Angiotensin type 1a receptors in the forebrain subfornical organ facilitate leptin-induced weight loss through brown adipose tissue thermogenesis

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ABSTRACT

Objective: Elevations in brain angiotensin-II cause increased energy expenditure and a lean phenotype. Interestingly, the metabolic effects of increased brain angiotensin-II mimic the actions of leptin, suggesting an interaction between the two systems. Here we demonstrate that angiotensin-type 1a receptors (AT₁aR) in the subfornical organ (SFO), a forebrain structure emerging as an integrative metabolic center, play a key role in the body weight-reducing effects of leptin via brown adipose tissue (BAT) thermogenesis.

Methods: Cre/LoxP technology coupled with targeted viral delivery to the SFO in a mouse line bearing a conditional allele of the Agtr1a gene was utilized to determine the interaction between leptin and SFO AT₁aR in metabolic regulation.

Results: Selective deletion of AT₁aR in the SFO attenuated leptin-induced weight loss independent of changes in food intake or locomotor activity. This was associated with diminished leptin-induced increases in core body temperature, blunted upregulation of BAT thermogenic markers, and abolishment of leptin-mediated sympathetic activation to BAT.

Conclusions: These data identify a novel interaction between angiotensin-II and leptin in the control of BAT thermogenesis and body weight, and highlight a previously unrecognized role for the forebrain SFO in metabolic regulation.

Keywords Leptin; Brown adipose tissue; Brain; Angiotensin; Sympathetic nervous system; Metabolic regulation

1. INTRODUCTION

The renin-angiotensin system (RAS), long known for its role in blood pressure and fluid balance regulation, is now emerging as a key regulator of metabolic control. Interestingly, while activation of the systemic RAS results in adipogenesis and body weight gain[1−3], recent evidence indicates that activation of the central nervous system (CNS) RAS has the opposite effect and promotes a lean phenotype. Brain infusion of angiotensin-II (Ang-II) in rats[4,5], or genetic overexpression of the RAS components in the CNS of mice[6], both result in an increase in thermogenic energy expenditure. While these findings highlight the importance of brain Ang-II in metabolic regulation, the underlying mechanisms and specific neural regions involved remain unclear.

Importantly, the energy expenditure effects of brain RAS activation are similar to the actions of the adipocyte-derived hormone leptin, suggesting that the metabolic influence of brain Ang-II may be due, in part, to an interaction with central leptin signaling. In line with this, a facilitatory leptin-RAS relationship has been demonstrated in the periphery[7,8]. Moreover, leptin and Ang-II type 1a receptors (AT₁aR) are co-expressed in a number of forebrain[9,10], hypothalamic[11,12] and brainstem[13−15] regions that are implicated in metabolism and energy expenditure[16]. Using AT₁R antagonists and global AT₁aR knockout mice, Hilzendeger et al. [16] previously identified a brain interaction between leptin and RAS in the regulation of sympathetic nerve activity (SNA), but the brain region(s) or metabolic effects of this interaction remains unknown. Given that a number of diseases, including obesity and diabetes, are characterized by altered Ang-II and...
leptin signaling in the CNS, investigation of brain sites that mediate a RAS-leptin metabolic interaction is critical. Here we tested the hypothesis that Ang-II signaling through AT1aR in the brain is involved in the metabolic actions of leptin. We show that the subfornical organ (SFO), a tiny forebrain structure situated outside the blood–brain-barrier, dense with AT1aR, and recently implicated as an integrative metabolic center [17–19], plays a previously unrecognized role in the control of body weight. Specifically, we demonstrate an interaction between SFO-AT1aR and CNS leptin in the regulation of brown adipose tissue (BAT) thermogenic metabolism and body weight. When AT1aR are selectively deleted from the SFO, sympathetically mediated BAT thermogenesis and decreases in body weight in response to leptin are significantly blunted, independent of changes in locomotor activity and food intake. In addition to revealing a previously unknown role for the forebrain SFO in metabolic regulation, these data identify a strong link between AT1aR in the SFO and CNS leptin in the control of energy expenditure and body weight.

2. METHODS

Detailed methods are available in the online supplement.

2.1. Animals

All animal protocols were approved by Animal Care and Use Committees at Cornell University and the University of Iowa. Studies were conducted in adult (8 weeks old) male AT1aR<sup>Rex/</sup> mice initially obtained from the colony of Dr. Thomas Coffman [20] and used to establish our own colony. Mice were fed standard chow and water ad libitum and were housed with a 12-h light/dark cycle.

2.2. Leptin administration

For experiments involving lateral ventricle (ICV) injection of leptin or vehicle (saline), mice were instrumented with an indwelling ICC cannula [21]. Murine leptin was injected ICV (2 μg daily) or i.p. (30 μg bi-daily) either over a 4-day period or acutely, as previously described [10,16,22].

2.3. Adenoviral targeting of Cre to the SFO and lateral ventricle cannulation

Targeting of the SFO with recombinant adenoviral vectors encoding AdCre (4 × 10<sup>10</sup> plaque-forming units/ml) or titer-matched AdLacZ was performed as previously described in detail by our laboratory [21,23,24]. Viral targeting and ICV cannulation were performed in the same surgical setting.

2.4. Sympathetic nerve recording

Mice were instrumented for multiferber recordings of BAT-SNA as previously described [10,16,22]. Briefly, following anesthesia, the nerves to BAT were identified, mounted on platinum–iridium recording electrodes and fixed with silicone gel. Following surgical procedures, the animals were allowed to stabilize prior to obtaining BAT measurements before and for up to 4 h following ICV leptin administration.

2.5. Quantitative real-time PCR

Micropunches of the SFO, organum vasculosum lamina terminalis (OVLT), accumbus nucleus, ventromedial hypothalamus, paraventricular nucleus of the hypothalamus and somatosensory cortex were obtained using brain atlas coordinates [25] as described [10]. Tissue from two mice was pooled per biological sample. Total RNA was also individually isolated from BAT for thermogenic mRNA evaluation.

2.6. Data analysis

Data are expressed as mean ± SEM and were analyzed by a two-tailed unpaired t-test or two-way repeated measures ANOVA, with appropriate post-hoc comparisons when applicable. A value of p < 0.05 was considered statistically significant.

3. RESULTS

3.1. Cre-mediated ablation of AT1aR selectively in the forebrain SFO

Previous investigations that have examined a role for the brain RAS in metabolic regulation have used global knockout models, whole brain overexpression of RAS components or central infusion of Ang-II or its antagonists [4–6,16]. While beneficial, these approaches are limited as they do not allow for specific dissection of the neural regions involved [26]. Within the CNS, the actions of Ang-II are mediated primarily through AT1aR, with very high expression of this receptor in the forebrain circumventricular SFO [15,17,18]. Interestingly, this CNS structure is emerging as an important in metabolic regulation [9,19,27]. To investigate the metabolic role of AT1aR specifically in the SFO, we utilized Cre/LoxP technology coupled with brain site-selective viral delivery in a mouse line bearing a conditional allele of the Agtr1a gene (AT1a<sup>Rex/</sup>) [20]. Targeting of an adenovector expressing Cre recombinase (AdCre) selectively to the SFO resulted in stable, robust, localized expression of Cre within this region (Figure 1A), consistent with our previous findings [10,23,24,28]. In line with this, quantitative real-time PCR of SFO micropunches demonstrated a ~95% reduction in AT1aR<sup>transcript</sup> levels in mice having undergone SFO-targeted transfer of AdCre compared to those that received a control vector (AdLacZ) (Figure 1B). Importantly, AT1aR levels remained unchanged in hypothalamic leptin-responsive regions, including the arcuate nucleus, ventromedial hypothalamus and paraventricular nucleus, as well as the circumventricular OVLT and somatosensory cerebral cortex (Figure 1B).

Since the long signaling form of the leptin receptor (LepRb) is also recognized role in the control of body weight. AT1aR in the SFO did not influence baseline body weight (AdCre vs. AdLacZ, p > 0.05). Next we administered leptin directly into the brain via an implanted ICV cannula [10,39], which allowed for investigation of brain leptin–AT1aR interactions without the confounding influence of leptin’s peripheral metabolic actions. Daily ICV leptin administration caused a progressive and robust decrease in body weight over a 4-day period in mice with intact AT1aR in the SFO (AdLacZ, Figure 1D). By comparison, mice in
which AT1aR had been ablated in the SFO showed markedly attenuated leptin-induced weight loss, such that they demonstrated 40% less of a decrease in body mass by 4 days. Similar findings were obtained with systemic (i.p.) leptin administration (Figure S1A) and long-term ICV leptin dosing over 7 days (Figure S1C), whereas daily administration of vehicle did not alter body weight (Figure 1D).

To assess whether the blunted weight loss response to leptin was due to changes in food intake, we evaluated the influence of SFO-AT1aR on the feeding responses to leptin. As expected, vehicle administration did not alter food intake, whereas central and systemic administration of leptin over a 4-day period caused a significant decrease in ad libitum food consumption. Interestingly, selective removal of AT1aR from the SFO did not significantly alter leptin-induced decreases in food intake [Figure 1E (ICV), Figure S1B (i.p.) and S1D (ICV)]. Similar findings were obtained when the data were analyzed as cumulative food intake over the study period (Figure 1F). Given the importance of angiotensinergic actions within the SFO on drinking responses [18], water intake was also evaluated during ICV leptin administration. Whereas locally increased production of Ang-II within the SFO promotes increases in drinking [34], water consumption was unchanged following Cre-mediated deletion of AT1aR (Figure S2A). This suggests that SFO-AT1aR is not necessary for tonic ad libitum regulation of fluid intake. Consistent with previous reports [35], daily water intake remained unchanged throughout leptin administration (Figure S2A).

We next sought to identify if circumventricular SFO-AT1aR mediate leptin-induced weight loss through an influence on energy expenditure. In normal mice, spontaneous locomotor activity occurs in a diurnal fashion, with low and high levels during the light and dark period of the day. In mice where AT1aR had been ablated in the SFO, locomotor activity was significantly attenuated by leptin administration (Figure 1G), with a 40% decrease in total activity by 4 days. Similar findings were obtained with systemic (i.p.) leptin administration (Figure S1A), whereas daily administration of vehicle did not alter locomotor activity (Figure 1D).

Figure 1: Ablation of AT1aR from the SFO blunts leptin-induced weight loss in a food intake-independent manner. (A) Representative immunohistochemical image of Cre staining in the SFO of a mouse with SFO-targeted delivery of AdCre (20×); LV, lateral ventricle. Quantitative real-time PCR measurements of AT1aR (B) or LepRb (C) mRNA from micropunches of the SFO, OVLT, arcuate nucleus, ventromedial hypothalamus (VMH), paraventricular nucleus of the hypothalamus (PVN) or somatosensory cerebral cortex (CTX) following SFO-targeted AdCre or control vector AdLacZ (n = 3–4; 2 brains pooled per sample). (D) Cumulative weight loss during daily ICV vehicle or leptin (2 μg/day) administration (n = 16–18/group). *p < 0.05 vs. AdLacZ, #p < 0.05 vs. respective baseline. Mean ± SEM. See also Figures S1 and S2.
This diurnal variation is lost in db/db mice, which exhibit leptin deficiency and ob/ob mice with a loss-of-function mutation in the leptin receptor [35–37]. Thus, given the importance of leptin signaling in the regulation of spontaneous locomotor activity, radiotelemetry was utilized to measure activity in AdLacZ- and AdCre-targeted AT1aR−/− mice before and during ICV leptin administration. As seen in Figure S2B, locomotor activity was similar between the two groups throughout the light–dark cycle under basal conditions. In line with previous findings [35,38], ICV leptin resulted in a modest and transient, albeit significant, increase in total spontaneous locomotor activity, primarily during the dark phase (p < 0.05). This was not influenced by deletion of AT1aR from the SFO (Figure S2B). Collectively, these findings demonstrate that AT1aR in the SFO are obligatory for the weight-reducing actions of leptin, but via a mechanism that is independent of food and water intake and activity-mediated energy expenditure.

3.3. AT1aR in the SFO mediate leptin-induced increases in body temperature and decreases in BAT mass

In addition to evoking a negative energy balance through appetite inhibition and increasing spontaneous locomotor activity, leptin acting within the CNS evokes an increase in body temperature due to mobilization of fat stores [39]. While basal core body temperature was similar between AdLacZ- and AdCre-treated mice (Figure 2A), ICV leptin caused a significant increase in 24-h body temperature measurements in control animals over a 4-day period (Figure 2B), in line with previous reports [40–42]. Following ablation of AT1aR in the SFO, leptin-induced increases in core body temperature were significantly

**Figure 2:** Deletion of AT1aR in the SFO attenuates leptin-induced increases in body temperature and decreases in brown adipose tissue mass. Baseline 24 h core body temperature (A) and the change (B) in body temperature in response to 4 days of ICV leptin (2 μg/day) administration in mice with SFO targeted AdCre or control vector AdLacZ (n = 5/group). *p < 0.05 vs. AdLacZ. Liver (C), gonadal white (D), subcutaneous inguinal white (E) and interscapular brown (F) adipose tissue weights following 4-day ICV vehicle or leptin administration (n = 5–8/group). *p < 0.05 vs. AdLacZ + ICV leptin, #p < 0.05 vs. respective vehicle group. Mean ± SEM. See also Figure S3.
attenuated (Figure 2B). These findings were also evident when considering the lower body mass (i.e., the increase in body temperature per gram of mass) of the AdLacZ animals during both the light and dark phases (Figure S3). This indicates that SFO-AT1aR is necessary for leptin-mediated increases in body temperature, and further suggests that the blunted weight loss in response to leptin in AdCre-targeted mice is due to an influence on activity-independent thermogenesis.

The pleiotropic actions of leptin within the CNS increase energy expenditure through neurohumoral regulation of peripheral tissue metabolism [39]. Therefore, we subsequently evaluated adipose tissue and liver mass following ICV leptin administration. Liver and perigonadal and subcutaneous white adipose tissue (WAT), as well as subscapular BAT weights, were similar between AdLacZ and AdCre-treated animals following daily ICV vehicle treatment (Figure 2C–F). Furthermore, robust decreases in liver and WAT masses were noted following ICV leptin, and these effects were not altered by deletion of AT1aR in the SFO (Figure 2C–E). In contrast, whereas ICV leptin elicited a marked reduction in BAT mass in control mice, selective removal of SFO-AT1aR abolished this response (Figure 2F). These data demonstrate that angiotensinergic signaling in the SFO is essential for leptin-induced reductions in BAT, but not for leptin-induced decreases in WAT or liver mass.

3.4. Ablation of SFO-AT1aR blunts leptin-induced upregulation of BAT thermogenic markers and sympathetic outflow to BAT

To evaluate the underlying mechanisms contributing to a blunted BAT response to leptin, quantitative real-time PCR profiling of thermogenic markers in BAT was performed. This was based on evidence that β-3 adrenergic receptor-mediated signaling promotes transcription factor-induced upregulation of uncoupling protein-1 (Ucp1), and thus thermogenesis. At the same time, lipolysis is increased through hormone sensitive lipase, and this free fatty acid release also serves to locally activate Ucp-1, while at the same time providing substrate for heat production [43]. Relative to ICV vehicle-treated mice, central leptin administration in SFO-targeted AdLacZ animals increased the expression of BAT thermogenic markers, including Ucp1, peroxisome proliferator-activated receptor-γ coactivator-1α (encoded by Ppargc1a) and hormone sensitive lipase (encoded by Lipe). In contrast, the ICV leptin-elicited increase in these markers was significantly blunted in mice with SFO-selective deletion of AT1aR (Figure 3A). Beta-3 adrenergic receptor expression (encoded by Adrb3) remained unchanged following daily leptin or vehicle in AdLacZ- and AdCre-treated mice (Figure 3A). Together, with the reduced BAT loss in response to ICV leptin, these findings demonstrate that SFO-AT1aR mediate leptin-induced decreases in body weight through modulation of BAT thermogenic energy expenditure.

The CNS is critical for the control of thermogenic responses through adjustment of sympathetic nervous system outflow to BAT [44]. In this context, mice with genetic brain RAS overexpression demonstrate increased SNA to BAT, in conjunction with elevated basal energy expenditure [6]. Furthermore, systemic or central administration of leptin induces large increases in sympathetic outflow to BAT, thereby promoting BAT thermogenesis [22,39,45]. Based on this, along with the observed increases in BAT Ucp1, Ppargc1a and Lipe mRNA (Figure 3A) and recent evidence that these markers are upregulated by increases in sympathetic tone [45], we investigated if an interaction between SFO-AT1aR and leptin signaling exists in the control of BAT SNA. Following selective deletion of SFO-AT1aR and recovery, mice were administered ICV leptin for one day. On the subsequent day, the time at which the body weight differences between the groups started to appear (Figure 1D), the effect of ICV leptin on the activity of the sympathetic nerves innervating subscapular BAT was tested. In response to ICV leptin, control AdLacZ-treated mice demonstrated remarkable...
increases in BAT SNA over a 4-h period (Figure 3B,C). Ablation of AT\textsubscript{1a}R in the SFO completely prevented leptin-induced increases in BAT SNA, and this response was evident with quantification of SNA either as a frequency or integrated voltage (Figure 3C). Importantly, the increase in BAT SNA in response to stepwise decreases in core body temperature was preserved in AdCre-targeted mice (Figure S4), illustrating that removal of SFO-AT\textsubscript{1a}R induces a selective effect on leptin-induced increases in sympathetic outflow to BAT.

4. DISCUSSION

To our knowledge, this is the first report of an obligatory role for SFO-AT\textsubscript{1a}R signaling in the weight-reducing effects of leptin. Further, while recent evidence suggests that the brain RAS induces energy expenditure through increases in thermogenesis [6,16], a specific brain site has not been identified. Our findings of an SFO-specific facilitatory action of AT\textsubscript{1a}R on leptin-mediated increases in sympathetically induced BAT thermogenesis advance these previous studies by pinpointing a key brain region involved. These results not only demonstrate an interaction between Ang-II signaling in the SFO and CNS leptin in the control of BAT thermogenesis and body weight, but also highlight a previously unrecognized role for forebrain SFO AT\textsubscript{1a}R in metabolic regulation. This does not rule out the possibility that this SFO AT\textsubscript{1a}R mechanism could be affecting leptin signaling at other CNS sites. The energy expenditure actions of the CNS are diverse and include effects on appetite, activity, and metabolism [39,46]. Despite a blunting of leptin-induced weight loss, removal of AT\textsubscript{1a}R from the SFO did not influence leptin-mediated food intake or locomotor activity. Furthermore, selective Cre-mediated removal of AT\textsubscript{1a}R from the SFO did not alter the effects of leptin on WAT and liver mass, whereas upregulation of BAT thermogenic markers and efferent sympathetic outflow to BAT were abolished by deletion of SFO-AT\textsubscript{1a}R. These findings illustrate a selective interaction between angiotensinergic signaling within the SFO and leptin to influence BAT thermogenesis, although the role of SFO-AT\textsubscript{1a}R in other metabolic actions of leptin (e.g. glucose metabolism, white adipose tissue browning) remain to be investigated. Interestingly, even though mice with global knockout of AT\textsubscript{1a}R also demonstrate a normal decrease in food intake and a blunted increase in BAT SNA in response to leptin, leptin-induced reductions in body weight are not affected [16]. This underscores the importance of considering the individual contribution of AT\textsubscript{1a}R signaling in specific brain regions. Given the diverse and widespread effects of RAS signaling, it is likely that a complex interaction exists in which global leptin-mediated weight loss actions can be influenced by AT\textsubscript{1a}R signaling in multiple neural regions and/or in the periphery [6,16]. Interestingly, we have recently shown that selective removal of the long form of the leptin receptor from the SFO did not influence leptin-induced decreases in body weight or increases in sympathetic outflow to BAT [10]. Taken together with the current findings, this would suggest that the influence of angiotensinergic signaling in the SFO is on downstream leptin sensitive sites. Indeed, the SFO contains extensive efferent connections to CNS metabolic control regions, including the hypothalamus and brainstem [27], and therefore removal of SFO-AT\textsubscript{1a}R may impact leptin action in these neural areas. Alternatively, leptin and AT\textsubscript{1a}R may signal through distinct cellular populations in the SFO, with AT\textsubscript{1a}R necessary for the complete thermogenic actions of leptin.

In the setting of obesity, the CNS develops resistance to the effects of leptin. While the mechanisms underlying partial leptin resistance in obesity remain to be elucidated, the overarching focus has been on disruptions in intrinsic leptin signaling [39]. The current findings suggest that alterations in angiotensinergic signaling within the SFO may also induce partial metabolic leptin resistance. Indeed, the blunted leptin-induced weight loss and abrogation of BAT sympathetically mediated thermogenesis following removal of SFO-AT\textsubscript{1a}R mimic the responses in diet-induced obese mice [22]. Although yet to be determined, disruptions in the brain RAS may therefore contribute to impairment in leptin function in pathophysiologic states.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.01.007.

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