically and presumably sympathetically mediated as indicated by augmented depressor response to ganglionic blockade. These data are further supported by a previous report documenting higher urinary norepinephrine levels in l/l mice.19 Third, the l/l mice have enhanced sensitivity of renal sympathetic and arterial pressure responses to leptin. Fourth, and importantly, the enhanced sympathetic nerve responses to leptin in the l/l mice are selective and not uniform as indicated by the fact that leptin-induced increases in renal SNA are enhanced, whereas increases in BAT SNA are attenuated. Such contrasting change in the sensitivity of regional SNA to leptin in the l/l mice is consistent with the differential regulation of SNA subserving various tissues.24,25 Together, these findings provide important insight into the molecular mechanisms underlying the influence of leptin on the sympathetic tone regulating cardiovascular function and arterial pressure.

Divergent signaling capacities of the leptin receptor are important for leptin control of physiological processes.14,27,28 Several intracellular pathways can be triggered by leptin through various tyrosine residues of ObRb. Our data indicate that selective loss of ObRb signaling emanating from Tyr985 (l/l mice) enhances the cardiovascular and renal sympathetic effects of leptin. Similarly, leptin effects on bone mass were found to be enhanced in l/l mice.19 Such increase in leptin sensitivity after loss of function mutation of Tyr985 of ObRb could best be explained by the inability of the mutant receptor to recruit suppressor of cytokine signaling-3, which antagonizes the signaling pathways activated by leptin.18 Suppressor of cytokine signaling-3 binds to Tyr985 to mediate inhibition of Janus kinase 2 tyrosine kinase activity through the N-terminal kinase inhibitory region.13 On the other hand, an inability of ObRb with a mutant Tyr985 to recruit SHP-2 and, therefore, fail to activate the extracellular signal-related kinase pathway may explain the attenuated BAT SNA response to leptin in l/l mice.26 We previously demonstrated the critical role of the ObRb–extracellular signal-related kinase axis in mediating the BAT sympathetic activation to leptin.23

Specific disruption of the ObRb–STAT3 pathway in s/s mice did not alter leptin-induced renal sympathetic activation, which excludes a role for STAT3 signaling in the control of renal sympathetic traffic by leptin. This finding adds renal SNA to the list of physiological processes regulated by leptin independent of the ObRb–STAT3 signaling. The ObRb–STAT3 pathway was previously shown to be critical in mediating leptin regulation of energy homeostasis as indicated by the severe obesity and hyperphagia in s/s mice with loss of function of the ObRb–STAT3 axis.17 In contrast, the ObRb–STAT3 pathway does not contribute importantly to leptin regulation of linear growth, reproductive function, and glucose metabolism.17

The fact that STAT3 signaling is not involved in the renal SNA response to leptin is consistent with the notion that PI3 kinase plays a major role in the transduction of leptin-induced changes in renal sympathetic outflow and arterial pressure.29

In mice, we found that both inhibitors of PI3 kinase, namely LY294002 and wortmannin, markedly attenuated the leptin-induced increase in renal SNA.22 These inhibitors did not affect the renal sympathetic activation to stimulation of the melanocortin system suggesting that their inhibitory effect on the responses to leptin was a specific effect. In a subsequent study in rat, we found that the role of PI3 kinase in leptin-induced sympathetic activation was specific to the kidney, because pretreatment with LY294002 prevented the effect of leptin on renal SNA without altering the SNA responses to other beds, including BAT, hindlimb, and the adrenal gland.23 Our finding in the present study that inhibition of PI3 kinase blocks the renal SNA response to leptin in the l/l mice confirms the importance of the PI3 kinase pathway in the transduction of leptin-induced renal sympathetic activation. Although the mechanism by which ObRb activates PI3 kinase remains unclear, it is known that in the hypothalamus, leptin phosphorylates and activates PI3 kinase pathway which results in the release of proinflammatory cytokines. This study also suggests that the PI3 kinase pathway may explain the attenuated BAT SNA response to leptin in l/l mice.26

We found that l/l mice have an attenuated leptin-induced increase in thermogenic BAT SNA, yet these mice remain lean. Activity of BAT is critical for thermogenesis and a reduction in leptin ability to regulate BAT activity may be expected to increase fat mass leading to an obese phenotype.20 Recently, You et al reported the development of a
knock-in mouse model resembling the l/l mice, in which Tyr₉₈₅ was replaced with a phenylalanine residue (Y985F).³⁰
Similar to the l/l mice, the Y985F mice were found to have a lean phenotype at a younger age, but as these mice get older, they tend to accumulate fat ultimately leading to the development of an obese phenotype. This observation prompted us to examine body weight in our l/l mice over time. In a preliminary study, we noticed a similar pattern of excess weight gain in l/l mice with aging. Although a larger study is needed to confirm this observation, this seems to indicate that the metabolic consequences of attenuated leptin-induced increases in BAT sympathetic nerve activity are manifest over time as an obese phenotype.

Perspectives
Despite the recent advances in understanding the pathophysiological role of leptin in obesity-induced hypertension and other cardiovascular disorders, little is known about the molecular mechanisms underlying the sympathetic and cardiovascular responses to leptin. Detailed characterization of the molecular mechanisms and individual ObRb signals in the control of arterial pressure, regional sympathetic activity, and metabolism will greatly enhance our understanding of the obesity-associated cardiovascular disorders. Such advances might make it possible to therapeutically modulate thermogenic and metabolic functions without altering cardiovascular function. It might also offer the possibility to selectively interfere with potentially deleterious renal/cardiovascular sympathetic actions of leptin at the same time as preserving the beneficial thermogenic, weight-reducing actions.

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Disclosures
None.

References
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Supplemental Figure I. Comparison of body weight (A) and weight of various fat pads (B) between l/l mice and wild type littermate controls. Relative to controls, l/l mice have normal body weight, but reduced visceral fat mass. *P<0.05 vs. WT, n=25-34 mice per group.
Supplemental Figure II. Comparison of locomotor activity during 24-hour period (A) or 12-hour day and night phases (B) between l/l mice wild type controls. Relative to controls, l/l mice have a slight, but not significant, increase in lococomor activity during the night phase. n=10 animals per group.
Supplemental Figure III. Comparison of body weight (A) and weight of various fat pads (B) between s/s mice and wild type littermate controls. Relative to controls, s/s mice have increased body weight and fat mass. *$P<0.05$ vs. WT, n=23-25 mice per group.