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Leptin “gates” thermogenic action of thyrotropin releasing hormone in the hindbrain

Richard C. Rogers, Maria J. Barnes, and Gerlinda E. Hermann

Laboratory of Autonomic Neurosciences, Pennington Biomedical Research Center, Baton Rouge, LA 70808

Abstract

Leptin, acting as a measure of metabolic fuel availability, exerts a powerful permissive influence on neurogenic thermogenesis. During starvation and an absence of leptin, animals cannot produce thermogenic reactions to cold stress. However, thermogenesis is rescued by restoring leptin. We have previously observed (Hermann et al., 2006) a highly cooperative interaction between leptin and thyrotropin releasing hormone [TRH] to activate hindbrain generated thermogenic responses. Specifically, exposure to both leptin and TRH elicited a 3.5°C increase in brown adipose tissue [BAT] thermogenesis, while leptin alone did not evoke any change, and TRH alone caused only ~1°C increase. The present study shows that the leptin-TRH synergy in controlling brown adipose [BAT] thermogenesis is order-specific and dependent on the feeding status of the animal. That is, fourth ventricular [4V] application of leptin to the food deprived animal, before TRH injection, yields a substantial increase in BAT; while the reverse order yields a significantly smaller effect. If the animal were fed within minutes of anesthesia, then exogenous leptin was not necessary for TRH to yield large increase in BAT temperature. The leptin-TRH synergy was uncoupled by pretreatment with the phosphoinositol-tris phosphate kinase [PI3K] inhibitor wortmannin and the Src-SH2 antagonist, PP2. The TRH transduction mechanism utilizes phospholipase C [PLC] potentially regulated by the SH2 site. Previous work in culture systems suggest that the product of PI3K activity [PIP3] potentially upregulates PLC by activating the SH2 domain of the PLC complex. Perhaps leptin “gates” the thermogenic action of TRH in the hindbrain by invoking this same mechanism.

Keywords

thermogenesis; synergistic interactions; TRH; leptin; brown adipose tissue

Introduction

Leptin is released from adipose tissue roughly in proportion to the amount of stored metabolic fuel. However, leptin apparently encodes both the level of body fat as well as the rate of change of fuel availability as the hormone’s secretion is highly sensitive to acute food deprivation and refeeding (Ahima and Flier, 2000; Marie et al., 2001). Leptin also increases thermogenesis (Trayhurn and Bing, 2006). Studies examining leptin’s thermogenic effects have focused on the forebrain; e.g., leptin action in the arcuate nucleus causes a sympathetically-mediated

Corresponding author: Gerlinda E. Hermann, Laboratory of Autonomic Neurosciences, Pennington Biomedical Research Center, LSU Systems, 6400 Perkins Rd, Baton Rouge, LA 70808, Gerlinda.Hermann@pbrc.edu, 225.763.2622.

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increase in thermogenesis through the activation of poly-synaptic projections to the hindbrain (Bamshad et al., 1999; Elmquist, 2001; Morrison, 2004).

Inputs from the forebrain are not always needed to produce thermogenesis. Within limits, the hindbrain has the capacity to control temperature. Decerebrate rats can increase heart rate, and to an extent, maintain core temperature in response to cold exposure (Harris et al., 2006). The hindbrain has the neural circuitry to both detect the need for heat and ameliorate that need through the release of stored fuels when metabolites are in abundance (DiRocco and Grill, 2003; I'Anson et al., 2003; Skibicka and Grill, 2009).

Our recent study has shown that administering leptin, alone, in the hindbrain did not have an effect on thermogenesis. However, in combination with thyrotropin-releasing hormone [TRH] leptin produced a significant increase in thermogenesis (Hermann et al., 2006). This synergistic effect of leptin and TRH was observed in both intact and decerebrate animals.

TRH containing pathways of the hindbrain are potentially important mediators of neurogenic heat production. Raphe projections to the spinal cord are responsible for triggering sympathetic and thermogenic inputs to brown adipose tissue [BAT] (Cano et al., 2003; Nagashima et al., 2000). Several lines of evidence suggest that these raphe projections are TRHergic and are responsible for initiating neurogenic heat production (Arancibia et al., 1996; Helke et al., 1986). These raphe neurons could, themselves, be influenced by descending hypothalamic TRHergic projections commanding an increase in heat production (Lechan and Fekete, 2006). Additionally, TRHergic projections from raphe to the NST are responsible for driving increased gastric motility that occurs in cold stress (Martinez et al., 2001; Palkovits et al., 1986; Rogers et al., 1996). This pathway provides the means by which a signal related to need for heat production [i.e., activation of the TRHergic projection to the NST] can converge with a signal related to metabolic fuel supply [i.e., circulating leptin] on NST neurons projecting to raphe and reticular areas controlling thermal homeostasis (Blessing, 1997).

The underlying mechanism[s] for this synergistic effect of leptin and TRH on thermogenesis is not known. One plausible theory is that TRH signaling is potentiated by events in the leptin's signaling cascade [see Figure 1]. Previous studies (Ahima and Flier, 2000; Marie et al., 2001) have shown that leptin levels are sensitive to acute fasting. Leptin can decline 75% in the course of a 16 hour fast. This decline in leptin could explain the gating effect exogenous leptin has on TRH in overnight deprived rats. If this is the case, then in animals fed ad libitum, TRH applied to the fourth ventricle should have a much larger effect on BAT thermogenesis.

Results

As we have previously reported (Hermann et al., 2006), microdrop application to the floor of the fourth ventricle [4V] of leptin followed by TRH causes a dramatic increase in BAT temperature, peaking at approximately +3°C within 60min of the application of TRH [Figure 2 and 3]. BAT temperature then declines to within one degree of baseline approximately six hours later [data not shown]. In contrast, reversing the order of application [i.e., TRH followed by leptin] did not elicit the same magnitude increase in BAT temperature as seen in the leptin/TRH group. In food deprived animals, the maximum change in BAT temperature elicited after the application of TRH and followed by leptin was only approximately 1°C above baseline. This effect is quite similar to that seen with the application of TRH alone in food deprived rats as we reported in our previous experiments (Hermann et al., 2006). Pretreatment with either PI3 kinase inhibitor [wortmannin] or the Src-SH2 antagonist [PP2] essentially eliminated BAT thermogenesis caused by 4V application of leptin followed by TRH [Figure 2 and 3]. Note that pretreatment with these antagonists had no independent effects on either thermogenesis or respiratory rates [data not shown].

The ability of TRH to elevate BAT temperature in fasted rats is “permitted” by preceding the TRH 4V injection with leptin 4V. However, TRH thermogenic effects are at least partially restored in animals that are freely fed. That is, in food deprived rats, 4V application of TRH, alone, produced a 1°C increase in BAT temperature [Hermann et al., 2006]; whereas, in ad libitum fed rats, TRH produced a $2.2 \pm 0.4^\circ\text{C}$ increase in BAT temperature [Figure 3].

These changes in BAT temperature are reflected in changes in core body temperature but at a smaller magnitude [Figure 3]. As expected, and reported previously [2006], peak changes in core body temperature tend to lag that of BAT temperature.

Discussion

We have previously demonstrated that leptin and TRH have a synergistic effect on sympathetically-controlled BAT thermogenesis (Hermann et al., 2006). Exposure of the hindbrain [via the fourth ventricle] to leptin prior to a TRH application markedly enhances the nominal thermogenic response to TRH administered alone. The current studies demonstrate that this effect is sensitive to the order of application in that, in food deprived animals, fourth ventricular TRH will not provoke significant BAT thermogenesis unless it is preceded by leptin. Leptin applied alone to the fourth ventricle also does not cause BAT thermogenesis (Hermann et al., 2006). This order effect strongly suggests that TRH signaling is gated by components of the leptin transduction pathway.

Substantial experimental effort has been directed toward the understanding of mechanisms of leptin action within the brain. Practically all of that effort has focused on leptin interactions with hypothalamic peptidergic neurons in the arcuate nucleus [ARC]; the results of which are reviewed in detail elsewhere (Ellacott and Cone, 2004; Grill and Kaplan, 2002; Sahu, 2003). OB-Rb utilizes the janus kinase 2 [JAK2] mechanism to phosphorylate the signal transducer and activator of transcription-3 [STAT3], and insulin receptor substrate-1 [IRS-1]. The phosphorylation of IRS-1 yields activation of phosphatidylinositol-3 kinase [PI3K].

There is strong evidence that leptin activates the production of hypothalamic TRH through the action of STAT3 (Guo et al., 2004; Harris et al., 2001) [Fig. 1]. This mechanism is probably responsible for the long-term depression of metabolic activity during starvation where low levels of leptin (Flier et al., 2000) cause a drop in TRH production and, ultimately, thyroid hormone release associated with fasting. Leptin administered during a fast restarts TRH transcription (Ahima and Flier, 2000; Harris et al., 2001).

In addition to transcriptional effects, leptin can also produce rapid changes in the excitability of neurons whose activity are correlated with rapid reductions in feeding behavior and increases in energy expenditure (Cowley, 2003; Cowley et al., 2001; Malcher-Lopes et al., 2006; Sahu and Metlakunta, 2005). Leptin can have divergent effects on neurons, i.e., activating some [e.g., POMC ARC neurons] and inhibiting others [e.g., NPY ARC neurons and neurons in the dorsal motor nucleus of the vagus - (Cowley, 2003; Cowley et al., 2001; Ellacott and Cone, 2004; Williams et al., 2007)]. Details about the mechanisms by which leptin can cause such immediate changes in hindbrain neuronal excitability are not completely understood. However, one possibility includes the rapid modulation of neuronal excitability through the action of PI3K. This kinase, acting through the intermediate of the Src homolog - 2 [SH2] can affect the function of cation conductances with direct effects to activate or inhibit neurons (Shanley et al., 2002; Williams et al., 2007). Furthermore, phosphorylation of SH2 regulatory sites on other transduction elements can potentially regulate cellular responses to agonists depending on those elements (Liu and Ye, 2005; Rameh and Cantley, 1999; Rhee, 2001).

The TRH transduction mechanism provides such an example. The neuronal TRH receptor is coupled with a Gq transduction element that, in turn, activates phospholipase C [PLC]. PLC,

in turn, produces inositol 1,4,5 trisphosphate [IP3] and diacyl glycerol [DAG]. IP3 causes the rapid release of calcium from intracellular stores, while DAG activates protein kinase C [PKC] (Barker et al., 1987; Gershengorn, 1989; Sun et al., 2003). The profound increase in cytoplasmic calcium induced by IP3 production has been linked to the initiation of calcium oscillations that can be used to control a wide variety of cellular functions (Somogyi and Stucki, 1991; Tse and Tse, 1999; Verkhatsky, 2002). This may include burst-type activation of neurons in the NST (Dekin et al., 1985; Hermann et al., 2005; Koshiya and Smith, 1999). Activation of PLC and liberation of ER calcium has also been linked to the opening of non-specific cation channels and the depression of [inhibitory] potassium currents (Bayliss et al., 1994; Ishibashi et al., 2003; Winks et al., 2005). These effects can lead to significant neuronal depolarization and excitation and have been observed in neurons in the dorsal medulla responding to TRH (Dekin et al., 1985; Travagli et al., 1992). TRH-PLC transduction can also inhibit DVC neurons (Browning and Travagli, 2001); the result depends on the phenotype of the cell in question.

Observations in culture systems suggest that the activity of PI3K and PLC is highly synergistic (Marshall et al., 2000; Rameh et al., 1998). A specific cross-talk mechanism linking PI3K and PLC activity in the brain has not been described until now. However, work in other cell-systems strongly suggests that the product of PI3K activity, [PIP3], potently upregulates PLC (Bae et al., 1998; Marshall et al., 2000; Rameh et al., 1998; Yang et al., 2001). A growing literature suggests that PIP3 positively modulates PLC by activating the SH2 domain of the PLC complex (Liu and Ye, 2005; Rameh and Cantley, 1999; Rhee, 2001). The results of this paper suggest that leptin gates the action of TRH in the hindbrain by invoking the same mechanism. Blockade of either PIP3 formation with wortmannin or SH2 activation with PP2 blocks the order-specific potentiation of TRH effects by leptin.

The neural circuitries involved in this hindbrain thermoregulatory phenomenon are as yet unknown, although the solitary nucleus [NST] is a prime candidate for inclusion. A speculative model can be constructed from the results of the present work as well as the literature [Figure 4]. Several investigators have reported the presence of the leptin receptor on limited numbers of NST neurons; leptin can gain access to these cells as they are in a weakened region of the blood brain barrier. Further, the NST is known to receive substantial, functionally significant TRHergic input from the raphe pallidus [Rp], a region critical to the maintenance of thermocontrol (Nagashima et al., 2000). We hypothesize that leptin and TRH interact in the hindbrain to change the sensitivity of thermogenic circuitry to descending central commands to produce heat. Descending hypothalamic projections carrying commands to increase heat generation terminate on sympathetic “premotor” neurons in the ventrolateral medulla [VLM] and the Rp. These ventral medullary neurons project to the intermediolateral cell column [IMLCC] in the cord that, in turn, control BAT thermogenesis and peripheral blood flow [i.e., heat conduction] (Nagashima et al., 2000). These ventral medullary neurons are under considerable local GABAergic inhibition; site specific injections of bicuculline produce rapid and dramatic increases in BAT and core temperature (Cao et al., 2004). Perhaps the NST acts to relieve this GABAergic inhibition, thus increasing the sensitivity of the thermogenic circuits? Such an arrangement is similar to those responsible for NST-mediated adjustments in cardiovascular function (Blessing, 1997). This could be accomplished by a convergence of leptin [hormonal] and TRHergic [neural] inputs onto NST neurons that are sensitive to both agonists. TRHergic input to the NST from the Rp is proportional to the demand for heat production (Martinez et al., 2001). Here, heat demand can be augmented by vagal afferents carrying information about core temperature and exposure to pyrogens. The gating mechanism at the NST could probably be similar to that depicted in Figure 4.

This model could explain why animals that are deficient in leptin [e.g., during starvation] cannot mount a thermogenic response to cold stress or to immune cytokine release unless leptin

is restored (Blumberg et al., 1999; Geiser et al., 1998). Further, our results contrasting the effects of TRH on BAT thermogenesis in fasted versus fed rats suggest that reductions in leptin levels caused by overnight fasts are sufficient to disrupt thermogenesis. Several previous reports show that circulating leptin levels are sensitive to food deprivation status (Marie et al., 2001; Ahima and Flier, 2000). While 48hr food deprivation essentially eliminates leptin secretion, even overnight deprivation can produce a 75% drop in leptin levels relative to levels seen in the fed state. Thus, leptin levels are an example of an ideal feedback control signal. Leptin is apparently a measure of the stored fuel supply combined with the derivative of the fuel supply [i.e., rate of use/loss]. As such, rapid rates of change in fuel supply would have an amplified impact on processes that are regulated by leptin. This makes good physiological sense in that an acute fuel shortage can be compensated for before it becomes an emergency. The present results and those of our previous paper (Hermann et al., 2006) are consistent with this view. If rats are allowed to free feed up to the moment of anesthesia, TRH can provoke a significant [$\sim 2^{\circ}\text{C}$] increase in BAT temperature. In animals deprived overnight, TRH, alone, produces a much smaller [$\sim 1^{\circ}\text{C}$] increase. Exogenous leptin “rescues” the TRH effect in a food deprived animal, which now produced $\sim 2.7^{\circ}\text{C}$ increase in BAT temperature. Regarding the potential for this mechanism to “waste” heat in the case of a surplus of stored fuel (Dulloo and Jacquet, 2001), high leptin levels alone will probably not cause significant thermogenesis unless this condition is paired with a central command or need [i.e., TRH] to do so.

Experimental Procedure

Long-Evans rats [300–500 g; 3–10months of age] of either sex, obtained from the breeding colony located at Pennington Biomedical Research Center, were used in these studies. All animals were maintained in a room with a 12:12 hour light-dark cycle with constant temperature and humidity, and given food and water *ad libitum* until the evening before experimentation. At this point, individual rats were food-deprived overnight [~ 16 hours]. All experimental protocols were performed according to the guidelines set forth by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees at the Pennington Biomedical Research Center.

Drugs

All drugs were reconstituted in phosphate buffered saline [PBS; pH = 7.4]: recombinant rat leptin [Preprotech Inc., Rocky Hill, NJ]; thyrotrophin releasing hormone [TRH; Sigma-Aldrich, St Louis, MO]; wortmannin [Sigma-Aldrich, St Louis, MO]; PP2 [4-amino-5-[4-chlorophenyl]-7-[t-butyl]pyrazolo[3,4-d] pyrimidine [CalBiochem, San Diego, CA].

Initial Surgical Techniques

Eighteen rats were food deprived overnight [approx 16hrs] and then anesthetized with thiobutabarbital [Inactin; 150mg/kg, IP; Sigma]. [Another group rats (N = 4) were fed *ad libitum* up to the time of anesthesia; all other preparations were the same as described for the primary experimental group.] Inactin is long-lasting and will not depress autonomic reflexes (Buelke-Sam et al., 1978). After induction of anesthesia, the trachea was cannulated for maintenance of an open airway. A jugular cannula was installed for intravenous drug infusion before the animal was placed in a stereotaxic frame. A midline incision was made between the parieto-occipital junction and the C1 spinus process. The exposed cervical musculature was retracted. The exposed foramen magnum was opened; removal of the dura and arachnoid membranes exposed the caudal portion of the floor of the fourth ventricle including the area postrema. The rat rested on a heating pad designed to maintain a constant temperature of 36°C . Note that a feedback-controlled temperature-regulating pad was not used here to avoid the problem of having the external temperature regulator attempt to compensate for our experimental manipulations.

Intrascapular brown adipose tissue [BAT] pads were exposed and a miniature thermistor probe [YSI-555; Yellow Springs Instruments, Yellow Springs, Ohio] was inserted into the pad using an 18 gauge trocar as a guide. The skin overlying the probe was then sutured closed. A YSI rectal thermistor probe was used to monitor core body temperature. Output from the thermistor probes was digitized, displayed on a monitor and stored using a RUN Technologies 2K2 PC-based data recorder [RUN Technologies, Mission Viejo, CA].

Experimental Design

All animals were allowed to stabilize for at least 30 minutes after surgery before temperature recording was started. BAT and body temperatures were recorded continuously. Food deprived rats were randomly assigned to one of four treatment groups:

- A. leptin [5 μ g in 5 μ l] followed by TRH [0.1 μ g in 2 μ l]; N = 5
- B. TRH followed by leptin; N = 5
- C. pretreatment with wortmannin [0.2 μ g in 2 μ l], then leptin followed by TRH; N = 4
- D. pretreatment with PP2 [0.2 μ g in 2 μ l], then leptin followed by TRH; N = 4

Ad libitum feed rats were only exposed to TRH [0.1 μ g in 2 μ l]; N = 4

All agonists were applied directly onto the exposed floor of the fourth ventricle. The doses of leptin and TRH are the same as those used in our previous studies (Hermann et al., 2006) which revealed the leptin-TRH synergy in BAT thermogenesis; 5 μ g leptin is considered to be in the “ineffective” to mid-range [1–10 μ g] of those widely used to inhibit feeding behavior and/or increase thermogenesis [e.g., (Cusin et al., 1996); also 0.1 μ g dose of TRH is frequently used in *in vivo* studies to activate dorsal vagal complex neurons that control autonomically mediated functions (Hermann and Rogers, 1995)]. Wortmannin and PP2 [0.2 μ g doses] are commonly used ICV to inhibit CNS PIP3kinase and Src kinases, respectively (Narita et al., 2006; Pittaluga et al., 2005; Rahmouni et al., 2003; Seyedabadi et al., 2001). Each agonist [or antagonist] was administered in succession at 15min intervals between each drug as indicated on the time line in Figure 2. That is, either saline, or one of the antagonists, was applied at “T -30min”; the first agonist was applied at “T -15min”; and the second agonist was applied at “T = 0”. For the ad libitum fed group, animals were allowed to stabilize for at least 30 minutes after surgery before temperature recording was started; TRH was applied at “T = 0”.

Statistical Analysis

BAT and core body temperatures immediately prior to any agonist or antagonist challenge [i.e., temperatures at “T -45min” provided the reference [baseline] temperatures. Depth of placement of the BAT probe as well as thickness of the BAT pads could have influenced the absolute recorded tissue temperature each day. Therefore, each animal served as its own control and ΔT [i.e., the difference between subsequent temperatures and the reference temperature] was plotted at 15min intervals. Peak difference values were extracted for each case and these were subjected to ANOVA followed by a Dunnett’s post hoc test of significant difference relative to the leptin-TRH case as a control. P values < 0.05 were considered significant.

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Figure 1. Potential intracellular interactions between leptin and TRH that could augment neuronal activation

[1]Leptin binding to its receptor; causes PIP3 production [2] through a JAK2, IRS-1 transduction mechanism. PIP3 activates Src-tyrosine kinase [Src-TK] [3] which, in turn, positively modulates transduction elements for other agonists such as TRH and cation channels responsible for regulating neuronal excitation [4]. TRH binds to its receptor and activates PLC [5], and this action can be gated by Src-TK. [6] PLC can dramatically augment the primary effects of leptin through intracellular calcium release, PKC activation and further upregulation of membrane excitability mechanisms. Therefore, we hypothesize that if leptin causes the augmentation of the TRH thermogenic response via these shared signal transduction elements, then this augmentation should be eliminated if Src-TK activation is blocked by wortmannin [i.e., loss of PIP3 production] or PP2 [blocks phosphorylation of PLC or cation channels].

Figure 2. Leptin and TRH interactions in the hindbrain to elevate BAT temperature are order-dependent

BAT [and core body temperature] was monitored continuously. Exposure of the hindbrain to leptin [at “T -15min”], followed by TRH [at “T = 0”] causes a dramatic increase in BAT thermogenesis; however, the reverse order of application [TRH followed by leptin] does not yield the same effects. Secondly, blockade [applied at “T -30min”] of either the formation of PIP3 [normally generated as a consequence of leptin binding to its receptor] via wortmannin or SH2 binding [required for activation of PLC] via PP2 also uncouples the leptin plus TRH BAT thermogenesis synergy. These results suggest that activation of the leptin transduction mechanism gates TRH transduction and downstream BAT thermogenesis.

Figure 3. Leptin and TRH interactions in the hindbrain to elevate BAT temperature are order-dependent and dependent on the leptin signal transduction cascade

[A] Maximal changes in BAT temperature elicited by the five different treatment groups. Overall one way ANOVA: $F_{4,17} = 8.5$; $P = 0.0006$; Dunnett's post hoc comparison to fasted leptin/TRH group: * = $P < 0.001$.

[B] Maximal changes in core body temperature elicited by the five different treatment groups. Overall one way ANOVA: $F_{4,17} = 4.5$; $p = 0.01$; Dunnett's post hoc comparison to fasted leptin/TRH group: * = $P < 0.05$.

Figure 4. Speculative network diagram showing how leptin and TRH together might affect the function of brainstem thermogenesis circuits

We hypothesize that leptin and TRH interact in the hindbrain to increase the sensitivity of thermogenic circuitry to descending central commands to produce heat. Descending hypothalamic projections carrying commands to increase heat generation terminate on sympathetic “premotor” neurons in the ventrolateral medulla and raphe nuclei, which, in turn, control BAT thermogenesis and peripheral blood flow. These ventral medullary neurons are under considerable local GABAergic inhibition. Perhaps the NST acts to relieve this GABAergic inhibition, thus increasing the sensitivity of the thermogenic circuits? This could be accomplished by convergent hormonal [leptin] and neural [TRHergic] inputs onto NST neurons that are sensitive to both agonists; TRHergic input to the NST from the raphe is proportional to the demand for heat production. Here, demand for heat can be augmented by vagal afferents carrying information about core temperature and exposure to pyrogens. The intracellular mechanics of this “gating” within the NST may be as depicted in Figure 1. This model could explain why animals deficient in leptin [e.g., during starvation] cannot mount a thermogenic response to cold stress or infection unless leptin levels are restored. It also suggests that excess stored fat [encoded as excess leptin release] could cause adaptive thermogenesis in individuals that are sensitive to leptin.