Knocking down the diencephalic thyrotropin-releasing hormone precursor gene normalizes obesity-induced hypertension in the rat

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OBESITY IS A MAJOR RISK FACTOR for essential hypertension. Conversely, hypertensive patients tend to be more obese than normotensive subjects (19, 20). On the other hand, weight reduction is an effective way to lower arterial blood pressure (ABP) in obese hypertensive patients, suggesting an important association between weight and ABP homeostasis (17). A cumulative body of evidence has also suggested that obesity-induced hypertension (OIH) may be due to an increased sympathetic outflow among other factors (32). However, the mechanisms of this association are poorly understood. Some light was shed when the position cloning of the ob gene by Friedman’s group in 1994 led to the discovery of its product, leptin, which regulates energy balance through the activation of specific hypothalamic receptors (40). Leptin effects include an increase in the overall sympathetic activity (2). As reported by Ahima et al. (1), leptin also counteracts the starvation-induced suppression of thyroid hormone apparently by upregulating the expression of the thyrotropin-releasing hormone (TRH, pyro-Glu-His-Pro-amide) precursor gene (prepro-TRH). In this way, leptin can act directly or indirectly by increasing the production of the MC4R ligand α-melanocyte-stimulating hormone (α-MSH) to regulate TRH expression (14, 31, 33, 37) as well as enzymes that convert pro-TRH in TRH (31, 35).

Besides its endocrine function, TRH also serves as a neurotransmitter in the central nervous system, and its presence in brain nuclei involved in cardiovascular regulation, such as the periventricular region and the preoptic area, suggests that this tripeptide may modulate cardiovascular function (4, 38). In 1997, we reported (12) that central overexpression of the TRH precursor in normal rats induces an increase in the diencephalic TRH content along with a long-lasting elevation of systolic arterial blood pressure (SABP) in a dose-dependent manner. These effects were specifically reversed by a prepro-TRH antisense (AS) treatment, indicating that the central TRH system effectively participates in cardiovascular regulation in the rat. Accordingly, we demonstrated that spontaneously hypertensive rats (SHR) present a hyperactivity of the TRH system activity (9). Consequently, we (8) found that intracerebroventricular (icv) prepro-TRH AS injection decreases both the elevated diencephalic TRH content and the SABP in the SHR independently of the thyroid status. Furthermore, we recently showed (11) that an icv leptin injection induced a long-lasting pressor effect that was not observed in prepro-TRH AS-pretreated rats. Hence, we proposed that obesity may raise ABP through TRH system activation, and we report here that Wistar rats made obese with a high-fat diet (HFD), compared with lean controls, showed elevated SABP that can be normalized by prepro-TRH AS treatment independently of thyroid status.

Although the discovery that small fragments of double-stranded RNA are able to silence gene expression was made only a few years ago, methods for experimentally silencing genes have already been extended to a broad diversity of organisms, including mammalian cells. RNA interference (RNAi) has also been discovered to function in physiological gene silencing (6). Then we also found that prepro-TRH RNAi induced a specific, potent, and prolonged decrease of both diencephalic TRH content and SABP of obese rats in a thyroid hormone-independent manner.

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METHODS

All reagents were from Sigma (St. Louis, MO) unless indicated.

**Animals.** Adults (10 wk old, 120–180 g) male Wistar rats were housed in a room with a controlled temperature (23 ± 1°C) under a 12:12-h light-dark schedule. Some animals were fed a HFD (40% wt/wt bovine and porcine fat added to the standard chow) for 6 wk and throughout the experiments, whereas control animals received the standard chow. Food and water were given ad libitum. The Institutional Animal Care and Use Committee approved animal experimentation protocols following ethical guidelines.

*Intracerebroventricular infusions.* For ivc infusion, rats were anesthetized and instrumented as described elsewhere (11). Briefly, a 25-gauge stainless steel cannula was directed to the third ventricle through a burr hole in the skull for injections. Coordinates were 1.3 mm posterior to the Bregma on the midline and 4.5 mm below the dura. At the end of each experiment, the position of the cannula was assessed by histological examination. All substances were dissolved in phosphate-buffered saline (PBS), and a total infusion volume of 5 μl (1 μl/min) was used.

Three groups of randomized control lean and obese animals (n = 6 per group) were ivc injected with vehicle or oligodeoxynucleotides (ODN, 100 μg). ODN were made resistant to nucleases by DNA backbone phosphothioation and were synthesized (Research Genetics) as 23-mers targeted to bases 20–42 (AS: 5′-AACCAA GGT CCC GGC ATC CTG GA-3′) of rat prepro-TRH gene encompassing the translation initiation codon (GenBank accession no. M23643). As control, we used an inverted ODN (INV AS: 5′-AGG TCC TAC GCC CTT GGA ACC AA-3′). The screening of known rat genes from the genomic database of the National Center for Biologival Information using the Blast program indicated specificity of the sequence used in ODN design and confirmed their 100% homology with rat prepro-TRH gene.

Two additional groups of obese animals (n = 6 each) were ivc treated with 0.5 μg of small interfering (si)RNA against prepro-TRH and green fluorescence protein (GFP), siRNA were prepared by cutting with ribonuclease III (DICER), double-stranded mRNAs obtained by in vitro transcription of the appropriate DNA constructs, according to manufacturer’s indications (Gene Therapy System, San Diego, CA). Animals were acclimated in a quiet room for 30 min before measuring of SABP by a tail cuff method twice a week during the feeding period.

The effect on SABP of the peripheral α-adrenergic receptor block-ade was studied in HFD-fed and control rats that were anesthetized with pentobarbital sodium (33–45 mg/kg) and chronically instrumented. The SABP was recorded in conscious animals throughout the experiment with a polyethylene cannula, previously inserted into the left carotid artery and connected to a Statham transducer, coupled to an A/D card installed in a personal computer. Prazosin (10 mg/kg body wt) was given via the tail vein.

In addition, twice a week during the feeding period and daily after icv siRNA treatments for the indicated time periods, animals were acclimated in a quiet room for 30 min before measuring of SABP by tail cuff method using a tail occlusor connected to a Hg manometer for calibration and a Statham transducer whose signal was digitized with an A/D card inserted in a personal computer. At the same time, a plethysmographic device also connected to the A/D card was used for registering the tail artery pulse. Each value corresponds to at least three independent measurements taken in a 5-min period. Then animals were killed by decapitation, brains were rapidly removed for diencphalic TRH and prepro-TRH mRNA determinations, blood samples were collected for leptin and thyroid hormone measurements, and intraperitoneal and retroperitoneal fat pads were measured by direct weighting.

**Assay of plasma leptin, thyroid hormones, TSH, and prolactin and 3-O-methyl metabolites of catecholamines.** Blood samples were collected with sodium EDTA. Plasma leptin and thyroid hormone levels were measured using an enzyme immunometric assay (Assay Designs) and an enzyme immunometric chemiluminescence assay (Roche, Buenos Aires, Argentina), respectively. TSH and prolactin were measured by RIA as previously described (34).

The O-methylated metabolites of norepinephrine (Normetaneph- rine, NMN) and epinephrine (Metanephrine, MN) were measured by a previously described method using high-performance liquid chromatography with electrochemical detection (25).

**Diencephalic TRH content determination by RIA.** The diencphalic region of each animal was rapidly dissected with the aid of the stereotaxic atlas, and TRH content determination was performed by a method published elsewhere (10).

**Ribonuclease protection assay for quantifying prepro-TRH mRNA abundance.** Briefly, total RNA was prepared from rat diencephalum by the modified method of Chomczynski et al., as previously described (9). Two radiolabeled antisense RNA probes were synthesized using SP6 RNA polymerase (Promega) in the presence of [α-32P]UTP (PerkinElmer Life Analytical Sciences, Boston, MA), using the plasmids pCMV-TRH (12) and GAPDH-pGEM-T (generously donated by Dr. O. Carretéro; Henry Ford Hospital, Detroit, MI) as templates given protected fragments of 345- and 168-mer corresponding to prepro-TRH and GAPDH mRNAs, respectively. Essentially, 10 μg of total diencephalic RNA were hybridized with 1 × 105 cpm of each labeled antisense RNA probe, treated with ribonucleases A and T1, and purified by proteinase K and phenol-chloroform-isoamyl alcohol (24:24:1), following the procedure of Davis et al. (5). The protected probes were resuspended in 5 μl of loading buffer (80% formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol) and fractionated on a 6% polyacrylamide containing 8 mol/l urea gel. Bands were quantitated using an image acquisition and analysis system (UVP Labworks).

**Statistical analysis.** Results are expressed as means ± SD. Statistical significance between means for the effects of treatments on body weight and SABP were determined by two-way ANOVA with repeated measurements on one factor. Where pairwise comparisons were made after ANOVA, Tukey’s test for individual differences was used; otherwise, we used Student’s t-test.

**RESULTS**

After a 6-wk period, HFD induced a significant (n = 30, P < 0.04) weight gain of ~35% compared with a standard diet in male Wistar rats (Fig. 1A). Indeed, the increase of body weight corresponded to an increase in adipose tissue, as a significant correlation was observed between body weight and peritoneal fat (Spearman R 0.69, n = 30, P < 0.05). In this model, unsurprisingly, we also observed a highly significant correlation between peritoneal fat mass and plasma leptin levels (Spearman R 0.791, n = 30, P < 0.001). In addition, a significant (n = 30, P < 0.04) higher increase in SABP was observed after 3 wk of feeding in overweight animals, compared with lean controls, that remained steady until the last week of the experiments (Fig. 1B). The increase in blood pressure seemed to be due to elevation of sympathetic activity, since we observed an increase in the plasma concentrations of catecholamine metabolites (MN and MN) in obese animals compared with lean controls (Fig. 2; see control and vehicle conditions). In addition, prazosin (10 mg/kg body wt iv), a specific α-blocker, significantly (P < 0.001, n = 6) induced a maximal decrease of SABP of 35 ± 7 mmHg over 40 ± 5 min. In contrast, lean animals showed a much smaller and shorter prazosin-induced hypotensive effect (23 ± 5 mmHg; Fig. 3).

In accord with the hypothesis, we found an increase in TRH labeling in cells surrounding the third ventricle in obese compared with lean rats (data not shown). This finding was con-
firmed by RIA (Fig. 4A; see control and vehicle conditions). Moreover, we found a significant correlation between diencephalic TRH levels and plasma leptin ($R = 0.5, P < 0.05, n = 16$).

To further investigate whether TRH participates in the elevation of SABP in this obesity-induced hypertensive model, we studied the effect of icv AS on diencephalic TRH levels and SABP of obese animals compared with lean controls. By RIA, we found that obese animals presented higher diencephalic TRH levels compared with lean controls either in basal con-

![Fig. 1. Time course of the effects of a high-fat diet (HFD) compared with a normal diet on body weight (top) and systolic arterial blood pressure (SABP; bottom) in Wistar rats. Results are expressed as means ± SD; $n = 30$. *$P < 0.04$ vs. normal diet at the same time point (ANOVA with repeated measures).](image)

![Fig. 2. Normethanephrine (NMN) and methanephrine (MN) concentrations in control (Con), vehicle (Veh), antisense oligodeoxynucleotides (ODN) against prepro-TRH (AS), and an ODN of the inverted AS sequence (Inv)-treated lean and obese animals. Results are expressed as means ± SD; $n = 6$. #P < 0.03 vs. vehicle in the same group; &P < 0.01 vs. INV in the same group; *P < 0.01 vs. lean animals in the same condition; ANOVA and Tukey's test.](image)

![Fig. 3. Time course of the effect on mean systolic arterial blood pressure (SABP) induced by iv injection of saline (50 μl) or prazosin (10 mg/kg body wt) in lean and obese rats. Animals were anesthetized with pentobarbital sodium and chronically instrumented with a polyethylene cannula inserted into the left carotid artery. SABP was recorded in conscious animals.](image)

![Fig. 4. A: diencephalic TRH levels in untreated lean and obese Wistar rats (Con) and 48 h after vehicle (Veh), an antisense ODN against prepro-TRH (AS), and an ODN of the inverted AS sequence (Inv) are shown. B: SABP in untreated lean and obese Wistar rats in basal and 24 and 48 h after no treatment (Con) and with vehicle (Veh), an antisense ODN against preproTRH (AS) and an ODN of the inverted AS sequence (Inv). Results are expressed as means ± SD; $n = 6$. #P < 0.03 vs. vehicle in the same group; &P < 0.01 vs. INV in the same group; *P < 0.01 vs. lean animals in the same condition; ANOVA and Tukey’s test.](image)
ditions or after treatment with vehicle. In addition, the elevated diencephalic TRH content observed in control and vehicle-treated obese rats was diminished by AS treatment, remaining unaffected by INV (Fig. 4A). No effect was observed in lean animals. We also observed that AS treatment significantly reduced the elevated SABP of obese animals at 24 and 48 h, whereas vehicle and INV had no effect (Fig. 4B). Again, no effect was observed in lean animals. These effects on SABP of obese animals seemed not to be due to changes either in circulating prolactin or thyroid status, since prolactin, TSH, and thyroid hormone levels were not altered either by HFD or by AS treatment (Fig. 5), but instead it was probably due to a reduction in sympathetic outflow, as shown by a decrease in circulating NMN and MN (Fig. 2). Again, there were no effects on lean rats. There were changes neither in food intake nor in body weight during the 48 h after icv treatments in any groups (data not shown).

To verify the specificity of prepro-TRH AS effect, we investigated whether another strategy of prepro-TRH gene knockdown might normalize blood pressure in this OIH model by using RNAi. Compared with GFP siRNA, 0.5 µg of siRNA against prepro-TRH decreased SABP in obese rats for up to 24 days (Fig. 6A), the time point when the animals were killed to measure diencephalic TRH content. Then, we found that the tripeptide level was decreased by prepro-TRH siRNA compared with what was found in obese animals treated with GFP siRNA as control (Fig. 6B). As shown in Fig. 6C, this effect of prepro-TRH siRNA on diencephalic TRH content was probably due to an inhibitory action on prepro-TRH mRNA abundance. As prepro-TRH antisense, the hypotensive effect of prepro-TRH siRNA was also independent of either circulating prolactin or the thyroid status, since prepro-TRH siRNA did not modify plasma prolactin, TSH, triiodothyronine (T_{3}), and thyroxine (T_{4}) levels (Fig. 6, D–F). Interestingly, there was a higher body weight gain in obese animals treated with prepro-TRH siRNA (433 ± 27 g) compared with those treated with GFP siRNA (393 ± 29 g, n = 6, P < 0.04) during the 24 days post-icv treatment period.

**DISCUSSION**

Obesity is the commonest nutritional disorder in Western societies and is considered to be an important public health problem because of its association with hypertension, among other conditions. Adipose tissue plays an important role in energy regulation via hormonal signals acting at multiple sites to control food intake and energy expenditure (22). In addition, leptin is an adipocyte-derived hormone that is involved in the regulation of food intake and body weight, with the hypothalamus as a primary target of its action (39). The effects of leptin on food intake and body weight balance are mediated, at least in part, by neuropeptides such as neuropeptide Y, corticotropin-releasing hormone, melanin-concentrating hormone and α-MSH, and cocaine- and amphetamine-regulated transcript, among others. In this sense, the effect of leptin on the metabolic rate seems to be mediated through TRH gene activation...
In addition, although OIH may be secondary to insulin resistance and/or hyperinsulinemia, the enhanced sympathetic outflow induced by leptin may also play a main pathophysiological role in this form of hypertension (13).

On the other hand, intravenous or icv injections of TRH increased ABP (38). This effect was blocked by destruction of the sympathetic system, indicating that the pressor effect could be mediated by catecholamines involving the modulations of diverse neurotransmitter system activities (29).

Therefore, we proposed that, as leptin increases central TRH synthesis and release, obesity may raise SABP through TRH system activation. Here, we show that, in rats made obese with a HFD, there was a correlation between the increased peritoneal adipose tissue and circulating leptin levels. Unsurprisingly, the higher levels of leptin were associated with an increase in SABP. These results are in agreement with the fact that acute and chronic leptin treatments can increase ABP in anesthetized and conscious rats and in ob/ob mice (2, 11).

Despite the fact that agouti yellow obese mice (C57BL/6J Ay) have milder obesity than ob/ob mice, the agouti mice also have elevated ABP (28). The effect of leptin on ABP seems to be due to an increase in sympathetic outflow (15). In fact, we observed that obese animals have elevated concentrations of plasma O-methyl metabolites of catecholamines such as NMN and MN, and SABP was normalized by an intravenous α-blocker treatment.

As hypothesized, we observed that, in obese rats, the increase in SABP was accompanied by an elevation in diencephalic TRH levels. It can be argued that this effect was related to an increase in leptin, since Harris et al. (14) reported that leptin directly upregulates TRH gene expression, acting on its promoter through the activation of either a cAMP response element or a STAT-response element. As recently pointed out by other groups, leptin action on TRH gene expression can be mediated by increasing α-MSH or decreasing neuropeptide Y (7, 36, 37). In fact, we found a significant correlation between diencephalic TRH levels and plasma leptin in addition to an increase in TRH labeling in the cells surrounding the third ventricle of the central nervous system in the obese rat compared with their lean controls.

To explore whether the increase in SABP was related to the elevated diencephalic TRH content, we treated obese animals with icv injections of either a prepro-TRH AS ODN made resistant to nucleases by phosphothioation or prepro-TRH siRNA. We observed that icv injections of both AS and siRNA against prepro-TRH normalized SABP for 48 h and 24 days.
posttreatment, respectively. It is important to note that siRNA appeared to be 200-fold more potent than the AS ODN on a microgram basis without considering its longer effect. Furthermore, at 48 h after AS and 24 days after siRNA treatment, we confirmed that the effect on SABP was due to an action of the prepro-TRH AS and siRNA on the TRH system by showing that the diencephalic TRH content also was diminished to the level of control animals. The observed actions of the TRH AS ODN are sequence specific and seem not to be caused by a nonspecific toxicity, since treatment with the INV ODN having an identical percentage-based composition showed no effects. Similarly, prepro-TRH siRNA seemed to exert specific effects, since siRNA against a non-animal protein, GFP, lacked any effect, indicating that a possible nonspecific toxic action of prepro-TRH siRNA, for instance, through the activation of the intracellular interferon-γ response (3), is very unlikely.

One possible site of the icv AS and siRNA actions is the hypothalamic-pituitary axis, where alterations in TRH synthesis might affect thyroid status indirectly influencing cardiovascular function. But this explanation seems unlikely, since we found no change in TSH and thyroid hormone levels after prepro-TRH gene knockdown. At first glance, these results may seem contradictory. We think that a possible explanation is that we measured diencephalic TRH, which involves several hypothalamic and septum prepro-TRH-containing neurons; therefore, we cannot be certain of specific TRH changes in TSH-regulating neurons. In fact, Perello et al. (33) have recently reported that, even in the hypothalamic-paraventricular nuclei, pre-TRH-expressing neurons with differential function, in addition to TSH regulation, may coexist. In addition, our knockdown experiments were relatively short-lasting, and we cannot rule out the possibility of acutely compensatory changes, particularly considering that T3 and T4 were measured at one time point only. Furthermore, we (8, 12) have consistently found that diencephalic TRH manipulation does not necessarily change thyroid status. This has been confirmed by other groups in different settings (23).

As TRH is a potent prolactin releaser (18) and prolactin has been shown to regulate ABP in rabbits and rats (30), it can be hypothesized that TRH gene knockdown may decrease ABP by affecting prolactin levels. Although we cannot reject that possibility completely, it is improbable, since prolactin does not alter ABP directly and we did not observe any change of circulating prolactin in any condition.

A note of caution should be added, even though in previous studies (8, 12) we reported that icv injections of a plasmid and AS oligos reached hypothalamic areas around the third ventricle and did not spread to posterior areas of the rat central nervous system, that we cannot be completely sure that knockdown of extradiencephalic TRH has not played a role in the hypotensive effects of icv AS and siRNA against prepro-TRH injections.

Although additional studies are necessary to delineate the complex interactions that may take place on the effect of humoral factors in obesity on cardiovascular regulation, our data suggest that, among others, some TRH-dependent cardiovascular effects are through sympathetic system activation, since TRH injections produce an increase in plasma catecholamine levels, and adrenolectomy avoids its hypotensive effects (29). Accordingly, in our hands, TRH AS treatment was effective and selective in decreasing the elevated concentrations of NMN and MN of obese animals, which added additional evidence to the existence of a TRH-dependent elevation of ABP mediated by sympathetic overflow. Interestingly, prepro-TRH siRNA treatment induced a significant increase in body weight, indicating that diencephalic TRH serves as a negative modulator of body weight gain, probably by acting on energy balance (21, 23, 24).

To conclude, we show here for the first time that obese animals develop a hypertensive state that depends, at least in part, on hyperactivity of the diencephalic TRH system. Then, knocking down the prepro-TRH gene by two different strategies normalizes ABP in this model of OIH. As leptin produces central TRH synthesis and release (11, 14), we propose that the obesity-related leptin elevation may induce hypertension through the TRH system activation which, in turn, increases sympathecic nerve activity. Recently, the concept has been raised that in some obese, leptin-resistant models there is a preservation of sympathoexcitatory actions of leptin despite resistance to the anorexigenic and metabolic action of leptin (27). If this concept proves to be true, TRH may be the mediator of this preserved pathway activated by leptin. At any rate, although more experiments are necessary to delineate this complex TRH-leptin interaction, it may contribute, at least in part, to the strong association between hypertension and obesity.

Although at first glance the role of TRH in human obesity-associated hypertension may seem speculative, some evidence reinforces the concept. Elevated leptin levels and sympathetic activation are common features of the disease, and both have been associated (13). In addition, leptin and TSH are tightly synchronized (26).

Finally, our study opens the intriguing possibility that elevation of ABP is a putative side effect of any treatment of obesity with fenfluramine-like drugs that may act by increasing the activity of the POMC-α-MSH system in the arcuate nucleus of the hypothalamus (16). Then the therapeutic management of obesity appears more challenging than ever.

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