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SiRNA-mediated silencing of the diencephalic thyrotropin-releasing hormone precursor gene decreases the arterial blood pressure in the obese agouti mice

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1. ABSTRACT

Obesity is associated with increased cardiovascular morbidity and mortality, in part through development of hypertension. Leptin promotes weight loss by reducing food intake and increasing energy expenditure through sympathetic stimulation. It also counteracts the starvation-induced suppression of thyroid hormone by up-regulating the expression of TRH. On the other hand, it is known that the extrahypothalamic TRH system participates in cardiovascular function modulating sympathetic system activity. In order to challenge the testable hypothesis that obesity may raise arterial blood pressure (ABP) through TRH system activation, we herein analyze the participation of the TRH system in the elevation of ABP in the obese agouti yellow mice. These mice are characterized by resistance to the weight reducing effect of leptin although they show a preserved sympathetic response to leptin along with a mild hypertension compared with the control strain (121+/-2 vs 102+/-2 mmHg, $p < 0.001$, n=10).We report here that hyperleptinemic agouti mice showed a 1.8-fold elevation of diencephalic TRH content compared with controls, and we demonstrate that a long lasting specific inhibition of TRH system by icv treatment with siRNA against *preproTRH* normalizes systolic ABP independently of the thyroid status. These results suggest that the interaction leptin–diencephalic TRH may be one of the mechanisms involved in the mild hypertension of the obese agouti mice.

2. INTRODUCCION

Several studies have demonstrated a strong association between obesity and hypertension (1). In addition, cumulative evidence points to visceral obesity as the most important risk factor for hypertension and cardiovascular disease. Then, obesity is associated with increased cardiovascular morbidity and mortality, in part through development of hypertension. Recent observations suggest that the cardiovascular actions of leptin may help to explain the link between excess fat mass and cardiovascular diseases (2). Leptin is an adipocyte-derived hormone that promotes weight loss by reducing food intake and increasing energy expenditure through sympathetic stimulation (3). These effects result from direct hypothalamic actions and are mediated by neuropeptide systems such as α-melanocyte stimulating hormone (alpha-MSH) and CART protein (4). As reported by Ahima *et al.*, leptin also counteracts the starvation-induced suppression of thyroid hormone by up-regulating the expression of the thyrotropin-releasing hormone (TRH, pyro-Glu-His-Pro-amide) precursor gene (*preproTRH*) (5). In this way, leptin can act directly or indirectly by increasing the production of the MC4R ligand, alpha-MSH to regulate TRH expression(6-9) as well as enzymes that convert proTRH in TRH(7, 10).

Besides its endocrine function, TRH also serves as a neurotransmitter in the central nervous system, and its

presence in brain nuclei such as in the periventricular region and the preoptic area suggests that this tripeptide may modulate cardiovascular function(11). In addition, Alexander *et al* have described the independence in the regulation of the TRH pools of the paraventricular nucleus (PVN) from the hypophysiotrofic one (12). We have reported that over-expression of TRH precursor in areas around the third ventricle of the central nervous system in normal rats induces long-lasting elevation of arterial blood pressure (ABP) as well as an increase in diencephalic TRH content in a dose-dependent manner (13). These effects were specifically reversed by a *preproTRH* antisense (AS) treatment, indicating that the hypothalamic TRH system effectively participates in cardiovascular regulation in the rat (13). Additionally, we have recently shown that an icv leptin injection induced a long-lasting pressor effect that was not observed in AS-pretreated rats (14).

To challenge the testable hypothesis that TRH participates in obesity-related hypertension, we used the obese agouti yellow mice. The agouti yellow mice is a rodent model of obesity associated with high levels of circulating leptin and elevated ABP (15). These mice are resistant to the weight reducing effect of leptin, but leptin contributes importantly to regulation of ABP in these mice (3).

As we proposed that obesity may raise ABP through TRH system activation, in the present study we analyze the participation of the TRH system in the elevation of the ABP in the obese agouti mice. We report here that the agouti mice showed an elevated diencephalic TRH content compared with control mice, and we demonstrate that the specific inhibition of TRH system by treatment with siRNA against *preproTRH* normalizes the systolic (SABP) independently of the thyroid status.

3. MATERIALS AND METHODS

3.1. Animals

We studied 17- to 20-week old male agouti yellow mice (C57BL/6J-A^y) and the wild type controls C57BL/6J a/a (Jackson Laboratories). The animals were housed in individual cages in a room with controlled temperature (23+1°C) under a 12-hour light/dark schedule with free access to food and water. The institutional Animals Care and Use Committee approved animal experimentation protocols following the Ethical Guidelines.

Before icv infusion mice were anesthetized with 90 mg/kg ketamine and 9 mg/kg xylazine intraperitoneally and placed in stereotaxic apparatus. A 25-gauge stainless steel cannula was directed to the third ventricle through a burr hole in the skull for injection. Coordinates were 1.0 mm posterior to the bregma on the midline and 4.0 mm below the dura. All substances were dissolved in phosphate-buffered saline, and a total infusion volume of 5 µl (1 µl/min) was used.

Two groups of male agouti and controls mice were treated intracerebroventricularly (icv) with 0.2 µl of siRNA against either *preproTRH* or green florescence protein (*GFP*). SiRNA were prepared by cutting double stranded mRNA obtained by *in vitro* transcription of the appropriate DNA construction using ribonuclease III (DICER) according to manufacturer's indications (Gene Therapy System, San Diego, CA, USA).

We measured systolic ABP (SABP) by a tail-cuff method twice a week during basal period, and daily after icv siRNA treatment for the indicated time periods. Each value corresponds to at least 3 independent measurements taken in a 5 min. period. Then, the animals were sacrificed by decapitation, the brains were rapidly removed and the diencephalum was dissected for TRH content, while blood samples were collected in sodium-EDTA contained tubes for leptin and thyroid hormones measurements.

3.2. Assay of plasma leptin and thyroid hormones

Plasma was immediately separated and frozen. Leptin and thyroid hormone levels were measured using an Enzyme Immunometric Assay (EIA) (Assay Desings, Inc, USA) and an Enzyme Immunometric ChemioLuminiscence Assay (EICLIA) (Roche, Buenos Aires, Argentina), respectively.

3.3. Diencephalic TRH content determination by RIA

The diencephalic region of each animal was rapidly dissected with the aid of stereotaxic atlas (16). TRH content determination was performed by a method published elsewhere (17).

3.4. Statistical analysis

Results are expressed as mean +/- SD. Statistical significance between means for effects of treatments on body weigh and SABP were determined by two-way ANOVA with repeated measures on one factor. Where pairwise comparisons were made after ANOVA, Tukey´s test for individual differences was used; otherwise, we used Student´s test.

4. RESULTS

As expected, plasma leptin levels were correlated with mouse body weight (r: 0.98, <0.01, n=20) and agouti mice showed higher leptin levels than controls (Table 1). Unsurprisingly, the agouti mice had higher SABP than lean controls under basal conditions (agouti: 121+/-2 mmHg vs controls: 102+/-2 mmHg, $p< 0.001$, n=10 per group) (Figure 1, upper panel). In accordance with the hypothesis, we found an increase ($p<0.01$) in the diencephalic TRH content in the agouti mice (768+/- 65 pg/mg protein, n= 10) as compared to controls (429 +/- 59 pg/mg protein, n=10) (Figure 1, lower panel). Accordingly, there was a correlation of diencephalic TRH with SABP (R: 0.56, $p < 0.01$, n=20).

To investigate further whether TRH participates in the elevation of SABP in this model of obesity, we studied the effect of the specific inhibition of diencephalic TRH production, by using siRNA against *preproTRH,* on SABP of agouti mice compared with the control strain. While *GFP* siRNA had no effects, 0.2 µg of siRNA against *preproTRH* decreased SABP in agouti obese mice for up to 19 days (Figure 2, upper panel), the time point when the

Table 1. Effect of *icv injection of GFP* and *preproTRH* siRNA on body weight, plasma leptin levels , serum T3 and T4 levels in obese agouti vs C57 black control mice

	Black C57BL/6J		**Agouti (C57BL/6J-Ay)**	
Treatment	RNAi-GFP	RNAi-TRH	RNAi-GFP	RNAi-TRH
Body Weight (gr)	32 +/- 1,9	34,5 +/- 1,9	50,1 +/- 1,9[1]	52,5 +/- 1,4 [1]
Plasma Leptin (pg/ml)	126,1 +/- 56,8	164 +/- 77	1844 +/- 231[1]	2094 +/- 232[1]
T3 (ng/ml)	4,46 +/- 0,29	4,24 +/- 0,26	4,14 +/- 0,29	3,56 +/- 0,26
T4 (µg/ml)	1,13 +/- 0,08	1,04 +/- 0,07	1,14 +/- 0,07	0,99 +/- 0,07

Results are expressed as media +/- SD (n=5 per group). [1] stands for p<0,001 vs control black mice in the same condition.

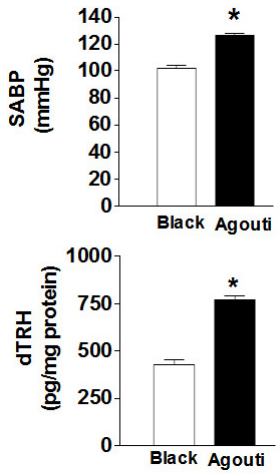

Figure 1. Systolic Arterial Blood Pressure and diencephalic TRH content in obese agouti yellow vs C57 Black control mice. Systolic Arterial Blood Pressure (SABP) (upper panel) and diencephalic TRH content (lower panel) in obese agouti yellow vs C57 Black control mice. Results are expressed as mean+/-SD (n=10), * stands for p< 0.01 with respect to lean control mice.

animals were sacrified to measure diencephalic TRH content. Then, we found that the tripeptide level was decreased by *preproTRH* siRNA with respect to that found in agouti obese animals treated with *GFP* siRNA as control (Figure 2, lower panel). The hypotensive effect of this treatment was independent of the thyroid status since *preproTRH* siRNA did not modify plasma T3 or T4 levels (Table 1).

5. DISCUSSION

Adipose tissue plays an important role in energy regulation via hormonal signals acting at multiple sites to control food intake and energy expenditure (18). Additionally, 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 (5). The effects of leptin on food intake and body weight balance are mediated directly or by diverse neuropeptides increasing sympathetic activity (19). In that sense, the rodent model of obesity -agouti yellow mice- shows a selective leptin resistance with preservation of the sympathetic actions despite loss of appetite and weight-reducing actions of systemic leptin (15). Rahmouni *et al* have described that icv leptin produces a significantly increase of the renal sympathetic nerve activity suggesting that selective leptin resistance in this model is not due to a defect in leptin transport across the blood brain barrier and elevated leptin levels may participate in the mild hypertension observed in the obese agouti mice (20). In that sense, hypertension in agouti mice has been attributed to hyperleptinemia, reinforcing the concept that these mice have selective leptin resistance (21).

On the other hand, it is known that TRH increased ABP and that this effect could be mediated by an increase in the sympathetic activity (22). Interestingly, it has been demonstrated that leptin increases central TRH synthesis and release(6-8). Based on the elevated leptin levels and the increased sympathetic activity observed in this model of obesity, we propose that leptin may raise SABP through TRH system activation in the obese agouti mice.

We showed that in agouti mice there was a correlation of the increased peritoneal adipose tissue and circulating leptin levels. Also, as expected, we found that the agouti mouse had higher ABP than lean black controls. These results are consistent with the ones found by other groups, which describe that these mice have milder obesity than the *ob/ob* mice but that, conversely, they have hyperleptinemia with elevated ABP (15).

In agreement with our proposal, we found that the elevated blood pressure was accompanied with an increased diencephalic TRH content suggesting that the elevated SABP could be mediated by the diencephalic TRH increase. In fact, TRH content was significantly correlated with the SABP values. This leads to the testable hypothesis that a long-lasting inhibition of the diencephalic TRH should normalize blood pressure in the obese agouti mice. SiRNA has emerged as not only an endogenous mechanism of gene expression regulation in several species but also as a potent tool of long lasting gene knocking down (23). Additionally, it has been successfully used in knocking down mice genes (24). On the basis of this evidence, we used icv siRNA against *preproTRH* in the hyperleptinemic obese agouti mice and, as expected, found that the specific inhibition of the diencephalic TRH production induces a decrease in the TRH content along with a decrease in blood pressure.

Although more experiments are necessary to define the precise siRNA site of action, we may speculate that icv siRNA targeted the hypothalamic paraventricular area since we have already reported that the icv antisense oligonucleotide action site was specific around the 3rd ventricle affecting PVN parvocellular neurons (13)

PreproTRH siRNA effects seemed to be specific

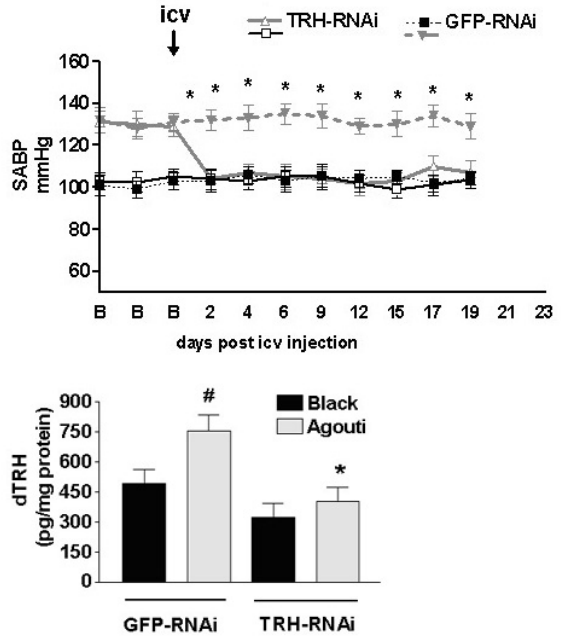

Figure 2. Effect of icv injection of *GFP* and *preproTRH* siRNA on systolic arterial blood pressure and diencephalic TRH content in obese agouti vs C57 black control mice. Upper panel shows a time course of systolic arterial blood pressure (SABP) during 19 days. At this time point, mice were sacrificed and diencephalic TRH was measured by RIA (lower panel). Results are expressed as media +/- SD (n=5 per group). * stands for p< 0,01 vs GFP siRNA-treated animals and # stands for p< 0,03 vs control black mice in the same condition.

since siRNA against GFP, a non-animal protein, lacked any effect indicating that a possible unspecific toxic action of *preproTRH* siRNA is very unlikely.

The siRNA effect on blood pressure was observed during all the experiment (19 days) indicating that the decrease of the diencephalic TRH content was sufficient to normalize blood pressure in the obese mice.

PreproTRH siRNA had no effect in control mice indicating that TRH does not play a tonic role in controlling ABP under basal conditions and suggesting an active role only in the development and/or maintenance of hypertension. These results are in agreement with our previous observations in rats (13).

PreproTRH siRNA may act on the hypothalamus-pituitary axis, where it is known that alterations in the TRH synthesis affects thyroid status and may indirectly influence cardiovascular function. However, as previously shown in rats (25), the decrease in blood pressure mediated by diencephalic TRH production inhibition seems not to be due to an alteration of the thyroid status. It is important to note that although thyroid hormones are not expected to change in a short period of time, the unchanged values found approximately 3 weeks after treatment point out that the diencephalic TRH pool involved in cardiovascular regulation could be different from the one of the hypophisiotrophic system in accordance with results published by Alexander *et al.* (12) and Perello *et al* .(8). In this regard, according to the model proposed by Perello *et al* (8),it is tempting to especulate that in the PVN of the Agouti mice there is a resistance to the action of alpha-MSH on proTRH neurons involved in the hypophysiotropic-thyroid axis regulation but the direct action of leptin through the STAT3 pathway is preserved on proTRH neurons involved in the sympathetic activation. Although we have no direct evidence of increased sympathetic activity, we propose that the main mechanisms by which TRH elevates ABP in the agouti yellow mice operate through an increase of sympathetic outflow. As recently shown by Knight *et al.*, this seems to be the case in the effects on metabolic rate and cardiovascular function of icv infusion of TRH in caloric restricted rats (26).

Our findings, together with results from other groups, suggest that in the agouti yellow mice the leptin sympathoexcitatory action is preserved although its anorexigenic and metabolic actions are absent (20, 21). Based on that, in this model, leptin-induced diencephalic TRH increase could be one of the hypertensive mechanisms involving a TRH-mediated sympathetic activity increase.

More experiments would be necessary to define the role of the TRH in this model, although our results suggests that the interaction leptin – diencephalic TRH may be one of the mechanisms involved in the mild hypertension of the hyperleptinemic obese agouti mice.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. V. Antic, A. Dulloo and J. P. Montani: Multiple mechanisms involved in obesity-induced hypertension. *Heart Lung Circ* 12, 84-93 (2003)
2. W. G. Haynes: Role of leptin in obesity-related hypertension. *Exp Physiol* 90, 683-688 (2005)
3. M. Aizawa-Abe, Y. Ogawa, H. Masuzaki, K. Ebihara, N. Satoh, H. Iwai, N. Matsuoka, T. Hayashi, K. Hosoda, G. Inoue, Y. Yoshimasa and K. Nakao: Pathophysiological role of leptin in obesity-related hypertension. *J Clin Invest* 105, 1243-1252 (2000)
4. M. S. Kim, C. J. Small, S. A. Stanley, D. G. Morgan, L. J. Seal, W. M. Kong, C. M. Edwards, S. Abusnana, D. Sunter, M. A. Ghatei and S. R. Bloom: The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. *J Clin Invest* 105, 1005-1011 (2000)

5. R. S. Ahima, D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell, E. Maratos-Flier and J. S. Flier: Role of leptin in the neuroendocrine response to fasting. *Nature* 382, 250-252 (1996)
6. M. Harris, C. Aschkenasi, C. F. Elias, A. Chandrankunnel, E. A. Nillni, C. Bjoorbaek, J. K. Elmquist, J. S. Flier and A. N. Hollenberg: Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. *J Clin Invest* 107, 111-120 (2001)
7. E. A. Nillni, C. Vaslet, M. Harris, A. Hollenberg, C. Bjorbak and J. S. Flier: Leptin regulates prothyrotropin-releasing hormone biosynthesis. Evidence for direct and indirect pathways. *J Biol Chem* 275, 36124-36133 (2000)
8. M. Perello, R. C. Stuart and E. A. Nillni: The role of intracerebroventricular administration of leptin in the stimulation of prothyrotropin releasing hormone neurons in the hypothalamic paraventricular nucleus. *Endocrinology* 147, 3296-3306 (2006)
9. S. Sarkar, G. Legradi and R. M. Lechan: Intracerebroventricular administration of alpha-melanocyte stimulating hormone increases phosphorylation of CREB in TRH- and CRH-producing neurons of the hypothalamic paraventricular nucleus. *Brain Res* 945, 50-59 (2002)
10. V. C. Sanchez, J. Goldstein, R. C. Stuart, V. Hovanesian, L. Huo, H. Munzberg, T. C. Friedman, C. Bjorbaek and E. A. Nillni: Regulation of hypothalamic prohormone convertases 1 and 2 and effects on processing of prothyrotropin-releasing hormone. *J Clin Invest* 114, 357-369 (2004)
11. M. J. Brownstein, M. Palkovits, J. M. Saavedra, R. M. Bassiri and R. D. Utiger: Thyrotropin-releasing hormone in specific nuclei of rat brain. *Science* 185, 267-269 (1974)
12. K. Alexander, M. Nikodemova, J. Kucerova and V. Strbak: Colchicine treatment differently affects releasable thyrotropin-releasing hormone (TRH) pools in the hypothalamic paraventricular nucleus (PVN) and the median eminence (ME). *Cell Mol Neurobiol* 25, 681-695 (2005)
13. S. I. Garcia, P. I. Porto, A. L. Alvarez, V. N. Martinez, D. Shaurli, S. Finkielman and C. J. Pirola: Central overexpression of the TRH precursor gene induces hypertension in rats: antisense reversal. *Hypertension* 30, 759-766 (1997)
14. S. I. Garcia, M. S. Landa, P. I. Porto, A. L. Alvarez, M. Schuman, S. Finkielman and C. J. Pirola: Thyrotropin-releasing hormone decreases leptin and mediates the leptin-induced pressor effect. *Hypertension* 39, 491-495 (2002)
15. A. L. Mark, R. A. Shaffer, M. L. Correia, D. A. Morgan, C. D. Sigmund and W. G. Haynes: Contrasting blood pressure effects of obesity in leptin-deficient ob/ob mice and agouti yellow obese mice. *J Hypertens* 17, 1949-1953 (1999)
16. G. Paxinos and K. B. G. Franklin, The Mouse Brain in Stereotaxic Coordinates: Compact Second Edition. San Diego, CA.: Elsevier Academic Press, (2004)
17. S. I. Garcia, S. M. Dabsys, D. Santajuliana, A. Delorenzi, S. Finkielman, V. E. Nahmod and C. J. Pirola: Interaction between thyrotrophin-releasing hormone and the muscarinic cholinergic system in rat brain. *J Endocrinol* 134, 215-219 (1992)
18. L. A. Tartaglia: The leptin receptor. *J Biol Chem* 272, 6093-6096 (1997)
19. J. E. Hall, D. A. Hildebrandt and J. Kuo: Obesity hypertension: role of leptin and sympathetic nervous system. *Am J Hypertens* 14, 103S-115S (2001)
20. K. Rahmouni, W. G. Haynes, D. A. Morgan and A. L. Mark: Selective resistance to central neural administration of leptin in agouti obese mice. *Hypertension* 39, 486-490 (2002)
21. M. L. Correia, W. G. Haynes, K. Rahmouni, D. A. Morgan, W. I. Sivitz and A. L. Mark: The concept of selective leptin resistance: evidence from agouti yellow obese mice. *Diabetes* 51, 439-442 (2002)
22. A. L. Sirenand I. Paakkari: Cardiovascular effects of TRH i.c.v. in conscious rats. *Clin Exp Hypertens A* 6, 2073-2077 (1984)
23. N. R. Dudleyand B. Goldstein: RNA interference: silencing in the cytoplasm and nucleus. *Curr Opin Mol Ther* 5, 113-117 (2003)
24. K. Miyawaki-Shimizu, D. Predescu, J. Shimizu, M. Broman, S. Predescu and A. B. Malik: siRNA-induced caveolin-1 knockdown in mice increases lung vascular permeability via the junctional pathway. *Am J Physiol Lung Cell Mol Physiol* 290, L405-L413 (2006)
25. S. I. Garcia, A. L. Alvarez, P. I. Porto, V. M. Garfunkel, S. Finkielman and C. J. Pirola: Antisense inhibition of thyrotropin-releasing hormone reduces arterial blood pressure in spontaneously hypertensive rats. *Hypertension* 37, 365-370 (2001)
26. W. D. Knight, S. J. Swoap, A. D. Parsons and J. M. Overton: Central Thyrotropin-Releasing Hormone Infusion Opposes Cardiovascular and Metabolic Suppression during Caloric Restriction. *Neuroendocrinology* 83, 69-76 (2006)

Abbreviations: ABP: arterial blood pressure, ICV: intracerebroventricular, SABP: systolic arterial blood pressure, TRH: thyrotropin-releasing hormone

Key Words: TRH, Thyroliberin, Antisense, siRNA, Blood Pressure, Hypertension, Agouti Mice, Obesity, Leptin

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