Leptin Impairs Cardiovascular Baroreflex Function at the Level of the Solitary Tract Nucleus

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Abstract—Circulating leptin is elevated in some forms of obesity-related hypertension, associated with impaired baroreflex function. Leptin receptors are present on vagal afferent fibers and neurons within the solitary tract nucleus, providing an anatomic distribution consistent with baroreflex modulation. Although solitary tract nucleus microinjection of 144 fmol/60 nL of leptin had no significant effect on baroreflex sensitivity for control of the heart rate in urethane/chloralose-anesthetized Sprague-Dawley rats, 500 fmol of leptin impaired baroreflex sensitivity for bradycardia in response to increases in pressure (1.15±0.04 versus 0.52±0.12 ms/mm Hg; P<0.01). Transgenic ASrAOGEN rats with low brain angiotensinogen have an upregulation of the leptin receptor and p85α mRNA in the dorsal medulla relative to Sprague-Dawley rats. Consistent with these observations, the response to leptin was enhanced in ASrAOGEN rats, because both the 144-fmol (1.46±0.08 versus 0.75±0.10 ms/mm Hg; P<0.001) and 500-fmol (1.36±0.32 versus 0.44±0.06 ms/mm Hg; P<0.05) leptin microinjections impaired baroreflex sensitivity. At these doses, leptin microinjection had no effect on resting pressure, heart rate, or the tachycardic response to decreases in pressure in Sprague-Dawley or ASrAOGEN rats. Thus, exogenous leptin at sites within the solitary tract nucleus impairs the baroreflex sensitivity for bradycardia induced by increases in arterial pressure, consistent with a permissive role in mediating increases in arterial pressure. Baroreflex inhibition was enhanced in animals with evidence of increased leptin receptor and relevant signaling pathway mRNA. (Hypertension. 2009;54:1001-1008.)

Key Words: leptin ■ solitary tract nucleus ■ transgenic rats ■ angiotensin ■ baroreflex

Leptin is secreted by adipose cells in direct proportion to adiposity and can cross the blood-brain barrier to activate hypothalamic pathways involved in satiety and energy expenditure. Leptin actions at key hypothalamic nuclei also mediate cardiovascular responses, including increases in sympathetic nervous system activity (SNA) and arterial pressure (AP), likely involving descending pathways to brain stem nuclei involved in direct control of AP and reflex modulation of autonomic function, such as the solitary tract nucleus (NTS). The active long form of the leptin receptor, Ob-Rb, has been localized to the nodose ganglion on vagal afferent fibers and on cells within brain stem areas, such as the NTS in normotensive rats, implicating leptin in direct actions on baroreflex sensitivity (BRS) for control of the heart rate (HR). Although the BRS is often impaired in conditions with elevated circulating leptin levels, a direct link between hypoleptinemia and brain sites mediating the effects on BRS is lacking.

Transgenic rats with low brain angiotensinogen (Aogen) resulting from glial overexpression of an ASrAOGEN exhibit a 90% reduction in brain Aogen and decreased hypothalamic tissue levels of angiotensin (Ang) I, with a similar trend for Ang II. ASrAOGEN rats have plasma leptin and insulin levels comparable with control Sprague-Dawley (SD) rats at 15 weeks of age but show enhanced sensitivity to both hormones, as detected with a glucose challenge. The sensitivity to leptin for cardiovascular actions in these rats is currently unknown.

We assessed the effect of acute, site-specific NTS microinjection of leptin on baroreflex function and indices of autonomic balance, as well as resting AP and HR in SD rats in comparison with ASrAOGEN rats, which might be expected to show enhanced sensitivity to leptin. The present study provides direct evidence that administration of exogenous leptin impairs BRS for control of HR in response to increases in AP within the NTS of SD rats and also alters autonomic balance. In addition, ASrAOGEN rats with down-regulation of the endogenous brain renin-Ang system exhibit increased sensitivity to exogenous leptin microinjection, consistent with an upregulation of leptin receptors and signaling pathways in this brain region.

Methods

The Institutional Animal Care and Use Committee approved all of the procedures. For a detailed Methods section, please see the online Data Supplement at http://hyper.ahajournals.org.

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Animals
Experiments were performed in 3- to 5-month-old male Hanover SD and transgenic (ASrAOGEN) 680 rats obtained from the Hypertension and Vascular Research colony at the Wake Forest University School of Medicine.

Surgical Procedures and Hemodynamic Measures
As reported previously,12–13 rats were anesthetized with combination urethane-chloralose (750 and 35 mg/kg, respectively) via IP injection with supplemental IV doses given as needed. Animals were instrumented with femoral artery and vein catheters and placed in a stereotaxic frame with the head tilted downward (45°) for surgical exposure of the dorsal medulla oblongata. Pulsatile AP and mean AP (MAP) were monitored, recorded, and digitized using a Data Acquisition System (BiOPAC System, Inc; Acknowledge software version 3.8.1), and HR was determined from the AP wave. After obtaining stable measures of MAP and HR, baseline responses to BRS were established by bolus IV randomized injection of 3 doses (2, 5, and 10 μg/kg in 0.9% NaCl) of phenylephrine (PE) or sodium nitroprusside, to determine the BRS for increases or decreases in AP, respectively. Assessment of BRS by bolus injections is more sensitive for the detection of alterations in the bradycardic BRS relative to infusion determinations.14 The BRS for bradycardia and tachycardia was determined for each animal as the slope of the relationship between changes in MAP and the pulse interval generated from the 3 doses of PE and sodium nitroprusside, independently.12,13 Reflex testing was completed within 30 minutes of leptin microinjection. Maximum transient changes in MAP and HR in response to NTS microinjection of leptin were measured, and BRS testing was repeated at 10 minutes after the leptin microinjection, with each animal serving as its own control. Indices of sympathovagal function were also analyzed using Nevrokard software (Nevrokard SA-BRS; Medistar).15 Consistent with the duration of recordings used in previous human and rodent studies,15–18 spontaneous BRS was determined from a minimum of 5 minutes of AP recordings obtained within 10 minutes of leptin injection, before the evoked baroreflex testing. Spontaneous BRS was calculated in the time (Sequence [Seq] Up, Seq Down, and Seq All) and frequency domains (low-frequency [LF] and high-frequency [HF] α indices). Time domain analysis was used to assess changes in HR variability (HRV), measured as the standard deviation of the beat-to-beat interval. Blood pressure variability (BPV) was measured in the time domain as the standard deviation of the MAP.

NTS Microinjections
Rat recombinant leptin (Sigma; 144 or 500 fmol [0.002 and 0.008 μg, respectively] in a 60-nL volume of 15.0 mmol/L of HCl and 7.5 mmol/L of NaOH diluted to pH 7.4 in artificial cerebrospinal fluid) or vehicle (60 nL) was microinjected bilaterally via pressure into the NTS (0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius [caudal tip of the area postrema], and 0.4 mm below the dorsal surface) using a glass micropipette connected to a syringe, as reported previously.12,13 The doses and volume of leptin were comparable to previous NTS microinjection studies in which the peptides effectively altered BRS.12,19,20 NTS microinjection of the vehicle solution had no significant effect on MAP, HR, or BRS for control of HR in SD or ASrAOGEN rats (Table S1 and Figure S1 in the online Data Supplement, available at http://hyper.ahajournals.org). Similar to previous studies in our laboratory,12,13 the vehicle had no effect on spontaneous BRS or BPV in SD and ASrAOGEN rats. However, HRV increased after injection of vehicle (1.81 ± 0.29 versus 2.32 ± 0.17 ms after vehicle; P < 0.05) in SD rats, with no effect of vehicle on HRV in ASrAOGEN rats. At the end of experiments, brains were removed, frozen, and sectioned (30 μm) for localization of microinjection sites (Figure S2). Only data from injections within the medial NTS at the rostro-caudal level −13.3 to −14.0 mm caudal to bregma were used in the analysis.

Quantification of Leptin Receptor and p85 α mRNA
Leptin receptor and phosphoinositide-3 kinase (PI3K) p85 α mRNA were measured in dorsal medullary tissue from separate groups of naive 15-week-old SD (n = 9) and ASrAOGEN (n = 7) rats. Brains were removed and placed on dry ice for excision of 2-mm3 dorsal medullary sections. The sections were obtained from 1 mm in front of to 1 mm behind the usual placement of the pipette, corresponding with the expected injectate spread20 and including portions of area postrema, dorsal motor nucleus, and nucleus gracilis. Isolation of RNA from excised tissue was assessed for concentration and stability. Total RNA (1 μg) was reverse transcribed using AMV reverse transcriptase in a 20-μL reaction mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in reverse-transcriptase buffer, as described previously.12,13 For real-time PCR, 2 μL of resultant cDNA were added to TaqMan Universal PCR Master Mix with the appropriate gene-specific primer/probe set for leptin receptor and p85 α (Applied Biosystems), and amplification was performed. All of the reactions were performed in triplicate. 18S ribosomal RNA served as the internal control. Results were quantified as Ct values, where Ct is the threshold cycle of PCR at which an amplified product is first detected, and was defined as relative gene expression (ratio of target:control).

Results
Effect of NTS Microinjection of Leptin on BRS for Control of HR in SD Rats
In SD rats, NTS microinjection of 144 fmol of leptin impaired BRS for control of HR measured as the bradycardic response to increases in AP produced by PE by 22%, an effect that did not reach significance (Figure 1A and 1D). In contrast, the 500-fmol leptin dose significantly impaired BRS by 63% in SD rats (Figure 1B and 1D), indicative of a dose-dependent response for leptin actions on BRS within the NTS. There were no differences in baseline BRS values or PE-induced increases in AP (Figure S3) among SD rats receiving various doses of leptin. Time-course experiments showed that the BRS was impaired at 10 and 60 minutes with partial recovery at 120 minutes after NTS microinjection of the 500-fmol leptin dose in SD rats (Figure 1C). BRS for control of HR measured as the tachycardic response to decreases in AP produced by sodium nitroprusside was not altered by NTS microinjection of 500 fmol of leptin (Figure S4).

Effect of NTS Microinjection of Leptin on BRS for Control of HR in ASrAOGEN Rats
The bradycardic BRS was significantly higher in anesthetized ASrAOGEN rats relative to SD rats at baseline (P < 0.01), with no differences in the PE-induced increases in AP between strains (Figure S3). NTS microinjection of both 144-
and 500-fmol leptin doses impaired the bradycardic BRS in ASrAOGEN rats corresponding with a 50% and 68% reduction, respectively (Figure 2A, 2B, and 2D). The 144-fmol leptin group shows data from younger (n=3) and older (n=5; 18 to 21 months) ASrAOGEN rats, because there were no differences in BRS values at baseline or in response to leptin in younger (1.56±0.15 ms/mm Hg baseline versus 0.81±0.19 ms/mm Hg after leptin; P<0.05) and older (1.40±0.09 ms/mm Hg baseline versus 0.72±0.12 ms/mm Hg after leptin injection; P<0.01) rats. The 500-fmol dose was only tested in younger ASrAOGEN rats. Similar to SD rats, there were no differences in baseline BRS or PE-induced increases in AP (Figure S3) among ASrAOGEN rats receiving varying leptin doses. In ASrAOGEN rats, the BRS remained suppressed at 10, 60, and 120 minutes after NTS microinjection of 500 fmol of leptin (C; n=3). In SD rats (D), the slope of the relationship between the increases in MAP produced by PE and the corresponding reflex bradycardia (expressed as pulse interval) shows graded reductions in the linear regression slope with increasing doses of leptin (1.05±0.14 ms/mm Hg baseline; 0.91±0.27 ms/mm Hg after 144 fmol of leptin; 0.38±0.10 ms/mm Hg after 500 fmol of leptin [r=−0.48 to 0.60]) in data from pooled SD rats. PI indicates pulse interval. *P<0.05 vs baseline, †P<0.01 vs baseline.

Figure 1. Effect of NTS leptin microinjection on BRS for control of HR evoked by PE in SD rats. NTS microinjection of 144 fmol of leptin impaired BRS by 22% in SD rats, an effect that did not reach significance (A; n=4). In contrast, 500-fmol leptin injection impaired BRS by ~63% (B; n=5). In SD rats, the BRS was impaired at 10 and 60 minutes, with evidence of recovery at 120 minutes after NTS microinjection of 500 fmol of leptin (C; n=3). In SD rats (D), the slope of the relationship between the increases in MAP produced by PE and the corresponding reflex bradycardia (expressed as pulse interval) shows graded reductions in the linear regression slope with increasing doses of leptin (1.05±0.14 ms/mm Hg baseline; 0.91±0.27 ms/mm Hg after 144 fmol of leptin; 0.38±0.10 ms/mm Hg after 500 fmol of leptin [r=−0.48 to 0.60]) in data from pooled SD rats. PI indicates pulse interval. *P<0.05 vs baseline, †P<0.01 vs baseline.

Figure 2. Effect of NTS leptin microinjection on BRS for control of HR evoked by PE in ASrAOGEN rats. In ASrAOGEN rats, NTS microinjection of 144 (A; n=8) and 500 (B; n=4) fmol of leptin significantly impaired BRS by 50% and 68%, respectively. The BRS was impaired at 10, 60, and 120 minutes after NTS microinjection of 500 fmol of leptin (C; n=3). In ASrAOGEN rats (D), the 144- and 500-fmol leptin doses produced equivalent reductions in the slope of the regression line (1.11±0.10 ms/mm Hg baseline; 0.45±0.19 ms/mm Hg after 144 fmol of leptin; 0.53±0.08 ms/mm Hg after 500 fmol of leptin [r=−0.61 to 0.89]). PI indicates pulse interval. *P<0.05 vs baseline; †P<0.001.
Table 1. Leptin Influence on Indices of Spontaneous BRS

<table>
<thead>
<tr>
<th>Group</th>
<th>Seq Up, ms/mm Hg</th>
<th>Seq Down, ms/mm Hg</th>
<th>Seq All, ms/mm Hg</th>
<th>LFα</th>
<th>HFα</th>
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<tr>
<td>SD 144 fmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>1.96±0.07</td>
<td>2.19±0.26</td>
<td>2.39±0.29</td>
<td>1.53±0.48</td>
<td>2.08±0.46</td>
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<tr>
<td>After leptin</td>
<td>2.05±0.06</td>
<td>2.70±0.09</td>
<td>2.56±0.14</td>
<td>2.48±0.23</td>
<td>2.25±0.21</td>
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<tr>
<td>SD 500 fmol</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.39±0.10</td>
<td>1.72±0.33</td>
<td>1.52±0.13</td>
<td>0.95±0.26</td>
<td>1.52±0.18</td>
</tr>
<tr>
<td>After leptin</td>
<td>1.06±0.10</td>
<td>0.97±0.16</td>
<td>1.05±0.12*</td>
<td>0.62±0.13</td>
<td>0.89±0.20*</td>
</tr>
<tr>
<td>AS 144 fmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.38±0.59</td>
<td>1.71±0.44</td>
<td>2.14±0.45</td>
<td>1.26±0.40</td>
<td>2.12±0.51</td>
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<tr>
<td>After leptin</td>
<td>1.28±0.35†</td>
<td>1.45±0.46</td>
<td>1.16±0.29†</td>
<td>1.39±0.39</td>
<td>1.18±0.36†</td>
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<tr>
<td>AS 500 fmol</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>2.14±0.46</td>
<td>1.58±0.07</td>
<td>2.05±0.33</td>
<td>1.83±0.20</td>
<td>1.9±0.32</td>
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<tr>
<td>After leptin</td>
<td>0.71±0.30†</td>
<td>0.79±0.33</td>
<td>0.77±0.32*</td>
<td>0.56±0.21*</td>
<td>0.50±0.24*</td>
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</table>

Values are mean±SEM and represent indices of spontaneous BRS measured in the time (Seq Up, Seq Down, and Seq All) and frequency (HFα and LFα) domains before and within 10 minutes of NTS microinjection of leptin. AS indicates ASrAOGEN.

*P<0.05 vs respective baseline.
†P<0.01 vs respective baseline.

Leptin Influence on Indices of Sympathovagal Function

There were no differences in baseline indices of spontaneous BRS, HRV, or BPV within groups of SD or ASrAOGEN rats. Indices of spontaneous BRS were also similar at baseline between anesthetized ASrAOGEN and SD rats (Table 1). However, baseline values of HRV, a measure of cardiac vagal tone, and BPV were significantly higher in ASrAOGEN rats relative to SD rats (Table 2; P<0.001 and P<0.05, respectively). Similar to the evoked baroreflex measurements, 144 fmol of leptin had no significant effect on spontaneous BRS indices, HRV, or BPV in SD rats. In contrast, the 144-fmol leptin dose significantly reduced spontaneous BRS (Seq All; P<0.01), as well as HRV, in ASrAOGEN rats. Specifically, vagal indices of the BRS were reduced (Seq Up and HFα), with no effect on sympathetic indices of the spontaneous BRS (Seq Down and LFα) in these animals. The 500 fmol of leptin reduced vagal spontaneous BRS indices (Seq Up and HFα) and HRV in both SD and ASrAOGEN rats. In addition, 500 fmol of leptin reduced the LFα sympathetic index in ASrAOGEN rats only (Table 1). Although 500 fmol of leptin significantly increased BPV in SD rats, there was no effect at either dose in ASrAOGEN rats on this parameter (Table 2).

MAP and HR Responses to NTS Microinjection of Leptin

There were no significant differences in MAP or HR within groups of SD or ASrAOGEN rats. However, as reported previously,12 the pooled baseline MAP was significantly higher in anesthetized ASrAOGEN rats relative to SD rats (112±5 versus 85±3 mm Hg, respectively; P<0.001; n=12 per group). The pooled baseline HR was also significantly higher in ASrAOGEN rats (345±14 versus 286±9 bpm; P<0.01). There was no significant effect of acute NTS microinjection of either 144 or 500 fmol of leptin on resting MAP in SD or ASrAOGEN rats (Table S1). Although leptin injection had no effect on resting HR in SD rats, HR was modestly reduced after 144 fmol of leptin with no effect of the 500 fmol of leptin in ASrAOGEN animals. Values of MAP and HR were not different from baseline at the time of reflex testing, at 10 minutes after the initial leptin microinjection (Table S1).

Differences in Leptin Receptor and PI3K mRNA in SD and ASrAOGEN Rats

Relative gene expression of the leptin receptor and PI3K regulatory subunit p85 α were measured in dorsal medullary tissue of naive SD (n=9) and ASrAOGEN (n=7) rats at 15 weeks of age (Figure 3). Leptin receptor mRNA was 3- to 4-fold higher and p85 α mRNA was 2-fold higher in the dorsal medulla of ASrAOGEN rats relative to SD rats. There were no differences in control 18S ribosomal Ct values...
Discussion

In the present study, we determined the effects of exogenous leptin on blood pressure and baroreceptor reflex regulation at the level of the NTS. Our results demonstrate that NTS microinjection of leptin impairs BRS for control of HR in response to increases in AP, an index of parasympathetic activity. The leptin-mediated impairment in BRS was associated with a shift in indices of sympathovagal balance toward a decrease in parasympathetic function, with no significant acute effect on resting MAP or HR. The novel finding that exogenous leptin impairs BRS for control of HR within the NTS may have implications for understanding the contribution of elevated leptin to baroreflex dysfunction. In addition, we examined whether leptin modulation of the BRS was altered in a model of enhanced leptin sensitivity to metabolic actions associated with basal differences in the central renin-Ang system by comparing leptin responses in control SD and transgenic AShAOGEN rats. AShAOGEN rats were more sensitive to BRS impairment in response to NTS microinjection of leptin. The enhanced sensitivity to exogenous leptin was associated with increased leptin receptor and PI3K p85 α mRNA in the dorsal medulla of AShAOGEN rats, suggesting that long-term reductions in brain Ang peptides or the subsequent consequences may be associated with upregulation of leptin signaling pathways.

Evidence suggests that key hypothalamic nuclei mediate increases in SNA and AP in response to exogenous leptin, likely involving descending pathways to brain stem nuclei involved in cardiovascular regulation. Independent of descending pathways, leptin receptors have been localized within the NTS and mediate both gastric and cardiovascular responses. NTS microinjection of a substantially higher leptin dose than used in the present study (1 μg) increases SNA and AP at 2 hours after the injection, consistent with baroreflex modulation. However, these studies did not examine the effect of NTS microinjection of leptin on baroreceptor reflex regulation. Indeed, the localization of leptin receptors to vagal afferent fibers and within the NTS implicates leptin as a direct modulator of BRS for control of HR. Although previous studies have shown that IV leptin does not acutely alter the sympathetically mediated baroreflex control of renal SNA, the contribution of circulating leptin to BRS for control of HR, a vagally mediated index that is often impaired in conditions with chronically elevated plasma leptin levels, has yet to be examined.

The results of the present study provide evidence for a direct action of exogenous leptin to modulate baroreflex function, because NTS microinjection of 500 fmol of leptin impaired BRS for control of HR in both SD and AShAOGEN rats. Leptin injection selectively altered BRS measured as the bradycardic response to increases in AP with no effect on BRS measured as the tachycardic response to decreases in AP in both SD and AShAOGEN rats, similar to actions of Ang II within the NTS. Although there are no published studies evaluating the tachycardic BRS in anesthetized AShAOGEN rats, the baseline tachycardic BRS values in SD rats are within the range of previously reported values using the same methods. NTS microinjection of 500 fmol of leptin did not alter depressor and bradycardic responses to cardiac vagal chemosensitive fiber activation (CVA) induced by IV phenylbiguanide in AShAOGEN rats (unpublished observation). The lack of alteration in CVA responses supports specificity of leptin actions, because these responses are mediated by chemoreceptor fibers that converge with baroreceptor inputs within the NTS. Leptin modulation of the BRS was transient in SD rats, with partial recovery at 120 minutes after the initial leptin microinjection. In contrast, there was no evidence of recovery in AShAOGEN rats at 120 minutes after the leptin injection. Although the mechanism for the lack of recovery of BRS in AShAOGEN rats is currently unknown, it may represent more sustained leptin actions within the NTS because of the upregulation of the leptin receptor and signaling pathways.

We cannot exclude the possibility that the spread of the leptin injection may have accessed the area postrema or the dorsal motor nucleus of the vagus for effects on baroreflex function. However, injection of 100 nL of 125I-Sar-Thr Ang II was mostly confined to the NTS. In addition, functional assessments show that 50 nL of an Ang II type 1 antagonist into the dorsal motor nucleus do not alter responses to NTS injection of Ang II. Because the injection of leptin accessed neuronal cell bodies, as well as presynaptic vagal afferents within the NTS, it is not clear which elements mediate the effects on BRS. However, Ang II is thought to exhibit its action on BRS primarily through vagal afferent fibers.

In SD rats, the 144-fmol leptin dose had no significant effect on BRS, whereas the 500-fmol dose impaired the BRS, suggesting a dose-response relationship. It appears that maximal suppression of the BRS was achieved with the lower dose of leptin in AShAOGEN rats, because both the 144-fmol and 500-fmol leptin doses impaired BRS to a similar degree. These results implicate an enhanced sensitivity of AShAOGEN rats to exogenous leptin within the NTS. Leptin impaired the BRS to ~0.5 ms/mm Hg in both strains, a level often observed in hypertension. As a possible mechanism for the enhanced sensitivity of AShAOGEN rats to exogenous leptin,
we observed a higher expression of the leptin receptor and PI3K p85 α mRNA in dorsal medullary tissue of ASrAOGEN rats relative to SD rats at 15 weeks of age. Although there are no differences in basal circulating leptin levels between strains at this age, sensitivity of leptin to a glucose challenge is enhanced in ASrAOGEN rats.11

The baseline values of MAP and HR were higher in anesthetized ASrAOGEN rats relative to SD rats, possibly because of an anesthesia-induced activation of the sympathetic nervous system observed in these animals.12 NTS microinjection of leptin resulted in no significant changes in resting MAP in either SD or ASrAOGEN rats, consistent with previous NTS microinjection studies using higher doses (8 to 31 pmol) of leptin.6,27 Only microinjection of 1 μg (63 pmol) of leptin within the NTS results in increases in SNA and AP in SD rats.6 However, these effects are delayed, requiring ≥2 hours for manifestation. Importantly, prolonged suppression of the BRS may contribute to the delayed modest increase in AP observed with NTS injection of higher doses of leptin.6 In the present study, differences in BRS after leptin administration are not attributable to differences in resting hemodynamics, confirming that the set point of the baroreflex is controlled independently from the sensitivity.12,13

Spontaneous and spectral analysis methods for measurements of BRS15 revealed no differences in spontaneous BRS values between strains, in contrast to the higher BRS in ASrAOGEN rats using the pharmacological approach. Although the classic method evokes changes in AP in an open-loop system, the spontaneous method measures changes over a smaller range (beat-to-beat) in a closed-loop model. Although a highly significant correlation exists between the 2 methods, differences have been reported in the BRS values obtained, perhaps because of differences in the sensitivity of the methods. Consistent with previous studies,28 baseline HRV was significantly higher in ASrAOGEN rats relative to SD rats, suggestive of an increased resting vagal tone in these animals. Interestingly, baseline BPV was also higher in anesthetized ASrAOGEN rats relative to SD rats, suggesting elevated sympathetic tone. Although in the conscious state there are no reported differences in BPV in ASrAOGEN rats,10,29 the state of anesthesia may result in an activation of the sympathetic nervous system in these animals.12

Similar to evoked BRS measurements, 144 fmol of leptin had no effect on spontaneous BRS indices, HRV, or BPV in SD rats. In ASrAOGEN rats, the 144 fmol of leptin impaired Seq All, Seq Up, and HFα and reduced HRV, providing further evidence for increased sensitivity to exogenous leptin in these animals. In both SD and ASrAOGEN rats, the 500-fmol leptin dose decreased Seq All, as well as vagal indices of spontaneous BRS (HFα and Seq Up) and HRV, further suggesting that leptin modulates BRS in response to increases but not decreases in AP. In ASrAOGEN rats, the 500 fmol of leptin also reduced the LFα index, with no effect in SD rats. Although this index is generally used as a marker of sympathetic activity, the spectral density of AP contained within this frequency is partially controlled by vagal tone.30 The 500-fmol leptin dose increased BPV in SD rats, further evidencing its role in altering cardiovascular autonomic balance. There was no effect of leptin on BPV in ASrAOGEN rats at either dose, perhaps because of the already high basal level of this index under anesthesia. Collectively, leptin altered blood pressure regulation, as assessed with either method and using a number of indices of autonomic function, similar to patterns observed in hypertension, obesity, and stroke,31 where the circulating peptide is often elevated.

Ang II increases leptin levels and promotes leptin production in vitro, suggesting a regulatory relationship between the peptides.32 Ang-converting enzyme inhibitors or Ang II type 1 receptor blockers reduce plasma leptin levels in patients with mild/moderate hypertension.33 Chronic Ang II type 1 receptor blockade prevents age-related increases in circulating leptin levels that are associated with decreases in dorsal medullary leptin receptor mRNA in Fischer 344 rats.34 Thus, chronic Ang II blockade maintains low endogenous leptin levels and increases leptin receptor mRNA. ASrAOGEN rats, with low endogenous brain Aogen, have decreased endogenous Ang II tone, contributing to BRS suppression within the NTS,12 and, therefore, may also have decreased leptin levels within the NTS. Although dorsal medullary leptin levels were not assessed in this study, leptin receptors and signaling pathways in ASrAOGEN rats appear upregulated on the basis of higher mRNA for the leptin receptor and PI3K p85 α relative to SD rats. The enhanced BRS suppression with exogenously administered leptin in ASrAOGEN rats is functional evidence consistent with this interpretation. Enhanced sensitivity to leptin could contribute to the overall enhanced metabolic phenotype observed in ASrAOGEN rats while maintaining low endogenous levels of leptin and, thus, a positive cardiovascular profile.10,12,28 Whether the upregulation of the leptin receptor and PI3K p85 α mRNA is attributed to a direct interaction with the renin-Ang system in the ASrAOGEN rats or an indirect effect is currently unknown; either low endogenous Ang II or leptin could contribute to the increased leptin receptor and PI3K mRNA expression in the dorsal medulla of ASrAOGEN rats.

We examined changes in BRS, MAP, and HR in response to acute, site-specific leptin administration. Effects of chronic peripheral or central leptin administration will need to be determined to further evaluate the role of leptin-mediated BRS impairments to pathophysiologies associated with elevated circulating, cerebrospinal fluid, or brain tissue leptin. Examination of the role of leptin in concert with other known modulators of cardiovascular and metabolic functions, such as Ang peptides, insulin, and glucose, may yield differing results, because recent studies show that central leptin infusion may improve glucose use in diabetic rats to have indirect beneficial effects on BRS and sympathovagal balance.35 Finally, examining the signaling pathways mediating the effects of leptin modulation of BRS within the NTS will help determine whether leptin uses different pathways for negative cardiovascular versus positive metabolic actions.

Perspectives
BRS for control of HR, a measure of vagal function, is often impaired in hypertension, obesity-related hypertension, and
stroke.\textsuperscript{7,31} Uncovering factors that modulate BRS may be important in understanding the predisposition to these conditions. Plasma leptin levels are elevated in obesity and independently in hypertension and stroke.\textsuperscript{36,37} and exogenous leptin contributes to sympathetically mediated elevations in AP.\textsuperscript{38} The present data suggest that leptin impairs BRS for control of HR, an index believed to precede and contribute to the development of hypertension. Therefore, leptin-mediated impairments in BRS may be permissive toward increases in AP observed in populations with elevated leptin levels. Studies suggest that resistance develops to the metabolic actions of leptin, with maintenance of sensitivity to the cardiovascular actions of leptin. Metabolic resistance to the leptin is associated with reduced transport into the brain and defects in intracellular signaling pathways.\textsuperscript{39,40} Reduction of leptin levels in patients with leptin resistance may prevent saturation of receptors to increase leptin transport, as well as to reduce negative regulators of leptin signaling to allow for maintenance of sensitivity to leptin’s positive metabolic effects. Concomitantly, low endogenous leptin levels may reduce activation of cardiovascular signaling pathways to prevent impairments in baroreflex function, as well as increases in AP and SNA. Understanding mechanisms to preserve leptin sensitivity, in the presence of low endogenous leptin levels, may be important for maintaining satiety effects while preventing negative cardiovascular effects of the peptide.

Acknowledgments

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Disclosures

None.

References


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LEPTIN IMPAIRS CARDIOVAGAL BAROREFLEX FUNCTION AT THE LEVEL OF THE SOLITARY TRACT NUCLEUS

By

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Short Title: Leptin-Mediated Impairments in Vagal Function

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Methods:
The institutional animal care and use committee approved all procedures. Animals: Experiments were performed in 3 to 5 month-old male Hannover Sprague-Dawley (SD) and transgenic TGR(ASrAogen)680 (ASrAOGEN) rats from the Hypertension and Vascular Research Center Colony, Wake Forest University School of Medicine, Winston-Salem, NC. The 144 fmol leptin group shows data from younger (n = 3) and older (n = 5; 18 to 21 months) ASrAOGEN rats as there were no differences in BRS values at baseline or in response to leptin in younger (1.56 ± 0.15 msec/mm Hg baseline versus 0.81 ± 0.19 msec/mm Hg after leptin; p < 0.05) and older (1.40 ± 0.09 msec/mm Hg baseline versus 0.72 ± 0.12 msec/mm Hg after leptin injection; p < 0.01) rats. The 500 fmol leptin dose was only tested in younger ASrAOGEN rats. Animals were housed in humidity- and temperature-controlled rooms in group cages (12-hour light/dark cycle) with free access to standard rat chow and water. Surgical Procedures and Hemodynamic Measures: As previously reported,1,2 rats were anesthetized with combination urethane-chloralose (750 mg and 35 mg per kg, respectively) via intraperitoneal injections, with intravenous (IV) supplemental doses given as needed. Animals were instrumented with femoral arterial and venous catheters for measurement of cardiovascular parameters and administration of drugs, respectively. Rats were placed in a stereotaxic frame with the head tilted downward at a 45° angle for surgical exposure of the dorsal medulla oblongata by incision of the atlanto-occipital membrane and breathed a mixture of 70% room air and 30% oxygen with body temperature maintained at 37.0 ± 1.0°C. Approximately 30 minutes was allowed after surgical procedures before baseline measurements were recorded. Pulsatile arterial pressure (AP) and mean AP (MAP) were monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1; Santa Barbara, CA) and heart rate (HR) was determined from the AP wave as previously reported.1,2 Baseline responses of baroreflex sensitivity (BRS) were established by bolus IV randomized injection of 3 doses (2, 5 and 10 µg/kg in 0.9% NaCl) of phenylephrine (PE) or sodium nitroprusside (NP), to determine the BRS in response to increases or decreases in AP, respectively. Bolus dose determinations were used as this method is more sensitive to parasympathetic alterations in the baroreflex relative to infusion determinations.3 A period of ≥ 30 minutes was allowed after baseline measurements before beginning microinjections. Maximum MAP responses (ΔMAP, mm Hg) and the associated reflex changes in HR (ΔHR, bpm) were recorded at each dose of PE or NP. ΔHR was converted to changes in pulse interval (ΔPI, msec) by the formula: 60,000/HR. BRS for bradycardia and tachycardia was determined for each animal as the slope of the relationship between changes in MAP and the corresponding PI generated from the 3 doses of PE and NP, independently as previously reported.1,2 Maximum changes in MAP and HR in response to NTS microinjection of leptin were measured and BRS testing was repeated within 10 minutes of leptin microinjection so that each animal was used as its own control. Reflex testing was completed within 30 minutes of leptin microinjection. Indices of sympathovagal function at baseline and in response to leptin were also analyzed using Nevrokard software (Nevrokard SA-BRS; Medistar, Ljubljana, Slovenia).4 Consistent with the duration of recordings used in previous human and rodent studies,4,7 we assessed a minimum of 5 min of AP recordings obtained from immediately after leptin injection until the 10 min time point when evoked baroreflex testing occurred. Spontaneous BRS was calculated using time and frequency domain analysis methods. For the sequence (seq) method, we quantified sequences of at least 3 beats in which systolic AP (SAP) consecutively increases (Seq Up) or decreases (Seq Down) and beat-to-beat interval (RRI) changes in the same direction on subsequent beats (n + 1). In the present
study, there was an average of 33 ± 4 up and 23 ± 2 down sequences utilized in the analysis of spontaneous BRS, well in excess of studies in humans in which the average spontaneous BRS values are based on 4 - 35 up and 4 - 11 down sequences. A linear correlation was calculated between RRI and SAP for each sequence. The mean of all individual regression coefficients was calculated as Seq All, which was used an index of spontaneous BRS. To measure BRS in the frequency domain, power spectral densities of SAP and RRI oscillations were computed, transformed and integrated over specified frequency ranges (LF = 0.25-0.75 Hz; HF = 0.75-3.0 Hz). The square root of the ratio of RRI and SAP powers were used to calculate LFα and HFα, indices of sympathetic and parasympathetic activity of the BRS, respectively. Time domain analysis was used to assess changes in heart rate variability (HRV) as measured by the standard deviation of the beat-to-beat interval in RRI duration. Blood pressure variability was measured by time domain analysis as the standard deviation of the MAP.

NTS Microinjections: Multi-barreled glass pipettes with an outer diameter of 30 to 50 µm were used as described previously. Rat recombinant leptin (Sigma; 144 or 500 fmol (0.002 and 0.008 µg, respectively) in a 60 nL volume of 15 mM HCl and 7.5 mM NaOH dissolved to pH 7.4 in artificial cerebrospinal fluid (CSF)) or vehicle (60 nL) was microinjected bilaterally via pressure into the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface] using a glass micropipette connected via PE 50 tubing to a syringe (1 mL; Becton, Dickinson and Company). Air pressure was generated by pushing on the syringe to displace the desired amount of leptin from the pipette into the NTS. This was visualized by movement of the fluid meniscus across the calibration line of the pipette barrel as previously reported. The doses and volume of leptin were comparable to previous NTS microinjection studies in which the peptides effectively altered BRS.

Histology: The brain was removed and frozen on dry ice at the end of each experiment for histological evaluation. Serial cryostat sections (30 µm) of the frozen medulla were used to assess the site of microinjections (Figure S2). Only data from injections within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were used in the analysis. The accuracy rate for injections was > 90%.

Quantification of leptin receptor and p85 alpha mRNA: Leptin receptor and phosphoinositide-3 kinase (PI3K) p85 alpha mRNA were measured in dorsal medullary tissue from separate groups of conscious 15-week old SD (n = 9) and ASrAOGEN (n = 7) rats. Brains were removed and placed on dry ice for excision of 2 mm³ dorsal medullary sections. The sections were obtained from 1 mm in front of to 1 mm behind the usual placement of the pipette, corresponding to the expected injectate spread and including portions of area postrema, dorsal motor nucleus and nucleus gracilis. Total RNA was isolated from dorsal medullary sections of using TRIZOL reagent (GIBCO Invitrogen, Carlsbad, CA). The RNA concentration and stability was assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Approximately 1 µg of total RNA was reverse transcribed using AMV reverse transcriptase in a 20 µL reaction mixture containing deoxyribonucleotides, random hexamers, and Rnase inhibitor in reverse transcriptase buffer. Heating the reverse transcriptase reaction product at 95°C terminated the reaction. For real-time PCR, 2 µL of the resultant cDNA was added to the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with the appropriate gene-specific primer/probe set (Applied Biosystems) and amplification was performed on an ABI 7000 Sequence Detection System. The mixtures were heated at 50°C for 2 minutes, at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in triplicate. 18S ribosomal RNA, amplified using TaqMan
Ribosomal RNA Control Kit (Applied Biosystems) served as the internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product is first detected, and was defined as relative gene expression (ratio of target/control).

**Analysis of Data:** Values are presented as mean ± standard error of the mean. A 2-way ANOVA was utilized to compare data between ASrAOGEN and SD strains. Comparisons of changes in BRS and indices of sympathovagal function in response to leptin or vehicle were compared to baseline using a one-sample paired t-test. Changes in resting MAP and HR over time and time-course experiments were analyzed by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. mRNA quantification was analyzed by an unpaired t-test between strains. The criterion for statistical significance was P < 0.05. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software, San Diego, CA).
Reference List:


Table S1. Values of MAP and HR in Response to NTS Microinjection of Leptin or Vehicle

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>MAP (mm Hg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SD 144 fmol Leptin</strong></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>81 ± 5</td>
<td>259 ± 11</td>
</tr>
<tr>
<td>Values at Peak Change</td>
<td></td>
<td>73 ± 10</td>
<td>248 ± 21</td>
</tr>
<tr>
<td>Value Prior to Reflex Testing</td>
<td></td>
<td>76 ± 7</td>
<td>244 ± 15</td>
</tr>
<tr>
<td><strong>SD 500 fmol Leptin</strong></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>91 ± 4</td>
<td>299 ± 14</td>
</tr>
<tr>
<td>Values at Peak Change</td>
<td></td>
<td>86 ± 5</td>
<td>287 ± 18</td>
</tr>
<tr>
<td>Value Prior to Reflex Testing</td>
<td></td>
<td>80 ± 9</td>
<td>282 ± 21</td>
</tr>
<tr>
<td><strong>AS 144 fmol Leptin</strong></td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>113 ± 8</td>
<td>324 ± 21</td>
</tr>
<tr>
<td>Values at Peak Change</td>
<td></td>
<td>103 ± 8</td>
<td>303 ± 28 *</td>
</tr>
<tr>
<td>Value Prior to Reflex Testing</td>
<td></td>
<td>113 ± 8</td>
<td>309 ± 25</td>
</tr>
<tr>
<td><strong>AS 500 fmol Leptin</strong></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>110 ± 10</td>
<td>373 ± 27</td>
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<tr>
<td>Values at Peak Change</td>
<td></td>
<td>103 ± 5</td>
<td>367 ± 34</td>
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<tr>
<td>Value Prior to Reflex Testing</td>
<td></td>
<td>110 ± 10</td>
<td>368 ± 31</td>
</tr>
<tr>
<td><strong>Vehicle in SD Rats</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>78 ± 1</td>
<td>302 ± 19</td>
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<tr>
<td>Values at Peak Change</td>
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<td>88 ± 10</td>
<td>295 ± 20</td>
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<tr>
<td>Value Prior to Reflex Testing</td>
<td></td>
<td>83 ± 13</td>
<td>293 ± 22</td>
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<tr>
<td><strong>Vehicle in AS Rats</strong></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>110 ± 4</td>
<td>366 ± 13</td>
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<tr>
<td>Values at Peak Change</td>
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<td>103 ± 6</td>
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<tr>
<td>Value Prior to Reflex Testing</td>
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<td>100 ± 7</td>
<td>351 ± 16</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Values represent baseline, peak changes in response to NTS microinjection of leptin and values at 10 minutes after the initial leptin microinjection (immediately prior to each series of reflex testing); N = number of animals; MAP = mean arterial pressure; HR = heart rate; SD = Sprague-Dawley; AS = ASrAOGEN

* = P < 0.01 versus Baseline
Figure S1. Effect of vehicle on BRS for control of HR
NTS microinjection of the vehicle solution (60 nL) had no effect on the bradycardic BRS for control of HR in response to increases in AP evoked by PE or the tachycardic BRS in response to decreases in AP evoked by NP in SD (A, n = 4) or ASrAOGEN (B, n = 3) rats.
Figure S2. Histological Analysis of Microinjection Sites
Photomicrography (5X magnification) of an unstained rat medullary section (30 µM) at approximately -13.8 mm caudal to bregma showing a typical microinjection site within the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface]. Only data from injections within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were used in this study. NG = nucleus gracilis, AP = area postrema, NTS = solitary tract nucleus, DMX = dorsal motor nucleus of the vagus, C = central canal.
Figure S3. α-adrenergic responsiveness in SD and ASrAOGEN rats at baseline and in response to NTS microinjection of leptin
Changes in mean arterial pressure (MAP) in response to randomized, intravenous graded doses of phenylephrine were assessed in anesthetized SD and ASrAOGEN rats at baseline and in response to NTS microinjection of 144 or 500 fmol leptin. There were no significant differences in phenylephrine-induced increases in MAP within groups of SD or ASrAOGEN rats.
Figure S4. BRS for control of HR in response to decreases in AP
BRS for control of HR was measured as the tachycardic response to decreases in AP evoked by NP before and after NTS microinjection of 500 fmol leptin in SD (A) and ASrAOGEN (B) animals. There were no significant differences in baseline responses to NP between SD and ASrAOGEN animals (n = 8 each group). In a subset of these animals (n = 4), leptin had no significant effect on responses to NP in either strain.