Leptin Mediates High-Fat Diet Sensitization of Angiotensin II–Elicited Hypertension by Upregulating the Brain Renin–Angiotensin System and Inflammation

Baojian Xue, Yang Yu, Zhongming Zhang, Fang Guo, Terry G. Beltz, Robert L. Thunhorst, Robert B. Felder, Alan Kim Johnson

See Editorial Commentary, pp 843–844

Abstract—Obesity is characterized by increased circulating levels of the adipocyte-derived hormone leptin, which can increase sympathetic nerve activity and raise blood pressure. A previous study revealed that rats fed a high-fat diet (HFD) have an enhanced hypertensive response to subsequent angiotensin II administration that is mediated at least, in part, by increased activity of brain renin–angiotensin system and proinflammatory cytokines. This study tested whether leptin mediates this HFD-induced sensitization of angiotensin II–elicited hypertension by interacting with brain renin–angiotensin system and proinflammatory cytokine mechanisms. Rats fed an HFD for 3 weeks had significant increases in white adipose tissue mass, plasma leptin levels, and mRNA expression of leptin and its receptors in the lamina terminalis and hypothalamic paraventricular nucleus. Central infusion of a leptin receptor antagonist during HFD feeding abolished HFD sensitization of angiotensin II–elicited hypertension. Furthermore, central infusion of leptin mimicked the sensitizing action of HFD. Concomitant central infusions of the angiotensin II type 1 receptor antagonist irbesartan, the tumor necrosis factor-α synthesis inhibitor pentoxifylline, or the inhibitor of microglial activation minocycline prevented the sensitization produced by central infusion of leptin. RT-PCR analysis indicated that either HFD or leptin administration upregulated mRNA expression of several components of the renin–angiotensin system and proinflammatory cytokines in the lamina terminalis and paraventricular nucleus. The leptin antagonist and the inhibitors of angiotensin II type 1 receptor, tumor necrosis factor-α synthesis, and microglial activation all reversed the expression of these genes. The results suggest that HFD-induced sensitization of angiotensin II–elicited hypertension is mediated by leptin through upregulation of central renin–angiotensin system and proinflammatory cytokines. (Hypertension. 2016;67:970–976. DOI: 10.1161/HYPERTENSIONAHA.115.06736.)

Key Words: blood pressure ■ high-fat diet ■ leptin ■ obesity ■ renin-angiotensin system

Obesity has become epidemic and is a serious health problem and a major risk factor for the development of hypertension. Accumulating evidence indicates that obesity causes chronic low-grade inflammation associated with systemic metabolic dysfunction that is accompanied by increased renin–angiotensin system (RAS) activity.1,2 Recent studies have demonstrated that the RAS and proinflammatory cytokines (PICs) in the central nervous system (CNS) play a critical role in mediating obesity-induced sensitization and obesity-related hypertension.3–5

The adipokine leptin is released in proportion to total fat and primarily acts on the hypothalamus to maintain energy homeostasis and normal body weight.6 Similar to the RAS and PICs, leptin can act in the CNS to enhance sympathetic nervous system activity (SNA) that contributes to the development of hypertension associated with obesity.7–9 Simonds et al9 demonstrated that obesity-induced elevation of circulating leptin increases blood pressure (BP) by acting on leptin receptors (LEPRs) in the dorsomedial hypothalamus. Furthermore, humans with loss-of-function mutations in leptin and the LEPRs have low BP, despite severe obesity. Prior et al,10 Armitage et al,11 and Lim et al12 recently reported that 3 weeks of high-fat diet (HFD) feeding led to increased mean arterial pressure (MAP), heart rate (HR), and renal SNA in rabbits.
This obesity-induced hypertension is accompanied by an increase in plasma leptin levels and can be blocked by central infusion of a leptin antagonist.

A recent study showed an interaction between leptin and the RAS in regulating SNA. Central infusion of leptin upregulates brain RAS components in specific brain regions. Conversely, central inhibition of angiotensin II (Ang II) type 1 receptor (AT1-R) or global genetic knockout of angiotensin-converting enzyme (ACE) reduces circulating leptin levels. These results suggest that the brain RAS plays a facilitatory role in sympathetic nerve responses to leptin. It has been shown that obesity can induce selective leptin resistance, whereby the anorexic effect of leptin is attenuated, but its action to increase SNA and BP remains intact. One recent study showed that the leptin resistance was induced by obesity-associated activation of the hypothalamic nuclear factor xB/IkB kinase-β pathway. Tumor necrosis factor (TNF)-α also can directly influence leptin signaling activity by modulating LEPR expression. These findings suggest that the interactions and synergism between leptin, the RAS, and PICs may be responsible for the obesity-induced increase in SNA, which occurs early in the process or secondary to long-standing obesity.

Hypothalamic nuclei such as dorsomedial hypothalamus and arcuate nucleus are the main sites of leptin action. However, the presence of the blood–brain barrier leads to the obvious question as to how leptin, a 16-kDa peripherally derived protein, gains access to the central sites to influence BP. Recently, the subfornical organ (SFO), a sensory circumventricular organ lacking the normal blood–brain barrier, has been demonstrated to be an important target of leptin that mediates leptin-induced increases in renal SNA. Our previous work demonstrated that short-term HFD feeding can upregulate the RAS and the PICs in the lamina terminalis (LT) including the SFO and sensitizes the hypertensive response to a pressor dose of Ang II. However, it is unclear what factors induce upregulation of the RAS and PICs in the SFO under the condition when there is no obvious increase in the plasma levels of Ang II or PICs after short-term HFD feeding. Given that the enhanced effect of leptin on SNA and increased plasma levels of leptin in obesity, it is reasonable to hypothesize that HFD feeding may first increase plasma leptin, which then acts on the brain to induce sensitization of the Ang II–elicited hypertensive response through upregulation of the brain RAS and PICs signaling pathways.

### Methods

#### Experimental Protocol

Male Sprague–Dawley rats (10- to 12-week old, Harlan, n=96) were used. Rats were prepared with a lateral ventricular cannula, osmotic minipumps for intracerebroventricular (ICV), and subcutaneous drug infusion, and with telemetry probes for continuous BP monitoring, as previously described. The ICV dose of leptin and doses of agents used to block LEPRs, AT1-R, TNF-α synthesis, or microglial activation were chosen on the basis of published in vivo studies. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by The University of Iowa Animal Care and Use Committee.

### Effects of Central Blockade of LEPR on HFD-Induced Sensitization and Molecular Changes in the LT and Paraventricular Nucleus of Hypothalamus

BP was monitored continuously by telemetry in rats that were fed either normal fat diet (NFD, 10% calories from lard, 5.85 kcal/g, D12450J, Research Diet, NJ) or HFD (60% calories from lard, 5.24 kcal/g, D12492, Research Diet, NJ) for 3 weeks (induction period, I), followed by NFD for 2 weeks, which coincided with an expression period (E). During I, rats received ICV vehicle (V) or rat superactive leptin antagonist (SHLA, 20 μg/d; Cat. SLAN-4, Protein Laboratories, Rehovot, Israel). This antagonist was delivered by osmotic pumps (model 2004, 0.25 μL/h for 4 weeks; Alzet). Total adipose tissue mass in 3 treatment groups (n=6 per group) was set at 2.5 g/d; Cat. SLAN-4, Protein Laboratories, Rehovot, Israel). Plasma, inguinal, retroperitoneal, and epididymal white adipose tissue (WAT) and brains were then collected for analysis of plasma levels of leptin, measurement of WAT mass, and RT-PCR for mRNA expression, respectively. The microdissected tissue samples for mRNA expression contained the structures lying along the LT (ie, the SFO, median preoptic nucleus, and organum vasculosum of the lamina terminalis) and the paraventricular nucleus (PVN). Food consumption, water intake and body weight were measured once a week.

### Effects of Brain RAS and Inflammation on Leptin-Induced Sensitization and Molecular Changes in the LT and PVN

Rats were fed normal rat chow (7013 National Institutes of Health-31 modified rat diet) ad libitum. BP was monitored continuously during an induction–delay–expression (I–D–E) protocol, as described previously. During I, ICV vehicle, leptin (20 ng/kg per minute), or leptin plus the TNF-α synthesis inhibitor pentoxifylline (PTX, 10 μg/h), the AT1-R blocker irbesartan (Irbe, 125 μg/d), or the inhibitor of microglial activation minocycline (Mino, 5 μg/h) was delivered by osmotic minipump (model 2001, Alzet) for 2 weeks. To ensure that any exogenous leptin and antagonists were metabolized, the rats then rested for 1 week (D). After this time, a second pump (model 2002, 0.5 μL/h; Alzet) was implanted to deliver a slow pressor dose of Ang II (120 ng/kg per minute) for 2 weeks (E). Thus, the primary study groups (n=6 per group) were (1) I-NFD+ICV V+E-Ang II, (2) I-HFD+ICV V+E-Ang II, (3) I-HFD+ICV V+I-ICV Ang II, (4) I-HFD+ICV V+I-ICV Irbe+E-Ang II, (5) I-HFD+ICV V+I-ICV PTX+E-Ang II, and (6) I-HFD+ICV V+I-ICV Mino+E-Ang II. Food consumption, water intake, and body weight were measured once a week.

Five additional groups (n=6 per group) underwent identical treatment during I (I-ICV V+E-Ang II, I-HFD+ICV V+E-Ang II, I-HFD+ICV V+IRBE+E-Ang II, I-HFD+ICV V+PTX+E-Ang II, and I-HFD+ICV V+Mino+E-Ang II) but were euthanized at the end of the D period to collect LT and PVN for analysis of mRNA expression.

### Data Analysis

The significance of differences in MAP and HR among groups was analyzed by 2-way ANOVAs followed by Tukey multiple comparison tests. One-way ANOVAs were used to analyze the differences in plasma leptin level, adipose tissue mass and in mRNA expression of brain leptin and its receptors, RAS components and PICs in all groups. All data are expressed as means±SE. Statistical significance was set at P<0.05.

### Additional Methods

Please see the online-only Data Supplement.
Results
Effects of HFD on Adipose Tissue Mass, Plasma Leptin Level, and mRNA Expression of Leptin and LEPRs
Total adipose tissue mass, WAT mass (Figure 1A), and plasma leptin concentration (Figure 1B) were significantly increased after 3 weeks of HFD when compared with those of NFD (P<0.05). Central infusion of the leptin antagonist SHLA slightly enhanced HFD-induced increase in total adipose mass and WAT mass, but had no influence on increased plasma leptin concentration. Likewise, significant increases in mRNA expression of leptin, LEPR-a, and LEPR-b were evident in the SFO (Figure 2A) and PVN (Figure 2B) of HFD-fed rats compared with rats fed NFD (P<0.05). Central infusion of the leptin antagonist SHLA during HFD had no influence on these increases in mRNA expression.

Effect of SHLA on HFD Sensitization of Ang II–Induced Hypertension
Baseline values for MAP (103.8±1.9 mm Hg) and HR (348.4±6.6 bpm) were comparable before and after application of HFD or HFD plus ICV infusion of leptin antagonist in all groups of rats. However, during the course of E, Ang II induced a greater increase in MAP in the rats that received the HFD pretreatment (Δ46.4±3.9 mm Hg, P<0.05; Figure 3A and 3B) when compared with the rats pretreated with NFD (Δ24.9±3.8 mm Hg). This augmentation of the pressor effect induced by Ang II was blocked by concurrent ICV infusion of leptin antagonist (Δ21.7±5.3 mm Hg) along with the HFD pretreatment (P<0.05). Systemic Ang II inductions produced slight, comparable decreases in HR in all groups (Figure S2).

Effect of ICV Leptin Antagonist on HFD-Induced mRNA Expression of RAS Components, PICs, and Microglial Marker in the LT and PVN
In LT tissue collected at the end of HFD feeding, HFD induced a significant increase in the mRNA expression of the RAS components (AT1-R and ACE1), the nicotinamide adenine dinucleotide phosphate oxide subunit (ie, NOX2) and the inflammatory elements (ie, TNF-α, interleukin [IL]-6, and the microglial marker CD11b) in the LT when compared with controls (P<0.05; Figure 4B). The expression of IL-1β in the LT was not higher after HFD (P>0.05, Figure 4A). Central infusion of SHLA significantly attenuated the increased mRNA expression of ACE1, NOX2, TNF-α, IL-6, and CD11b (P<0.05, Figure 4A).

In PVN tissue collected at the end of HFD feeding, HFD also induced a significant increase in the mRNA expression of the RAS components (AT1-R and ACE1), the nicotinamide adenine dinucleotide phosphate oxide subunit (ie, NOX2) and the inflammatory elements (ie, TNF-α, IL-1β, IL-6, and the microglial marker CD11b) when compared with controls (P<0.05, Figure 4B). Central infusion of SHLA normalized the increased mRNA expression of ACE1, NOX2, TNF-α, IL-1β, and CD11b, but had no effect on the increased expression of AT1-R and IL-6 (P<0.05, Figure 4B).

Effect of ICV PTX, Irbe, or Mino on ICV Leptin Sensitization of Ang II Hypertension
To confirm the direct sensitizing actions of central leptin and the RAS and the PICs mediation of central leptin-induced sensitization, leptin alone or leptin combined with TNF-α synthesis inhibitor, AT1-R blocker or inhibitor of microglial activation was infused ICV for 1 week during I. These treatments resulted in a mild but significant increase in MAP during D, which gradually returned to the basal level by the end of D (Figure 5A). Subsequent Ang II–induced hypertension during E was greater in the groups receiving leptin during I in comparison with those groups receiving vehicle (leptin Δ39.3±3.8 mm Hg versus vehicle Δ21.2±5.2 mm Hg, P<0.05; Figure 5A and 5B), which mimicked the HFD sensitization of Ang II–induced hypertension (Δ46.4±3.9 mm Hg). This augmentation of the pressor effect induced by leptin was prevented by concurrent ICV infusion of PTX, Irbe, or Mino along with the ICV leptin administrated during
I (PTX Δ9.5±4.6 mm Hg, Irbe Δ21.9±3.8 mm Hg, Mino Δ20.9±3.1 mm Hg, *P<0.05; Figure 5A and 5B).

ICV leptin infusion during I produced a significant increase in HR, which was sustained throughout I and D and during most of E, but then gradually returned to the basal level by the end of E. Concomitant ICV infusion of either PTX or Mino had no effect on this leptin-induced increase in HR, whereas Irbe blocked it (Figure S3).

**Effect of ICV PTX, Irbe, or Mino on Leptin-Induced mRNA Expression of RAS Components, PICs and Microglial Marker in the LT and PVN**

In LT and PVN tissue collected at the end of D, the ICV leptin given during I produced a significant increase in the mRNA expression of AT1-R, ACE1, NOX2, TNF-α, IL-1β, IL-6, and CD11b when compared with controls (*P<0.05, Figure 6A and 6B). Central concurrent infusion of PTX, Irbe, or Mino along with a sensitizing dose of leptin blocked the enhanced increase in mRNA expression in most cases with the exception of IL-6 in the LT and of TNF-α in the PVN (*P<0.05, Figure 6A and 6B).

**Discussion**

The major findings of this study are (1) WAT mass, plasma leptin levels, and mRNA expression of leptin, and its receptors in both the LT and the PVN were elevated in rats by 3 weeks of HFD feeding; (2) ICV administration of leptin mimicked HFD sensitization of Ang II-induced hypertension, and ICV leptin antagonist abolished this HFD-induced sensitizing action; (3) central inhibition of AT1-R, TNF-α synthesis, or microglial activation significantly attenuated leptin-induced upregulation of RAS activity, inflammation in the LT and PVN, and leptin sensitization of Ang II-induced hypertension. These observations indicate that HFD can predispose rats to display an enhanced Ang II–elicited hypertensive response through leptin-mediated upregulation of RAS components and PICs in the LT and PVN.

Leptin is primarily produced in WAT and secreted into the circulation. Leptin exerts its biological action through binding to and activating the long form of LEPR (LEPR-b), but not through the short form of LEPRs (a, c–f).24 Recently, the SFO, a sensory circumventricular organ devoid of the blood–brain barrier, has been shown to possess functional LEPRs and its neurons can directly be activated by leptin.25-27 Young et al17 demonstrated that leptin acts within the SFO to selectively increase sympathetic outflow to the kidney and that depletion of SFO LEPRs abolishes arcuate nucleus LEPR-mediated increase in renal SNA response to leptin. This highlights an emerging role for the SFO in the regulation of circulating energy signals such as leptin. Therefore, it is likely that information regarding large circulating peptides such as leptin can reach brain metabolic and cardiovascular nuclei inside the blood–brain barrier through sensory circumventricular organs, including the SFO. Furthermore, the PVN, a nucleus downstream from the SFO, has recently been demonstrated to be a site responsible for leptin-induced increases in lumbar SNA, MAP, and HR; bilateral inhibition of the PVN with muscimol completely reversed the effects of central leptin.28 Beside LEPRs, the SFO and PVN also possesses AT1-R and PIC receptors that respond to the corresponding agents.29,30
Taken together, these findings are consistent showing that the increased by week 3.31 This timing of changes in leptin lev-
tations were unaltered by an HFD at day 3 but were markedly
feeding and further showed that circulating leptin concentra-
the PICs in the SFO and PVN suggest that these structures
are sites for the convergence of leptin signaling with other car-
diovascular active agents.

In this study, we found that 3 weeks of HFD feeding increased WAT mass, plasma leptin levels, and mRNA expression of leptin and LEPR-a and b in both the LT and PVN. Central administration of leptin mimicked HFD sen-
sitization of Ang II–induced hypertension, whereas ICV
leptin/IRbe, central concurrent treatment with leptin and AT1-R blocker irbesartan during I; I-ICV leptin/MinO, central concurrent treatment with leptin and inhibitor of microglial activation minocycline during I; I-ICV leptin/PTX, central concurrent treatment with leptin and TNF–α synthesis inhibitor PTX during I; and IVC V, central treatment with vehicle during I.

The functional and anatomic coincidence of leptin, the RAS and the PICs in the SFO and PVN suggest that these structures are sites for the convergence of leptin signaling with other cardiovascular active agents.

Although there are strong correlations between plasma leptin concentration and renal SNA, hypertension, and tachy-
cardia in rabbits within 3 weeks of fat feeding, the enhanced cardiovascular responses still remained high after the withdrawal of HFD and after plasma leptin concentration was rapidly normalized.10–11 This finding raises a possibility that HFD-induced leptin may activate other sensitizing pressor factors that act centrally to maintain the elevated renal SNA and BP. Hilzendeger et al13 recently demonstrated that ICV injection of leptin can upregulate the brain RAS in the SFO and that the brain RAS plays a facilitatory role in the sym-
pathetic nerve response to leptin. Similarly, Thieme et al14 reported that chronic systemic infusion of leptin resulted in elevated renin and Ang II plasma levels and increased sys-
tolic BP. Concurrent infusion of AT1-R antagonist blocked the
leptin-induced increase in BP. Moreover, blockade of AT1-R
inhibited leptin resistance and restored leptin sensitivity in
diet-induced obese rats.33 These results indicate that obesity-
associated hyperleptinemia contributes to the development of hypertension through cross talk between leptin and the RAS.

Either HFD feeding or systemic infusion of leptin significa-
tly increased hypothalamic and renal PIC expression, including IL-1β, IL-6, and TNF–α, and several components of the RAS, such as AT1-R. The increased gene expression for RAS components and PICs were accompanied by increased microglial activation in the hypothalamus, including the arcu-
ate nucleus, SFO, and PVN.32,34,35 Conversely, TNF–α upregu-
lates LEPR-b protein level and cell surface expression, which can lead to an increased cellular response to leptin and soluble LEPR production in cultured cells.15 These results suggest that
in both the periphery and CNS, PICs also mediate the cardiovascular and metabolic effects of leptin. In this study, we found that 7 days of central leptin infusion during I upregulated the expression of the RAS components and several PICs in the LT and downstream in the PVN, which was accompanied by the elevated expression of a microglial marker and nicotinamide adenine dinucleotide phosphate oxidase. Central inhibition of AT1-R, TNF-α synthesis, and microglial activation during I reversed the leptin-enhanced increase in gene expression and the sensitization of Ang II–elicited hypertension. This study extends our previous studies and provides novel evidence that obesity-associated leptin primarily augments brain RAS activity and inflammation, which further sensitizes Ang II–elicited hypertension. This leptin-dependent increase in RAS activity and PICs within the CNS may offer a causal link between obesity and hypertension.

Although our results suggest a causal role for leptin in HFD sensitization of Ang II–induced hypertension through upregulation of brain RAS and PICs, there still are several issues that need to be addressed. (1) The alterations of the mRNA expression of RAS and PICs occurred not only in the LT but also in the downstream PVN during HFD feeding. Given the roles of hypothalamic nuclei such as the arcuate nucleus, dorsomedial hypothalamus, and ventromedial hypothalamus and hindbrain nucleus such as the nucleus tractus solitarii in mediating leptin actions,9,16,25,36 the involvement of hypothalamic and hindbrain nuclei in the sensitizing effects of leptin cannot be excluded. (2) The HFD feeding increased plasma leptin levels. Whether this increased circulating leptin induces the central production of leptin that contributes to the sensitizing effects of circulating leptin needs to be studied in the future. (3) In animal experiments, the ranges of effective doses of leptin to produce changes in hemodynamic and sympathetic activity are 2 to 10 μg for ICV injection and roughly 100 to 150 μg for intravenous bolus injection.10–13,16,17,32 Also, it is important to note that the sensitivity to leptin is altered by diet.10–12 In light of these results, it is assumed that a pharmacological dose of leptin would have been needed for ICV or systemic infusion of leptin to induce a sensitization effect rather than the physiological concentration produced in rats fed HFD. In this study, we used a dose of 20 ng/kg per minute for ICV infusion of leptin, which is also higher than that in the plasma after HFD feeding. ICV infusion of this pharmacological dose of leptin produced a slight increase in BP, but gradually returned to the basal level by the end of D, whereas the increased HR gradually returned to the basal level by the end of E although a pressor dose of Ang II was infused during E. Therefore, it is not likely that the changes of BP and HR produced by leptin pretreatment have an influence on leptin’s sensitizing actions. Moreover, an increasing number of studies indicate that the CNS is mainly responsible for the leptin-mediated overactivity of the hemodynamic parameters produced by HFD feeding. This study used the central infusion of the pharmacological doses of leptin peptide or leptin antagonist to give further insight into the findings from our HFD study.

Perspectives
This study investigated the mechanisms responsible for HFD upregulation of brain RAS and PICs. The results indicate that leptin mediates HFD-induced upregulation of the brain RAS and PICs both of which can exert sensitizing actions. The LT including the SFO is a primary site for convergence of cardiovascular and metabolic signals. These observations suggest that there is a vicious cycle involving leptin, RAS, inflammation, and sympathetic activation triggered by feeding an HFD to produce a progressive rise in BP. A diet-induced increase of leptin elicits a state of RAS activation and inflammation, which in turn leads to leptin resistance. This type of mutually enhanced signaling eventually results in both obesity and hypertension. Furthermore, the present findings imply that the sensitizing effects of increased leptin and upregulated RAS and PICs on the development of hypertension will predispose obese individuals to a greater risk of hypertension when they are exposed to many other types of hypertension-eliciting challenges (ie, stressors). However, because of involvement of multiple brain regions and pressor factors in the sensitization process, the precise central mechanism underlying these interactions that ultimately leads to obesity-related hypertension still need to be determined.

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Disclosures
None.

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**Novelty and Significance**

**What Is New?**
- These studies demonstrate that high-fat diet (HFD)-induced sensitization of angiotensin II-elicited hypertension is mediated by leptin through upregulation of central renin–angiotensin system and proinflammatory cytokines.
- Central infusion of leptin mimics HFD-elicited sensitization of angiotensin II hypertension, whereas central blockade of leptin receptors abolished the sensitization produced by HFD feeding, which involves regulation of brain proinflammatory cytokines and renin–angiotensin system activity.

**What Is Relevant?**
- HFD-induced increase in leptin elicits a state of renin–angiotensin system activation and inflammation, which in turn leads to leptin resistance.

**Summary**

The study indicates that HFD-induced increase of leptin acts on the brain to sensitize the hypertensive response to angiotensin II and that sensitization is associated with altered expression of renin–angiotensin system and proinflammatory cytokines within forebrain neural network controlling cardiovascular function.
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Running head
Leptin sensitization of Ang II hypertension

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Methods

Physiological Studies
Under Ketamine-xylazine anesthesia, rats were chronically instrumented with telemetry probes (TA11PA-C40; DSI) placed in the femoral artery for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). Beginning seven days after recovery from surgery, MAP and HR data collection was initiated. In the first set of experiments, to study effect of central blockade of leptin receptor on high fat diet (HFD, 60% calories from lard, 5.24 kcal/g, D12492, Research Diet, NJ) -induced sensitization, the rats were fed with HFD or normal fat diet (NFD, 10% calories from lard, 3.85 kcal/g, D12450J, Research Diet, NJ) for three weeks. These treatments were combined with central administration of agents through a brain lateral ventricle cannula (the coordinates 1.0 mm caudal, 1.5 mm lateral to bregma, and 4.5 mm below the skull surface) connected to an osmotic pump (model 2004, 0.25 µl/h for 4 weeks, Alzet) containing vehicle (sterile water) or rat super-active leptin antagonist (SHLA, 20 µg/d, Cat. SLAN-4, Protein Laboratories, Rehovot, Israel). These pumps were disconnected before Ang II infusion. The HFD was then replaced by NFD for two weeks and a second osmotic pump (model 2002, 0.5 µl/h for 2 weeks, Alzet) containing Ang II (120 ng/kg/min) was implanted on the back of rats under isoflurane anesthesia. Food consumption, water intake and body weight were measured once a week. Total food consumption, water intake and gain of body weight before, during HFD or Ang II infusion were averaged by daily. Daily calorie intake was calculated: average daily food consumption x diet energy density. Feed efficiency, the ability to convert caloric intake into BW, was determined by: mean BW gain (mg) / total caloric intake (kcal).

Total adipose tissue mass in three treatment groups (n=6/group) (I-NFD + ICV V, I-HFD + ICV V and I-HFD + ICV SHLA) following I but prior to E were determined by NMR spectroscopy using a Bruker mini-spec LF 90II instrument (Bruker Corporation, Billerica, MA). Plasma, inguinal, retroperitoneal and epididymal white adipose tissue (WAT) and brains were then collected for analysis of plasma level of leptin, measurement of WAT mass and RT-PCR for mRNA expression, respectively. The microdissected tissue samples for mRNA expression contained the structures lying along the lamina terminalis [LT, i.e., the subfornical organ (SFO), median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT)] and the paraventricular nucleus of hypothalamus (PVN).

To study the direct sensitizing action of central leptin on Ang II-induced hypertension, the second set of experiments followed an Induction-Delay-Expression (I-D-E) experimental design. During I, ICV vehicle (artificial cerebrospinal fluid for leptin, PTX and Mino or 0.84% NaHCO$_3$ for Irbe), leptin (20 ng/kg/min), or leptin plus the TNF-α synthesis inhibitor PTX (10 µg/h), the AT1-R blocker irbesartan (Irbe, 125 µg/d) or the inhibitor of microglial activation minocycline (Mino, 5 µg/h) was delivered by brain cannulas connected to an osmotic minipump (model 2001, 1µl/h for 1 week, Alzet) for 1 week. To assure that any exogenous leptin and antagonists were metabolized, the rats then rested for 1 week (D). After this time, a second pump (model 2002, Alzet) was implanted to deliver a slow pressor dose of Ang II (120 ng/kg/min) for 2 weeks (E). In this set of experiments, all rats were fed normal rat chow (7013 NIH-31 modified rat diet) ad libitum. Food consumption, water intake and body weight were measured once a week.
**Leptin Concentrations**
Plasma leptin was determined using a rat leptin Quantikine ELISA Kit (MOB00, R&D systems). Plasma from the NFD groups required no dilution. Plasma samples from the HFD groups were diluted 1:4 to avoid exceeding the standard curve.

**Body Composition**
Total body, fat, lean, and fluid mass were determined by NMR spectroscopy using a Bruker mini-spec LF 90II instrument (Bruker Corporation, Billerica, MA). To analyze body composition, rats were placed into a restraint tube and inserted into the rodent-sized NMR apparatus, adjusting the volume of the chamber based on the size of the animal.

**Measurement of mRNA Expression in the LT and PVN**
Total RNA was isolated from LT or PVN using Trizol method (Invitrogen) and treated with DNase I (Invitrogen). RNA integrity was checked by gel electrophoresis. Total RNA was reverse transcribed using random hexamers following the manufacturer’s instructions (Applied Biosystems). Real time PCR was conducted using 200-300 ng of cDNA and 500 nM of each primer in a 20 μl reaction with iQ SYBR Green Supermix (Bio-Rad). Amplification cycles were conducted at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at 60°C for 30 s. Reactions were performed in duplicate and analyzed using a C1000 thermocycler system (Bio-Rad). Samples that did not yield homogenous melt curves were excluded. Changes in mRNA expression levels were normalized to GAPDH levels and calculated using the ΔΔCt method. Results are expressed as relative fold change, mean of fold change ± SE. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the primers are shown in Table S1.

**Data Analysis**
Baseline MAP and HR data were collected for 5 days and then for 28-35 consecutive days. MAP and HR are presented as mean daily values averaged from daytime and nighttime measurements. Difference scores for MAP and HR were calculated for each animal based on the mean of the 5-day baseline subtracted from the mean of the final 5 days of treatment. Two-way ANOVAs for the experimental groups (1. among NFD group, HFD groups and HFD groups with central SHLA; 2. among groups with central vehicle, leptin, or leptin combined with antagonists) were then conducted on the means of calculated difference scores. After establishing a significant ANOVA, post-hoc analyses were performed with Tukey multiple comparison tests between pairs of mean change scores. One-way ANOVAs were used to analyze the differences in plasma leptin level, adipose tissue mass and in mRNA expression of brain leptin and its receptors, RAS components and PICs in all groups. All data are expressed as means ± SE. Statistical significance was set at $P < 0.05$. 
Results

Caloric Intake, Feed Efficiency, Body Weight and Water intake during HFD and Systemic Ang II

During I, rats fed the HFD had slight increases in caloric intake (73.2±3.8 Kcal/day, \(p>0.05\), Fig S1A), but exhibited significant higher feed efficiency (36.4±2.3 mg BW/Kcal, \(p<0.05\), Fig S1B) when compared with the group of NFD fed rats (65.6±3.2 Kcal/day and 27.7±1.8 mg BW/Kcal). Accordingly, 3 week HFD feeding resulted in a significant increases in body weight (HFD, 57.4±4.2 gm vs. NFD 35.9±3.3 gm, \(p<0.05\), Fig S1C). During E, with all groups receiving the NFD and the Ang II infusion, caloric intake was comparable in rats that had been induced with NFD or HFD (\(p>0.05\)) and the rates of body weight gain were significantly reduced in all groups (\(p<0.05\), Fig. S1A,B,C). Central infusion leptin antagonist SHLA slightly enhanced the increase in caloric intake (77.7±3.9 Kcal/day), feed efficiency (42.7±3.5 mg BW/Kcal) and body weight (69.5±6.2 gm) when compared with the group of rats fed HFD alone (\(p>0.05\)). Consistent with the increased total adipose mass, percentage of fat was significantly higher in HFD treated rats compared with NFD treated rats (HFD, 8.2±0.15 % vs. NFD 6.8±0.14 %, \(p<0.05\), Fig S1D) while the percentages of fluid and lean were similar.

There was no difference in water intake between NFD (23.3±1.9 ml/d) and HFD (24.1±1.5 ml/d) fed rats before Ang II infusion. Systemic Ang II resulted in a significant increase in water intake in NFD rats (31.2±2.9 ml/d, \(p<0.05\)), in HFD rats (30.1±2.1 ml/d, \(p<0.05\)) and in HFD rats concurrently treated with ICV SHLA (28.9±1.3 ml/d, \(p<0.05\)) (Fig. S1E).

Effects of ICV leptin and systemic Ang II on Food Consumption, Body Weight and Water Intake

ICV infusion of leptin during I elicited significant decreases in food consumption and body weight, which were sustained during I and D and restored during E. ICV concomitant infusion of PTX or Mino had no effect on these ICV leptin-induced changes. However, concomitant ICV infusion of Irbe significantly attenuated the leptin-induced inhibitory effect on food consumption and body weight during I, and further normalized these changes during D. (\(p<0.05\), Fig. S4A, S4B).

ICV infusion of leptin and inhibitors of the RAS and PICs had no effect on water intake during I, but increased it during D only in leptin alone treated rats. Systemic Ang II resulted in a further enhanced increase in water intake in rats pretreated with ICV leptin alone but not in other groups (\(p<0.05\), Fig. S4C).
<table>
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LEPR, leptin receptor; AT-R, angiotensin receptor; ACE1, angiotensin converting enzyme 1; NOX2, NADPH oxidase 2; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; CD11b, cluster of differentiation molecule 11b.
Figure S1. Changes in caloric intake (Fig. S1A), feed efficiency (Fig. S1B), body weight (Fig. S1C), body composition (Fig. S1D) and water intake (Fig. S1E) in normal fat diet (NFD) rats and high fat diet (HFD) rats treated with central (ICV) leptin receptor antagonist (SHLA) before and after systemic infusion of a pressor dose of Ang II. (* $p<0.05$ vs. before Ang II; # $p<0.05$ vs. NFD rats; § $p<0.05$ vs. HFD groups).
Figure S2. Daily heart rate (HR) before and during normal fat diet (NFD) or high fat diet (HFD) feeding and systemic infusion of angiotensin II (Ang II) in ICV vehicle or leptin receptor antagonist SHLA treated rats. I-NFD = pretreatment with normal fat diet during I; I-HFD = pretreatment with high fat diet during I; ICV V = central treatment with vehicle during I; I-HFD+ICV SHLA = pretreatment with HFD plus central treatment with leptin receptor antagonist SHLA during I; E-Ang II = peripheral treatment with a pressor dose of Ang II during E.
**Figure S3.** Daily heart rate (HR) during ICV vehicle, leptin or leptin plus PTX, Irbe or Mino in Induction (I) period and during systemic infusion of angiotensin II (Ang II) in Expression (E) period. ICV V = central treatment with vehicle during I; ICV leptin = central treatment with leptin during I; I-ICV leptin/PTX = central concurrent treatment with leptin and TNF-α synthesis inhibitor pentoxifylline during I; ICV leptin/Irbe = central treatment with leptin and angiotensin II type 1 receptor antagonist irbesartan during I; I-ICV leptin/Mino = central concurrent treatment with leptin and inhibitor of microglial activation minocycline during I; E-Ang II = peripheral treatment with a pressor dose of Ang II during E. (* p<0.05 vs. baseline)
**Figure S4.** Changes in food consumption (Fig. 5A), body weight (Fig. 5B) and water intake (Fig. 5C) in rats treated with central (ICV) vehicle (V), leptin or leptin plus synthesis inhibitor pentoxifylline (PTX), AT1-R blocker irbesartan (Irbe) or inhibitor of microglial activation minocycline (Mino) before and after systemic infusion of a pressor dose of Ang II. (* p<0.05 vs. ICV V group; # p<0.05 vs. ICV leptin/PTX and ICV leptin/Mino groups; § p<0.05 vs. groups of rats concurrently treated with ICV vehicle, PTX, Irbe or Mino).

**Figure S5.** Schematic representation of high fat diet sensitizing effect on angiotensin (Ang) II-elicited hypertension through upregulation of renin-angiotensin system (RAS) and inflammatory elements in the subfornical organ (SFO) and paraventricular nucleus of hypothalamus (PVN).