Endogenous leptin contributes to baroreflex suppression within the solitary tract nucleus of aged rats

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Arnold AC, Diz DI. Endogenous leptin contributes to baroreflex suppression within the solitary tract nucleus of aged rats. Am J Physiol Heart Circ Physiol 307: H1539–H1546, 2014. First published September 26, 2014; doi:10.1152/ajpheart.00282.2014.—The decline in barovagal baroreflex function that occurs with aging is accompanied by an increase in circulating leptin levels. Our previous studies showed that exogenous leptin impairs the baroreflex sensitivity for control of heart rate in younger rats, but the contribution of this hormone to baroreflex dysfunction during aging is unknown. Thus we assessed the effect of bilateral leptin microinjection (500 fmol/60 nl) within the solitary tract nucleus (NTS) on the baroreflex sensitivity in older (66 ± 2 wk of age) urethane/chloralose anesthetized Spraque-Dawley rats with elevated circulating leptin levels. In contrast to the 63% reduction observed in younger rats, leptin did not alter the baroreflex sensitivity for bradycardia evoked by phenylephrine in older rats (0.76 ± 0.19 baseline vs. 0.71 ± 0.15 ms/mmHg after leptin; P = 0.806). We hypothesized that this loss of sensitivity reflected endogenous suppression of the baroreflex by elevated leptin, rather than cardiovascular resistance to the peptide. Indeed, NTS administration of a leptin receptor antagonist (75 pmol/120 nl) improved the baroreflex sensitivity for bradycardia in older rats (0.73 ± 0.13 baseline vs. 1.19 ± 0.26 at 10 min vs. 1.87 ± 0.32 at 60 min vs. 1.22 ± 0.54 ms/mmHg at 120 min; P = 0.002), with no effect in younger rats. There was no effect of the leptin antagonist on the baroreflex sensitivity for tachycardia, responses to cardiac vagal chemosensitive fiber activation, or resting hemodynamics in older rats. These findings suggest that the actions of endogenous leptin within the NTS, either produced locally or derived from the circulation, contribute to baroreflex suppression during aging.

THE ARTERIAL BARORECEPTOR reflex is critically involved in short-term regulation of blood pressure through modulation of central autonomic pathways that control sympathetic and parasympathetic outflow to peripheral cardiovascular organs. During aging, there is impairment of the baroreflex sensitivity (BRS) for control of heart rate (HR) (13, 15, 32), a marker of parasympathetic tone mediated at the level of the solitary tract nucleus (NTS) in the dorsal medulla (2), due to changes in central neuronal and peripheral vascular function. Importantly, baroreflex dysfunction is permissive to chronic sympathetic activation to increase blood pressure and its variability and is an independent risk factor for cardiovascular morbidity and mortality (25, 42). The precise mechanisms involved in age-related baroreflex impairments, however, are not fully understood. The identification of hormones or other factors that modulate autonomic brainstem regions, such as the NTS, may therefore improve our understanding of the decline in baroreflex function that occurs with aging.

Aging is also associated with increased levels of leptin, in part attributed to the development of obesity (1, 22, 38). Leptin is an adipocyte-derived hormone that acts primarily in the hypotalamus to reduce food intake and increase energy expenditure (18). Leptin can also elicit deleterious cardiovascular effects such as sympathetic-mediated elevations in blood pressure, through receptors distributed to brainstem and hypothalamic nuclei regulating autonomic outflow (29, 35, 44). Since the active long form of the leptin receptor (Ob-Rb) is also localized to the nodose ganglion, to vagal afferent fibers, and on cell bodies within the NTS (29, 31), it is likely that the sympathoexcitatory actions of leptin involve baroreflex suppression. Indeed, our previous studies show that leptin administration within the NTS impairs the BRS for control of HR in younger rats (4). There is no information, however, on the contribution of leptin to baroreflex dysfunction during aging.

Thus the present study assessed the role of leptin in modulation of baroreceptor reflex function in older Sprague-Dawley (SD) rats, an animal model of aging that exhibits baroreflex impairment and increases in body weight and adiposity that are associated with metabolic resistance to elevated levels of insulin and leptin (22, 23). First, we examined the effect of acute, site-specific microinjection of leptin within the NTS on the BRS for control of HR, and other hemodynamic and reflex measures, in older SD rats. We provide evidence that exogenous leptin does not alter the BRS in older rats, when given at a dose previously shown to suppress baroreflex function in younger rats. We hypothesized that this loss of sensitivity reflects endogenous suppression of the baroreflex by elevated leptin levels, rather than a cardiovascular resistance to this hormone. To test this hypothesis, we examined the effect of NTS microinjection of a leptin receptor antagonist on the BRS and resting cardiovascular function in a separate group of animals.

METHODS

Animals. Experiments were performed in younger (19 ± 1 wk) and older (66 ± 2 wk) male Hannover SD rats obtained from the Hypertension and Vascular Research Center colony at the Wake

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The pipette tip was localized to the medial NTS at rostrocaudal level end of each experiment to determine the accuracy of microinjection. BRS at 60 and 120 min after injections to establish a time course of been previously documented, and therefore, we also measured the effects of the leptin antagonist on baroreflex function have not rats (4), and thus this time point was used for older rats in this study.

Surgical procedures. Rats were given an intraperitoneal bolus of combination urethane and α-chloralose anesthesia (750 and 35 mg/kg, respectively), with subsequent intravenous doses given as needed. Femoral arterial and venous catheters were inserted for measurement of systemic hemodynamics and drug infusions, respectively. Rats were placed in a stereotaxic frame with the head tilted downward (45° angle) for surgical exposure of the dorsal medulla oblongata. A recovery period of at least 30 min was allowed after surgical procedures before baseline measurements were obtained.

Hemodynamic measures and reflex testing. A strain gauge transducer was connected to the femoral artery catheter to monitor, record, and digitize pulsatile arterial pressure (AP) and mean AP (MAP) using a data acquisition system (BIOPAC; Acknowledge Software Version 3.8.1). HR was derived from the AP wave. The evoked BRS was measured by the Oxford method in which randomized, intravenous boluses of graded doses of phenylephrine or sodium nitroprusside (2, 5, and 10 μg/kg in 0.9% NaCl) are given to increase or decrease pressure, respectively (26). The BRS was determined for each animal as the slope of the relationship between changes in MAP and the pulse interval (60,000/HR). Since leptin selectively suppresses the BRS for bradycardia in younger SD rats (4), we used bolus injections that are more sensitive to detect changes in this component compared with ramp infusions (24). The average r value for each individual rat showed a very tight fit of the data (>0.95), although r values for the line of best fit shown for the group data are generally lower. An intravenous bolus of the 5-HT1 receptor agonist phenylbiguanide (10 μg/kg in 0.9% NaCl) was given to determine reflex responses to cardiac vagal chemosensitive fiber activation. Immediate depressor and bradycardic responses to phenylbiguanide were assessed, to avoid direct activation of NTS neurons, and were expressed as percent change from baseline to account for differences in resting MAP and HR among animals. All reflex testing was completed within 30 min.

NTS microinjections. At least 30 min after baseline measurements, we performed bilateral injections via pressure into the NTS (0.4 mm lateral from the dorsal surface). Since the resting level of blood pressure can confound baroreflex function, continuous changes in MAP and HR were measured following microinjections to ensure that these hemodynamic parameters stabilized before reflex testing. The reflex testing was performed at 10 min after the injection, to avoid potential transient hemodynamic effects in the first several minutes after drug administration (4, 6, 8), with each animal serving as its own control. Leptin suppressed the BRS at 10 min after NTS injection in younger rats (4), and thus this time point was used for older rats in this study. The effects of the leptin antagonist on baroreflex function have not been previously documented, and therefore, we also measured the BRS at 60 and 120 min after injections to establish a time course of action. The dorsal medulla was frozen and cryostat sectioned at the end of each experiment to determine the accuracy of microinjection sites, as previously illustrated (4, 6, 7, 30). Data were only included if the pipette tip was localized to the medial NTS at rostrocaudal level −13.3 to −14.0 mm caudal to bregma. There was one missed injection during leptin antagonist administration in an older rat, resulting in a 94% success rate for correct NTS localization in this study. In this older rat, the injection tip was localized to the dorsal motor nucleus of the vagus, which resulted in no change in the BRS for bradycardia evoked by phenylephrine following leptin antagonist administration (0.61 at baseline vs. 0.56 ms/mmHg leptin antagonist). Our previous studies have shown that injection of a similar volume of monoiodinated angiotensin II was primarily limited to the NTS (6). In addition, cardiovascular responses to NTS microinjection of angiotensin II were not prevented by AT1 or AT2 receptor blockade in the dorsal motor nucleus (16). Thus functional and anatomical evidence supports a site of action within the NTS for the baroreflex effects observed. Finally, NTS vehicle injections of similar volumes produced no effect on hemodynamic measurements or reflex function in younger and older SD rats over the time frame studied (4, 36). Thus vehicle injections were not repeated in this study.

Drugs. Rat recombinant leptin was injected at a dose and volume previously shown to suppress the BRS in younger rats (500 fmol in 60 nl of 0.9% NaCl; Sigma-Aldrich) (4). The leptin receptor antagonist is a triple mutant of the full-length rat peptide that competitively binds Ob-Rb to prevent activation of intracellular signaling pathways (L39A/D40A/F41A; Raybiotech). This antagonist has been used in numerous in vivo studies to determine the role of endogenous leptin in metabolic and cardiovascular regulation. Specifically, intracerebroventricular administration: 1) increases body weight, adiposity, and food intake (45); 2) inhibits leptin-mediated signaling in hypothalamus (43, 45); 3) inhibits anorexie and weight-reducing effects of leptin (45); 4) reverses hypertension due to central leptin overexpression (43); and 5) reduces blood pressure and renal sympathetic nerve activity (27). The leptin receptor antagonist was given at a dose derived from these intracerebroventricular studies (75 pmol in 120 nl of artificial cerebrospinal fluid) (45). A larger volume of antagonist was used to block the entire NTS area where incoming afferents mediate the reflex.

Circulating hormone measurements. Separate groups of younger (n = 7) and older (n = 6) anesthetized SD rats were subjected to rapid decapitation for collection of trunk blood. Serum leptin and insulin levels were measured by radioimmunoassay analysis (Linco Research, St. Charles, MO). Serum glucose was measured using a Freestyle glucose monitor (TheraSense).

Statistical analysis. Values are presented as means ± SE. Analyses were performed using Prism 4.0 (GraphPad Software), with a two-tailed P < 0.05 defined as statistical significance. Differences in resting reflex function and hemodynamics between younger and older SD rats were compared using an unpaired t-test. The effects of leptin injection on BRS and responses to cardiac vagal chemosensitive fiber activation in older rats were compared with baseline using a paired t-test. Changes in outcomes over time (i.e., MAP and HR in response to injections, BRS responses to leptin antagonist) were compared within each group using a one-way repeated-measures ANOVA with post hoc Student Newman-Keuls multiple comparisons.

RESULTS

Cardiovascular reflex function during aging. Older SD rats had higher body weight compared with younger rats (608 ± 13 vs. 434 ± 17 g, respectively; P < 0.001). In addition, older anesthetized SD rats had higher circulating insulin (3.67 ± 1.20 older vs. 1.48 ± 0.31 ng/ml younger; P = 0.032) and leptin (2.48 ± 0.25 older vs. 1.54 ± 0.30 ng/ml younger; P = 0.039) levels, with no significant differences in glucose (143 ± 25 older vs. 129 ± 25 mg/dl younger; P = 0.721). Resting MAP was lower in older rats under anesthesia (Table 1; P = 0.022), with no significant difference in resting HR (P = 0.89). As expected, the resting BRS for bradycardia evoked by phenylephrine was lower in older rats (Fig. 1, A and B; P = 0.008), with no differences in phenylephrine-induced increases in MAP (Fig. 1C). There were no differences in the resting BRS for tachycardia evoked by sodium nitroprusside (Fig. 1D;
In contrast to the 63% reduction observed in younger rats in our previous study (Fig. 2, 4), there was no effect of NTS leptin microinjection on the BRS for tachycardia in younger rats. Leptin injection produced a transient decrease in resting MAP in older rats (Table 1; \( P = 0.033 \)) which recovered to baseline levels before reflex testing, with no significant effect on HR (\( P = 0.128 \)). There was no effect of leptin injection on the BRS for tachycardia in younger rats (0.22 \pm 0.07 baseline vs. 0.18 \pm 0.14 mmHg after leptin) or older (0.18 \pm 0.04 baseline vs. 0.17 \pm 0.09 mmHg after leptin; \( P = 0.932 \)) rats or on responses to activation of cardiac vagal chemosensitive fibers (Table 2).

**Endogenous leptin contribution to baroreflex suppression during aging.** Representative MAP and HR responses to evoked BRS testing with phenylephrine, at baseline and following NTS leptin receptor antagonist injection, are shown in Fig. 3. The leptin receptor antagonist produced a significant facilitation of the BRS for bradycardia in older SD rats, an effect that peaked at 60 min, and returned towards baseline at 120 min after the NTS injection (Fig. 4A; \( P = 0.002 \)). In contrast, leptin antagonism within the NTS did not significantly alter the BRS for bradycardia over the same time course.
in younger rats (Fig. 4B; $P = 0.171$). There was no effect of the leptin receptor antagonist on the BRS for tachycardia in either younger (0.07 ± 0.05 baseline vs. 0.31 ± 0.13 at 10 min vs. 0.16 ± 0.09 at 60 min vs. 0.16 ± 0.08 ms/mmHg at 120 min; $P = 0.561$) or older (0.10 ± 0.06 baseline vs. 0.11 ± 0.04 at 10 min vs. 0.25 ± 0.08 at 60 min vs. 0.34 ± 0.21 ms/mmHg at 120 min; $P = 0.423$) rats or on responses to cardiac vagal chemosensitive fiber activation (Table 2). The leptin receptor antagonist transiently reduced MAP in younger rats, an effect that recovered to baseline levels before reflex testing, with no effect in older rats (Table 1). There was no effect of the leptin receptor antagonist on HR in either group (Table 1).

**DISCUSSION**

The overall goal of this study was to determine the role of leptin within the NTS in baroreceptor reflex regulation during aging. Our findings suggest NTS microinjection of leptin in older SD rats results in no effect on the BRS for bradycardia, the BRS for tachycardia, or responses to cardiac vagal chemo-
sensitive fiber activation. These findings contrast the ~63% reduction in the BRS for bradycardia in response to leptin injection in younger rats (4) and suggest that there is loss of sensitivity to endogenous leptin modulation of cardiovagal baroreflex function during aging. We further administered a leptin receptor antagonist within the NTS to determine if this loss of sensitivity reflects increased endogenous leptin tone for BRS suppression. Indeed, leptin receptor antagonism improved the BRS for bradycardia in older rats with elevated circulating leptin but not in younger rats with normal levels of the hormone. These findings provide new evidence that elevations in endogenous leptin, acting at the level of the NTS, can contribute to age-related baroreflex dysfunction.

Cardiovascular reflex function during aging. The pattern of metabolic and cardiovascular derangements observed with aging in SD rats in this study is consistent with our previous reports (22, 23, 36). In addition, we observed a selective impairment of the BRS for bradycardia, an established measure of parasympathetic tone, with no difference in baroreflex control of sympathetic activity to the heart in older anesthetized rats. The resting BRS for tachycardia was lower than that for bradycardia but was within the range of previously reported values in anesthetized rats (4, 6). A similar selective impairment of the parasympathetic components of the BRS has been observed in conscious older SD rats (20), in diet-induced obese rats (5), and in human hypertension (17). The lower BRS for bradycardia in older rats was not due to differences in α-adrenergic responsiveness, as pressor responses to phenylephrine were similar compared with younger rats. A similar finding was observed in coronary vasculature from aged Fischer 344 rats (41) but reduced vascular α-adrenergic sensitivity has also been shown in older anesthetized SD rats (36). Systolic blood pressure increases during aging in conscious SD rats (22), but the use of anesthesia can confound resting blood pressure in these animals. Our previous study showed no difference in MAP between older and younger anesthetized rats (36), with the present study showing modest hypotension. While we do not know the precise mechanism underlying this phenomenon, the preparations were stable throughout the study for both older and younger rats, and a decrease in resting blood pressure with anesthesia would be expected to improve baroreflex function, which was not observed. Finally, responses to stimulation of cardiac vagal chemosensitive afferents were normal in this study when compared with younger rats (4, 37), but enhanced depressor responses to phenylbiguanide have been observed in conscious older rats (10).

Exogenous leptin modulation of baroreflex function. Acute leptin administration within the NTS impairs the BRS for bradycardia in younger rats (4, 9), providing evidence that leptin directly modulates cardiovagal baroreflex function. Our findings suggest loss of sensitivity to exogenous leptin for baroreflex modulation in older rats, as we observed no effect on the BRS at a dose producing profound impairment in younger rats. There are, however, several potential alternative explanations for this finding. First, a higher dose of leptin may have suppressed the BRS in older rats, but this was not assessed. Second, it is possible that the baroreflex was already maximally suppressed in older SD rats, thus preventing any further effect of exogenous leptin. This is unlikely as our previous studies show that the BRS can be reduced to levels of 0.4 ms/mmHg with pharmacologic interventions, which is lower than the level of resting BRS observed in this study (~0.75 ms/mmHg) (3, 4). Finally, the time course for effects of leptin administration within the NTS on the BRS may differ between younger and older rats.

In younger SD rats, NTS leptin injection produced baroreflex suppression independent of acute blood pressure changes, providing evidence that different neural mechanisms are involved in the modulation of resting pressure and the BRS (4). In contrast, higher doses of leptin within the NTS can evoke pressor and sympathoexcitatory responses in younger rats (9, 29). The finding that leptin transiently decreased blood pressure in older SD rats, an action not seen in the younger rats in the previous study, may suggest that the neural pathways activated by exogenous leptin within the NTS are altered with aging. The mechanism underlying this depressor response is unclear, and this was not the focus of our study. Similar transient depressor responses to angiotensin II injection within the NTS have been observed, in part due to glutamate and substance P release (12, 16). While our collective findings suggest that leptin does not play a role in modulation of cardiac vagal chemosensitive fibers, this hormone has been shown to modulate neuronal circuits within the NTS controlling respi-
Endogenous leptin tone for baroreflex suppression during aging. Older SD rats exhibit elevated circulating leptin levels (22, 23), which could access autonomic pathways either through leptin transporters or via blood-brain barrier deficient circumventricular organs to contribute to baroreflex suppression. Alternatively, leptin is produced within the brain and could be elevated locally within the NTS for baroreflex modulation. While the source is unknown, our results suggest that endogenous leptin contributes to age-related baroreflex impairment at the level of the NTS. Consistent with findings for delayed improvement in the BRS for bradycardia, central administration of this leptin receptor antagonist produces delayed depressor and sympathoinhibitory effects in high-fat-fed rats (27). The selectivity for endogenous leptin to suppress the BRS for bradycardia is consistent with effects of exogenous angiotensin II and leptin within the NTS (4, 6). As further evidence of specificity, responses to activation of cardiac vagal chemosensitive fibers were not affected by leptin antagonism, which are mediated by chemoreceptor afferents that converge with baroreceptor inputs in the NTS (21, 34). It does not appear that endogenous leptin contributes to baroreflex modulation within the NTS under normal conditions in younger rats. A similar pattern is reported for the role of the endogenous cannabinoid system in BRS regulation during aging. In older SD rats, elevated endogenous expression of the endocannabinoid 2-arachidonoylglycerol is associated with the BRS impairment, and blockade of CB1 receptors in the NTS restores the BRS to normal levels. In contrast, the CB1 antagonist has no effect on the BRS in young animals (40). Finally, injection of the leptin receptor antagonist within the NTS transiently decreased blood pressure in younger but not older SD rats. The significance of this finding with respect to support of resting pressure in younger vs. older animals is not known. Since the resting level of pressure attained following either leptin or leptin antagonist injection was similar in older rats (e.g., 68 ± 6 vs. 67 ± 8 mmHg; respectively), it is possible that these effects represent random differences in baseline pressure. Alternatively, different effects in the older and younger rats may reflect varying effects of endogenous leptin at the two ages.

While not explored in this study, it is important to note that the loss of sensitivity to exogenous leptin and the actions of endogenous leptin to suppress the BRS could also be associated with cardiovascular resistance to leptin actions during aging. Leptin resistance has been shown to develop in vagal afferent neurons in high-fat-fed rats (11), and aging is associated with peripheral and central metabolic leptin resistance that involves transporter saturation, defective intracellular signaling, and receptor downregulation (14, 33, 39). Leptin sensitivity for cardiovascular actions during aging, however, is unknown. It is possible to observe divergent effects in this regard as previous studies have shown that resistance is selective to the metabolic, but not cardiovascular, actions of leptin in obese animal models perhaps due to brain–signaling pathway-specific mechanisms (28). Interestingly, the leptin injection did produce a transient decrease in blood pressure in older rats suggesting at least some responsiveness in cardiovascular pathways in the dorsal medulla during aging. Further studies are needed, however, to assess age-related changes in leptin signaling pathways for cardiovascular actions.

There are some potential limitations to this study. First, these studies were performed while the animals were under anesthesia, which can impact resting cardiovascular function. We employed a combination anesthesia widely used for studies investigating the neural control of the circulation given its relative preservation of baroreflex function, but this may have confounded resting blood pressure. Second, resting levels of metabolic hormones were not measured in microinjection animals to avoid potential confounding effects from drug administration or prolonged anesthesia. Thus we were not able to examine relationships among resting leptin levels, blood pressure, and BRS. Third, we examined the BRS at 10, 60, and 120 min after drug administration, and it is possible that we missed differences in the time course for effects of either leptin or the leptin antagonist with aging. Fourth, we examined the effects of acute, site-specific administration and further studies are needed to determine chronic peripheral or central effects. Finally, the effects of leptin on the BRS in older rats were compared with our previous publication in younger rats, which may limit our ability to interpret exogenous leptin actions with aging. Direct comparisons were made, however, for the main observation with the leptin receptor antagonist showing that endogenous leptin contributes to age-related baroreflex dysfunction.

Conclusions. There is an increasing need to understand mechanisms underlying the decline in BRS for control of HR that occurs during healthy aging, given the increasing prevalence of elderly individuals worldwide. We present new evidence that aging is associated with a loss of sensitivity to exogenous leptin for baroreflex modulation within the NTS, which is at least in part due to endogenous suppression of the baroreflex by elevated leptin. Rather than a true loss of sensitivity to the hormone, the evidence we provide argues that elevated endogenous leptin within the NTS may be exerting maximal effects to impair baroreflex modulation of HR in older animals. Thus these findings provide an alternate interpretation of leptin resistance, whereby elevated endogenous actions of the hormone may negate further actions of exogenously administered leptin, rather than altered signaling as the sole mechanism for reduced responsiveness of the hormone. Importantly, leptin-mediated impairments in the BRS may be permissive to increases in blood pressure and sympathetic activation, especially in individuals with elevated leptin levels. Interactions with the renin-angiotensin or endocannabinoid systems and changes in nitric oxide bioavailability and neurotransmitters (i.e., GABA, glutamate) are all possible mechanisms for endogenous suppression of baroreflex function by leptin during aging. Elucidation of these precise mechanisms will be the focus of future studies, as will determining whether leptin acts on receptors localized to vagal afferents, neurons or glia for baroreflex modulation. Regardless, these collective findings suggest that endogenous leptin contributes to baroreflex dysfunction in older but not younger animals. Clinically, impaired control of HR control predicts target-organ damage independent of elevated arterial pressure. Thus our findings reveal the potential consequences of elevated leptin and suggest that interruption of leptin actions at the level of the NTS may be a novel strategy to preserve BRS during aging.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: A.C.A. and D.I.D. conception and design of research; A.C.A. performed experiments; A.C.A. analyzed data; A.C.A. and D.I.D. interpreted results of experiments; A.C.A. prepared figures; A.C.A. drafted manuscript; A.C.A. and D.I.D. approved final version of manuscript; D.I.D. interpreted results of experiments; A.C.A. prepared figures; A.C.A. drafted manuscript; A.C.A. and D.I.D. approved final version of manuscript; D.I.D. edited and revised manuscript.

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