Central Leptin and Tumor Necrosis Factor-α (TNFα) in Diurnal Control of Blood Pressure and Hypertension*

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Leptin and TNFα can individually work in the brain to affect blood pressure; however, it remains unknown whether these two cytokines might have an interactive role in this process and, if so, how. In this work, we found that leptin stimulation led to TNFα production under both in vitro and in vivo conditions, and diurnal fluctuation of leptin concentrations in the cerebrospinal fluid predicted the circadian changes of TNFα gene expression in the hypothalamus. Signaling analysis showed that leptin stimulation led to a rapid and strong STAT3 activation followed by a second-phase moderate STAT3 activation, which was selectively abolished by anti-inflammatory chemical PS1145 or TNFα antagonist WP9QY. Physiological study in normal mice revealed that diurnal rise of blood pressure was abrogated following central administration of PS1145 or a leptin receptor antagonist. Central TNFα pretreatment was found to potentiate the effect of leptin in elevating blood pressure in normal mice. In pathophysiology, dietary obesity mimicked TNFα pretreatment in promoting leptin-induced blood pressure rise, and this effect was blocked by central treatment with either PS1145 or WP9QY. Hence, central leptin employs TNFα to mediate the diurnal blood pressure elevation in physiology while enhancement of this mechanism can contribute to hypertension development.

In coordination with physical and physiological requirements, blood pressure (BP) levels are tightly regulated to rise and fall in circadian manners, while alterations in these regulatory processes can chronically lead to the development of hypertension. From both physiological and pathological perspectives, BP changes involve peripheral contributions as well as neural inputs. Recently, there was an increasing amount of research in addressing the molecular and neuronal types in the central nervous system (CNS) mechanisms of hypertension (1–5). Leptin, an adipose tissue-derived cytokine which is over-produced in obesity, has been shown to increase BP when chronically administrated centrally (4, 6–9), and the underlying physiological basis involves up-regulation in the sympathetic outflow from the brain to the peripheral tissues (6, 10). Interestingly, the central effects of leptin on BP versus feeding do not necessarily follow the same line (8, 11, 12), for example, while leptin’s regulation on feeding is compromised (namely “leptin resistance”) in obesity, its action in raising BP is well reserved, leading to a notion that leptin resistance is physiologically selective (8, 9, 13). Taken together, the action of leptin on BP is probably mediated by certain mechanisms which are less clear compared with the mechanisms in mediating metabolic regulation. Also, an acute excess of leptin, e.g. via a single injection, has a prominent effect on feeding but little influences on the BP in normal animals (14, 15), leaving it unsolved regarding if leptin is involved in BP control under normal physiology and if so, how.

In addition to leptin, inflammatory cytokines were recently found to play crucial roles in the neural and hypothalamic mechanisms of hypertension (16). These findings agree with the general appreciation that inflammatory cytokines can interact with many important BP-regulating factors, such as renin-angiotensin system and sympathetic nervous system in participating in the development of hypertension (17, 18) and related cardiovascular diseases (19). Our recent research indicated that the hypothalamus is crucial for the action of pro-inflammatory cytokine TNFα in increasing BP in light of promoting the development of obesity-related hypertension (20, 21). In this background and also hinted by evidence showing that leptin can influence immune response (22), we carried out the current study to investigate if there exists an interactive action between leptin and TNFα in the central control of BP in normal physiology and also whether this potential mechanism is important for the development of obesity-related hypertension.

Results

Leptin-stimulated Hypothalamic TNFα and the Association with Diurnal Rhythms—Considering that leptin can influence immune response (22), we recently questioned that leptin could have a direct effect on hypothalamic TNFα production. Indeed, based on human monocytes, it has been reported that leptin stimulation led to synthesis of immune cytokines such as TNFα (23). However, it remains unclear if this relationship might be biologically important in a non-immune system. We were interested in the hypothalamus since it is classically known as a major site for exerting the actions of leptin. But, before directly analyzing the hypothalamus, we did an in vitro experiment to...
evaluate if leptin could affect TNFα production in cultured cells, using a cell line which is unrelated to the immune system. In this experiment, we employed HEK293 cells which do not express leptin receptor but were genetically engineered with the long form of leptin receptor, a suitable in vitro model established for studying leptin signaling in the research (24). These cells were treated with leptin or the vehicle, and TNFα mRNA levels were determined at various time points during the treatment. We obtained data confirming that leptin stimulation led to increases in TNFα mRNA levels for ~5 h, which we followed up while the peak effects occurred at 1–3 h during the stimulation (Fig. 1A).

Next, using mice, we examined if leptin-induced TNFα production might occur in the hypothalamus. To do so, we injected a single dose of leptin (2.5 μg) into the hypothalamic third ventricle through pre-implanted cannula, and 2 h later, the hypothalami were harvested for measuring TNFα mRNA. Data showed that hypothalamic TNFα mRNA increased >2-fold by
leptin stimulation (Fig. 1B). Also, to gain an insight into the potential physiological relevance of this relationship, we studied if hypothalamic TNFα mRNA levels might physiologically correlate with the circadian profile of leptin release, since the release of leptin fluctuates according to the circadian rhythms of feeding behaviors (25, 26). To do so, we collected the hypothalamus as well as the cerebrospinal fluid (CSF) from normal C57BL/6 mice at different circadian time points over a 24-h period. We found that circadian rhythms displayed both in hypothalamic expression of TNFα and in leptin concentrations in the CSF (Fig. 1, C and D). Also, there was 3~4-h phase shift between the curves of leptin and TNFα, with the peak concentration of leptin occurring 3~4 h after the peak level of hypothalamic TNFα. In summary, leptin-induced hypothalamic TNFα production in the hypothalamus is associated with nighttime phase (active phase for mice) of the circadian rhythms in physiology.

Biphasic STAT3 Phosphorylation in Response to Leptin Stimulation—Subsequently, we profiled if the classical molecular signaling of leptin might have a component that is affected by leptin-induced TNFα. As established, leptin-induced STAT3 phosphorylation is critical for many functions of leptin (27–30); however, there seems to be a lack of sufficient details on the long-term kinetics of STAT3 phosphorylation in response to leptin. Here, we examined leptin-induced STAT3 phosphorylation over frequent time points during a 4-h period in HEK293LEPR cells. We treated HEK293LEPR cells with leptin at the concentration of 100 ng/ml, a standard dose for studying leptin signaling established in previous research of using this in vitro model (24, 31, 32). As expected, leptin stimulation strongly and rapidly increased STAT3 phosphorylation within 15 min following the treatment (Fig. 1E). Compared with the baseline level, leptin-induced STAT3 phosphorylation was robust at 15 min, declined at 45~75 min, and substantially dropped at ~90 min during leptin stimulation. Interestingly, as we continued to monitor STAT3 phosphorylation, we observed that there was a second-phase elevation of STAT3 phosphorylation which was evident from 105 to 210 min (Fig. 1E). Also, as revealed in this experiment, the second-phase induction in STAT3 phosphorylation was moderate and tonic, in contrast with the dramatic and rapid induction of STAT3 phosphorylation in the first phase. Therefore, leptin-induced STAT3 activation include an acute-phase and a tonic, second-phase stage of effects.

Second-phase STAT3 Activation via Chronic Action of Low-dose Leptin—As established in research, leptin rapidly activates the JAK2–STAT3 cascade, which is immediately downstream of leptin receptor, and this process is the molecular basis for leptin-induced acute phosphorylation of STAT3 (30). In light of second-phase STAT3 phosphorylation triggered by leptin shown in Fig. 1E, we predicted that it was likely a result of a molecular event that is secondary to leptin stimulation. To test this hypothesis, we decreased the dose of leptin to a low concentration (10 ng/ml) which was insufficient to result in an acute induction of STAT3 activation. As shown in Fig. 1F, we barely detected STAT3 phosphorylation over 15 to 45 min of leptin treatment at this low dose. In contrast, after 60 min of this treatment, a moderate but chronic induction of STAT3 phosphorylation was observed (Fig. 1F). This time course of STAT3 activation by low-dose leptin agreed with the second-phase profile of leptin-induced STAT3 phosphorylation shown in Fig. 1F. By comparing these two sets of observations, it can be suggested that leptin can induce an acute phase followed by a second phase of STAT3 activation, and these two processes involve different molecular mediators.

Reduction of Second-phase STAT3 Activation of Leptin by Inhibiting TNFα or Downstream—Given the biphasic STAT3 activation of leptin, we re-considered the results that the circadian changes of leptin concentrations in the CSF were followed by the circadian changes of hypothalamic TNFα in mice (Fig. 1, C and D), and also the results that leptin increased hypothalamic TNFα (Fig. 1, A and B), and thus questioned how TNFα might be involved in biphasic STAT3 activation induced by leptin. To justify this question, we confirmed that TNFα stimulation indeed induces an appreciable level of STAT3 phosphorylation in these cells (data not shown), similarly as shown in the literature although based on other cell types (33, 34). To specifically address if there exists a cause-effect relationship, we did experiments to test if leptin-induced STAT3 activation could be reduced by inhibiting TNFα signaling pathway. As known, PS1145 is a potent anti-inflammatory chemical through inhibiting the IKKβ/NF-κB pathway (35) which is an important downstream of TNFα. We found that, while PS1145 did not affect acute-phase STAT3 phosphorylation of leptin (data not shown), it substantially abrogated leptin from inducing the second-phase STAT3 activation (Fig. 2A, left panel). In addition, using a TNFα antagonist, WP9QY (36, 37), we found that it similarly diminished chronic STAT3 activation of leptin (Fig. 2A, right panel). Thus, according to STAT3 signaling, leptin can exert an acute-phase and a chronic-phase action, the latter process was mediated at least by TNFα which, of note, is also produced from leptin simulation.

Role of Central Leptin and TNFα in Mediating Diurnal BP Rise in Mice—Considering that central leptin and TNFα both increased during the active phase (nighttime) in mice (Fig. 1), and this diurnal phase features elevations in many physiological and physical functions supported by BP rise, we wondered if central leptin and TNFα are both required for the diurnal rhythm of BP rise occurring during the active phase of a diurnal cycle. To answer this question, we investigated if blocking TNFα or leptin in the brain could affect BP rise during the active phase (nighttime) in normal mice. Standard, chow-fed C57BL/6 mice were implanted with a telemetric BP probe in the carotid artery and an injection cannula in the third ventricle, using the surgical procedure described previously (20). Following post-implantation recovery, we injected PS1145 to therefore inhibit the action of central TNFα during the nighttime or daytime. While this treatment did not affect daytime BP levels (data not shown), it prevented the nighttime rise of BP levels over ~4 h (Fig. 2, B–G). Also, nighttime increases in heart rates were abrogated by PS1145 treatment (Fig. 2H). In analogy, we employed the similar design but centrally injecting a leptin receptor antagonist to inhibit the action of central leptin. However, a single injection of leptin receptor antagonist did not evidently affect BP, probably because this peptide-based intervention is less efficient compared with the chemical approach.
of administering PS1145; thus we decided to repeat the injection over 3 diurnal cycles to potentially increase the efficacy of the treatment. Despite that the treatment did not change the daytime BP, as similarly appreciated in the literature (10), we found that 3-midnight treatment indeed prevented the nighttime rises across systolic, diastolic and mean BP levels and heart rates (Fig. 2, B–H). Also, the effect from 3-midnight injections of leptin receptor antagonist occurred earlier after the last injection, compared with the effect from a single midnight injection of PS1145 (Fig. 2, B–H). Through superimposing the time-course curves of BP levels from mice that received a single midnight PS1145 treatment versus 3-midnight leptin receptor antagonist treatment (Fig. 2, B–G), it further supported the point that central inhibition of leptin and TNFα can similarly blunt the BP rise during the nighttime in normal mice.

Central TNFα Potentiates BP-elevating Effect of Leptin—Following the loss-of-function results above suggesting that leptin and TNFα are involved in BP rise during the nighttime, we
asked if this relationship of leptin and TNFα in affecting BP could be experimentally separated from nighttime condition. To answer this question, standard C57BL/6 mice received a single injection of either leptin or TNFα in the hypothalamic third ventricle via the pre-implanted cannula during the daytime. To target the daytime was more meaningful for studying if exogenously delivered cytokines have an effect on BP, because loss-of-function study suggested that daytime BP is not affected by the endogenous levels of these cytokines. The injection dose of leptin was set at 2.5 μg, as it represents an established dosage condition for studying leptin effects on metabolic physiology as well as BP (8, 38, 39). Injection dose of TNFα was set at 10 pg, a low-dose option which we employed previously to show that it led to BP increase (20), although it was unclear whether an acute action of this low dose could have a role in normal physiology of BP control. We found that a single injection of TNFα resulted in BP increases which sustained ~30 min (Fig. 3, A–D), a single injection of leptin did not evidently increase BP (Fig. 3, E–H). The lack of an evident effect from single leptin injection was consistent with the literature, which suggested that the hypertensive effect of leptin relied on repeated injections (14, 40). Given the results in Figs. 1 and 2, we wondered if a single
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Injection of leptin might be insufficient to induce the amount of TNFα production and its signaling required for leading to a detectable effect on BP. To explore this possibility, we pretreated mice with low-dose TNFα (10 pg) and 2 h later after BP returned the normal baseline levels (but TNFα downstream signaling was mobilized), we performed an injection of leptin (2.5 μg) and continued to monitor BP in these mice. As shown in Fig. 3, E–H, leptin injection led to strong and sustained BP increases in mice that were pretreated with TNFα. These data indicate that central TNFα is an important mediator for central leptin in mediating physiological BP rise of normal mice.

Central Leptin Excess Potentiates Hypertension Development in Obesity—While results in Figs. 1 to 3 suggested that central TNFα and leptin are involved in mediating the diurnal BP rise in physiology, we further studied if the augmented relationship of these two cytokines could be a basis for the development of obesity-related hypertension, especially since obesity is associated not only with circadian disorders but with excess of both cytokines. Also, because the effect of single leptin administration in increasing BP is modest (14), it suggests that leptin probably require other factor(s) to act together for a potential role in hypertension. To address this question, we profiled the effect of central leptin administration on BP in mice with a moderate degree of obesity induced by ~3-month high-fat diet (HFD) feeding; these mice were still normotensive, but expression of pro-inflammatory genes including TNFα already increased in the hypothalamus as appreciated in recent research (20, 42).

These HFD-fed mice were pre-implanted with a telemetric BP probe in the carotid artery and a cannula in the third ventricle. After post-surgery recovery, mice were injected centrally with leptin (2.5 μg) or the vehicle via the cannula, and BP signals were monitored continuously prior to and post injection, as presented in Fig. 4, A–G. As discussed above, all these injections were performed during the daytime, which was more suitable for studying the effect of exogenously delivered leptin. On Day 1, we found that systolic BP increased at ~40 min following the completion of leptin injection, and this increase remained about 45 min and then declined (Fig. 4, A and D). Notably, systolic BP resurged after an interval (~30 min), seemingly leading to a second-phase increase, which was more long-lasting than the increases in the initial phase (Fig. 4, A and D). Leptin injection on Day 1 did not significantly increase diastolic or mean BP or heart rate (Fig. 4, B–G). On Day 2 following the injection, the BP-raising effects of leptin injection became evident across systolic, diastolic and mean BP as well as heart rate (Fig. 4, A–G), and the effect on diastolic BP further increased on Day 3 following the injection (Fig. 4, B and E). Bi-phasic BP increases continued to be observed (Fig. 4, A–F), suggesting that leptin employs different mechanisms to affect BP over time. Altogether, in contrast to that the acute effect of leptin excess in increasing BP is modest in normal physiology, obesity condition can potentiate the effect of central leptin excess in elevating BP, which can contribute to hypertension development.

Reduction of Leptin-mediated Hypertension in Obesity by Central WP9QY Treatment—To further evaluate if TNFα was important for the BP-raising effect of central leptin in obesity condition, we carried out a study in which mice received 2-day injection of leptin (2.5 μg) in the hypothalamic third ventricle. Since 2-day leptin injections during the daytime was sufficient to increase BP (Fig. 4), we performed 2-day injections of leptin in the daytime, each preceded by an injection of TNFα antagonist, WP9QY (5 μg) versus the vehicle at 2 h before leptin administration. Mice were continuously recorded under telemetry for BP. As shown in Fig. 5, WP9QY treatment significantly prevented leptin from increasing BP. These protective effects by WP9QY against the second-phase action of leptin seemed to be more evident compared with the effect against the first-phase action of leptin. In addition, WP9QY treatment blunted the effect of leptin in increasing the heart rate of these mice (Fig. 5G). Taken together, these results supported that central excess of leptin and TNFα act together to play a critical role in hypertension development in obesity condition.

Prevention of Leptin-mediated Hypertension in Obesity by Central PS1145 Treatment—Next, we investigated if a general inhibition of hypothalamic inflammation by PS1145 could also be useful to counteract against leptin-mediated BP increase in the condition of obesity. As similarly described in Fig. 5, we employed a group of C57BL/6 mice with moderate obesity induced through ~3-month HFD feeding. Under telemetric monitoring, these mice were pre-injected with PS1145 (1 μg) at 2 h before each of 2-day leptin injections in the daytime. Telemetric recording revealed that PS1145 pretreatment significantly prevented leptin from increasing BP in these obese mice, and these protective effects of PS1145 were observed across systolic, diastolic and mean BP (Fig. 6, A–F). In addition, PS1145 pretreatment blocked the effect of central leptin excess from increasing heart rate in these mice (Fig. 6G). Hence, these results corroborated the therapeutic effects of WP9QY treatment presented above, further supporting the point that suppression of hypothalamic inflammation is generally valuable for treating obesity-related hypertension.

Discussion

In this work, we investigated the molecular relationship between leptin and TNFα, examined their role through the CNS in the physiological control of diurnal BP rise, and also studied the potential contribution of central leptin-TNFα connection, when augmented, to obesity-related hypertension (Fig. 7). Indeed, leptin and TNFα are both released significantly from the fat, and cell culture experiments have shown that leptin stimulation led to increased TNFα mRNA and protein levels in cells such as macrophages and monocytes (43, 44). Furthermore, TNFα inhibition was shown to diminish the effect of leptin in up-regulating CD11b expression in neutrophils (45). However, it is still unclear regarding whether there is a relationship between these two cytokines in the brain and especially in the hypothalamus for a physiological function or disease. Here, we demonstrated that increases in leptin concentrations in the CSF over a 24-h circadian period are associated with increases in hypothalamic TNFα production. Also, in vitro and in vivo models both indicated that leptin stimulation leads to TNFα production, a process mostly likely mediated by leptin-induced MAPK activation, which can sensitively increase TNFα gene expression (44). Through signaling analysis, we demonstrated that leptin-induced STAT3 activation were biphasic, including
an initial, acute phase and a second, tonic phase. As known, leptin-induced STAT3 activation during the initial phase is mediated through leptin receptor activation-induced JAK2-STAT3 cascade (30). This initial phase of leptin signaling is fast and excites neurons to rapidly induce biological actions (46), some of which might be independent of STAT3 transcriptional program (47–49). In contrast to the short time window of initial leptin signaling, the second phase of leptin-activated STAT3 is moderate but tonic, presumably being involved in certain chronic functions of leptin, such as immune regulation. This second phase of STAT3 activation is attributed, at least, to leptin-mediated TNFα production, since it is reversed by not only TNFα antagonist WP9QY but also anti-inflammatory chemical PS1145.

Our subsequent physiological experiments focused on comparatively analyzing the effects of leptin and TNFα on BP. Given these two cytokines in the CNS are associated with diurnal rhythms in a coordinated manner, we directed our attention to circadian changes of BP between daytime and night, and found that both cytokines are required to induce BP rise during the night time phase of mice. Leptin is a fat tissue-derived cytokine which is essential for the hypothalamic control over feeding and metabolic physiology, and release of leptin is associated with circadian rhythms of feeding activities (50, 51). It has been

**FIGURE 4.** Rapid BP-raising effect of central leptin in mice with dietary obesity. C57BL/6 mice (adult males) with moderate dietary obesity through 3-month HFD feeding were implanted with a BP radio transmitter in the carotid artery and an injection cannula in the hypothalamic ventricle. After post-surgery recovery, mice received an injection of leptin (Lep, 2.5 μg) during the daytime per day for 3 days. All mice were continuously recorded under telemetry for SBP (A, D), DBP (B, E), MBP (C, F), and HR (G) prior to and post injection. Curves show the time-course changes over a 3-h follow-up according to SBP (A), DBP (B), and MBP (C). Bar graphs show the levels of SBP (D), DBP (E), MBP (F), and HR (G) averaged over the early phase or second phase outlined by broken lines following leptin injection on each day. BP profiles of mice injected with vehicle (Veh) on each day were comparable, and data presented were obtained from day 1. *, p < 0.05; **, p < 0.01, n = 4–5 mice per group. Error bars reflect mean ± S.E.
documented that central delivery of leptin can lead to BP increase in rodents (8); however, it is still unclear regarding how leptin is relevant in the control of BP in physiology. A recent study showed that patients with congenital leptin deficiency or leptin receptor deficiency manifested a decrease in systolic BP despite the condition of severe obesity (10). On the other hand, leptin treatment in normotensive patients with lipodystrophy, a disease associated with low level of leptin due to shortage of fat tissue, did not cause hypertension (52). In context of these clinical observations and supported by our findings, we speculate that in physiology, leptin has a role in inducing a normal level of BP rise during diurnal cycles, which is meant to prevent hypotension rather than causing hypertension. In light of TNFα, despite that it is often appreciated as a pro-inflammatory cytokine which participates in the inflammatory mechanism of disease, acute induction of this cytokine in a controlled manner can be physiologically relevant, and we recently reported that short-term induction of hypothalamic TNFα signaling is required for the initial phase of adaptive immune response (36). Taken together, by extending a previous view that TNFα is a mediator of metabolic syndrome including hypertension (20), the current study suggests a conceptual model (Fig. 7) that a well-regulated production of hypothalamic TNFα in the context of physio-

**FIGURE 5. Reduction of leptin-mediated hypertension in obese mice by central TNFα inhibition.** C57BL/6 mice (adult males) that were maintained HFD feeding for ~3 months received 2-day injection of leptin (2.5 μg) in the hypothalamic third ventricle, each preceded by an injection of WP9QY (WP, 5 μg) versus vehicle (Veh) at 2 h before leptin administration. Mice were continuously recorded under telemetry for SBP (A, D), DBP (B, E), MBP (C, F), and HR (G) prior to and post injection. Curves show the time-course changes over a ~3-h follow-up according to SBP (A), DBP (B), and MBP (C) following leptin injection on Day 2. Bar graphs show the levels of SBP (D), DBP (E), MBP (F), and heart rate (HR) (G) averaged over the early phase or second phase outlined by broken lines following leptin injection on Day 2. *, p < 0.05; **, p < 0.01, n = 4–5 mice per group. Error bars reflect mean ± S.E.
logical leptin stimulation can participate in the physiological control of BP fluctuations.

From the disease perspective, our work in this study suggests that hyperleptinemia in the context of TNFα excess is chronically important for the development of obesity-related hypertension. To mimic the hypothalamic inflammatory changes in obesity, we employed central TNFα pretreatment and found that it remarkably potentiates the effect of subsequent leptin administration in elevating BP. Like leptin, TNFα is secreted prominently from the fat and is excessively produced in obesity (53). Also, it is known that early stage of obesity is already associated with sustained inflammation in the hypothalamus (20, 54), and it was elucidated that hypothalamic NF-κB pathway downstream of TNFα signaling is an important factor for the development of obesity-associated metabolic and cardiovascular disorders (20, 54). Agreeing with the fact that TNFα is an activator of NF-κB, we recently reported that TNFα excess can activate hypothalamic NF-κB to mediate the hypothalamic mechanism of obesity-related hypertension (20). The findings in this work provide information suggesting that hyperleptinemia is a chronic condition involved in inflammatory mechanism of hypertension.

Considering the recent report showing that leptin administration in patients with leptin deficiency did not increase the BP

![Figure 6](image_url)

**FIGURE 6. Reversal of obesity-associated hypertensive effect of leptin by PS1145 treatment.** C57BL/6 mice (adult males) that were maintained HFD feeding for ~3 months received 2-day injection of leptin (2.5 μg) in the hypothalamic third ventricle, each preceded by an injection of PS1145 (1145, 1 μg) versus vehicle (Veh) at 2 h before leptin administration. All mice were continuously recorded under telemetry for SBP (A, D), DBP (B, E), MBP (C, F), and heart rate (HR) (G) prior to and post injection. Curves show the time-course changes over a ~3 h follow-up according to SBP (A), DBP (B), and MBP (C) following leptin injection on Day 2. Bar graphs show the levels of SBP (D), DBP (E), MBP (F), and heart rate (HR) (G) averaged over the early phase or second phase outlined by broken lines following leptin injection on Day 2. *, p < 0.05; **, p < 0.01; n = 4–5 mice per group. Error bars reflect mean ± S.E.
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![Proposed model of central leptin and TNFα in BP control and hypertension](image)

**Physiology**
- **Leptin release and hypothalamic action**
  - Hypothalamic TNFα production
  - Controlled leptin-TNFα diurnal rhythms
  - BP circadian rhythm

**Hypertension**
- **Leptin excess and TNFα excess**

(52), we predict that central TNFα excess should be taken into account for the role of central leptin excess in hypertension development. As it has been raised in the research that the mechanisms for the hypertensive effect exerted by leptin under obesity and lean conditions are different (55, 56), our study indicates that cytokines such as TNFα could be a key point for appreciating the role of leptin in mediating pathological BP increase. This concept is also supported by our result showing that treatment with anti-inflammatory chemical PS1145 and TNFα antagonist WP9QY were both effective in reducing the hypertensive effect of leptin in obesity. Therefore, compared with the strategy of blocking leptin or leptin receptor, use of anti-inflammatory approach can represent an alternative option of breaking the leptin-TNFα mechanism to treat hypertension and related diseases.

Finally, it is worth discussing that, although high-dose TNFα is often known as a cachectic cytokine in diseases like cancers and infections, low-dose TNFα in the hypothalamus at the level of obesity-associated inflammation has been implicated to reduce the sensitivity of leptin in the control of feeding (57, 58). This effect of TNFα in decreasing leptin sensitivity in feeding is in contrast with its augmentation in the BP-raising effect of leptin. The underlying basis can be related to different formulas of how TNFα-induced STAT3 activation is involved in these two processes. As we learned in other studies of ours, STAT3 activation induced specifically by TNFα and that induced specifically by leptin (the portion that is TNFα-independent) can be added up, resulting in more levels of STAT3 activation, which have additive effects on BP. On the other hand, feeding regulation by leptin probably more relies on leptin-specific, TNFα-independent STAT3 activation, while the latter leads to a decrease in the signal to noise ratio of such leptin-specific STAT3 activation. These formulas further imply that the network as well as downstream programs of STAT3 in affecting metabolic physiology versus BP can be differential, which are also in agreement with the fact that the neuroendocrine versus sympathetic nervous system are differentially required in these different physiological functions. This complexity further increases, due to the scenario that leptin can lead to TNFα production and thus contribute to TNFα-dependent STAT3 activation. Hence, future research is much needed to decipher the overlapping versus the differential programs of leptin, TNFα and their downstream molecules (including STAT3) in physiology and diseases.

**Experimental Procedures**

**Experimental Animals**—All studies were conducted in normal or obese adult male C57BL/6 mice obtained from the Jackson Laboratory. All the procedures were approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. All mice were housed in a pathogen-free, standard animal facility and fed a standard chow or high-fat diet (Research Diet, Inc).

**Cannulation and Infusion**—As we previously described (20, 41), under an ultra-precise small animal stereotaxic apparatus (10 μm resolution, David Kopf Instruments), guide 26-gauge guide cannula (Plastics One, Inc.) were implanted into the hypothalamic third ventricle of anesthetized mice at the midline coordinates of 1.8 mm posterior to the bregma and 5.0 mm below the bregma. After 1–2-week post-implantation recovery, mice were injected with chemicals, including leptin (2.5 μg, R&D), TNFα (10 pg, Sigma), PS1145 (1 μg, Sigma), WP9QY (5 μg, Santa Cruz Biotechnology), and mouse leptin receptor antagonist (2.5 μg, Protein Laboratories). Stock solution of chemicals were finally dissolved in 0.5-μl artificial cerebrospinal fluid and infused via pre-implanted cannula over 5-min period using a 33-gauge internal injector (Plastics One, Inc.) that was connected to a 5-μl Hamilton Syringe.

**Telemetric Probe Implantation and Recording**—Radiotelemetric catheters (model TA11PA-C10, Data Sciences International, DSI) were implanted in the carotid artery, using the procedure we described previously (20). Briefly, mice were anesthetized, a ventral midline skin incision was made and the left common carotid artery was isolated under a binocular surgical microscope. The proximal end of the artery was ligated right below the carotid bifurcation, and the distal end was occluded with a microclip. Using a microscissor, a small incision was made near the proximal end, and pressure transmission catheter was guided into the artery, advanced to the aorta, and secured in place with sutures. The transmitter device was placed subcutaneously on the right flank, as close to the hindlimb as possible. Subsequently, neck incision was closed using 5–0 sutures (Ethicon), and mice were allowed for 1–2 weeks of post-surgical recovery, and the pressure signals of each animal under conscious, free-moving conditions were recorded using the computerized method (DSI). BP data were
sampled continuously with a sampling rate of 1,000 Hz with 1-min segment duration.

Cell Culture—HEK293LEPR cells (kindly provided by Dr. L. Rui at University of Michigan) were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplied with 10% fetal bovine serum (Hyclone), 100 units of penicillin, and 0.1 mg/ml streptomycin (Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO2. Cells were fasted in serum-free medium for an overnight period and then were incubated with indicated chemicals including leptin, PS1145, and WP9QY for various time periods as described in the text.

Tissue Harvesting, Western Blot Analysis, and ELISA—Hypothalamus was dissected from the brain as we described previously (54). Collection of the CSF was performed in anesthetized mice at indicated circadian time points, according to an established procedure (36). Protein lysates were prepared from tissues or cultured cells, dissolved in a lysis buffer, separated by SDS-PAGE, immunoblotted with primary antibodies including rabbit anti-p-STAT3, anti-STAT3, and anti-β-actin (Cell Signaling), and reacted with HRP-conjugated secondary antibody (Pierce). Quantification of Western blots was performed with Image J, and p-STAT3 levels were normalized according to the protein levels of total STAT3. Leptin concentration in the CSF was determined using an ELISA Kit (Crystal Chem).

RNA Extraction and qPCR—Total RNA was extracted with Trizol (Invitrogen), the Moloney Leukemia Virus Reverse Transcriptase system (Promega) was used to synthesize complementary DNA, and qPCR was performed using SYBR Green MasterMix (Applied Biosystems) Data were normalized according to mRNA levels of housekeeping gene β-actin.

Statistics—Two-tailed unpaired t test was used for studies which involved only two groups. ANOVA and appropriate post-hoc test were used for experiments comprising more than two groups. All data are presented as mean ± S.E. p < 0.05 was considered statistically significant.

Author Contributions—D. C. conceived the hypothesis and designed the project and structures of experiments. C. H. co-designed and performed experiments represented in Figs. 3 to 6, data interpretation and figure preparation. W. W. and M. K. did experiments in Figs. 1 and 2. A. A. performed implantation surgeries for the study. D. C. wrote the paper, and C. H. provided assistance for writing.

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