Thyrotropin-Releasing Hormone as a Mediator of the Central Autonomic Pathway Controlling Ovarian Function

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Key Words

Ovary · Sympathetic nerves · Catecholamines · Thyrotropin-releasing hormone · Celiac ganglia · Tyrosine hydroxylase · Thyroid hormones

Abstract

We studied the effect of thyrotropin-releasing hormone (TRH) applied centrally on the sympathetic activity of the ovary in female rats. Intracerebroventricular (i.c.v.) administration of a dose of 25 ng/kg weight produced an increase in noradrenaline (NA) content at the ovary after 5 days of hormone administration. However, higher doses in a range up to 500 ng/kg weight decreased NA content at the ovary. At the celiac ganglia (where the cell bodies of sympathetic neurons projecting to the ovary originate) there was an accumulation of NA in spite of a decrease in tyrosine hydroxylase activity (T-OH). After cold exposure, opposite effects on T-OH activity and no effects on NA in ganglia and in ovary were obtained. Besides, i.v. injection of TRH only induced a decrease in ovarian NA. In contrast to the increase in T_3 plasma levels obtained after the cold-stress procedure, none of the i.c.v. doses of TRH used produced changes in T_3 plasma levels, strongly suggesting that the effect on sympathetic activity is mediated by a central effect of TRH acting as a putative activator of ovarian sympathetic nerves.

Supported by Fondecyt 102-0581, ECOS and DID Sal 002/2.

Introduction

Increasing evidence leads to the assumption that, in addition to its classical hormonal influences, the brain exhibits a neural control over ovarian activity [1]. Since the first report of Kawakami et al. [2], data has accumulated in the rat, suggesting a direct neural connection between the ovary and the brain [3, 4]. Recently, using a viral transneuronal tracing technique, Gerendai et al. [5] showed that local administration of a viral tracer into the ovary produced intense cell-body labeling in the hypothalamic paraventricular nucleus (PVN). This nucleus (mainly its dorsal cap) represents a conspicuous forebrain region from which cells project to preganglionic sympathetic neurons either directly [6] or through the dorsome-

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dial nucleus (DMN) [7]. Whatever the intermediate steps, sympathetic neuronal inputs impinge on the celiac postganglionic cells whose efferents innervate the ovary [8].

Thyrotropin-releasing hormone (TRH), a tripeptide mainly synthesized in the PVN [9] has been associated with the sympathetic pathway linking the hypothalamus to the autonomic nervous system [7, 10] and is presumably involved in the control of body temperature [11, 12]. As a hypothalamic neurohormone, it also participates in the thermogenic response via an activation of the thyrotropic axis [13, 14]. TRH plays a key role as a ubiquitous molecule that integrates both neuronal and hormonal elements of the thermogenic response [15], both of which are activated by cold-stress exposure [16, 17]. In recent years, we [18] and others [19] have documented a sympathetic mediation in stress-induced ovarian hyperactivity. Since cold stress activation of autonomous pathways involves TRH [20, 21], the purpose of this work was to investigate whether the neuropeptide may be involved in ovarian sympathetic control. Three parameters were studied: tyrosine hydroxylase activity, noradrenaline content in celiac ganglia (origin of the neurons controlling steroidogenic cells of the ovary) and noradrenaline content of the ovary. To discriminate between a central and peripheral, thyrotropin-mediated stimulation of sympathetic nerves, the animals were subjected to either intracerebroventricular or intravenous injection of TRH. Finally, a mixed paradigm of central and peripheral effects, chronic exposure to cold was also used. It appears from our results that TRH could act as a mediator in pathways connecting the brain to the ovary.

Materials and Methods

Animals

Cycling virgin rats (220–250 g) were obtained from a stock of Sprague-Dawley animals maintained at the University of Chile. The animals were kept on a 12-hour light, 12-hour dark photoperiod (lights on from 07.00 to 19.00 h) and allowed free access to pelleted rat chow and tap water. Thirty-six rats were divided into four groups: saline controls (n = 4); central administration of TRH (n = 16); peripheral administration of TRH (n = 8; 4 saline-treated and 4 TRH-treated); cold-stressed rats (n = 8; 4 controls and 4 treated). There were no changes in estrous cyclicity of any experimental group. One hour after the final stress session, control and experimental rats were sacrificed by decapitation, as this procedure has the least effect on corticosterone (CORT) plasma levels [18]. Rats were transported, one-by-one, from an adjoining room to the sacrifice location.

Animals showing regular 4-day estrous cycles were used for the experiments. All animal procedures were performed using protocols previously approved by the Institutional Ethics Committee, Faculty of Chemical and Pharmaceutical Sciences, University of Chile.

Central TRH Treatment

Rats were anesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus. A cannula was inserted into the third ventricle (3V) (coordinates: –0.28 mm anteroposterior, 0 mm mediolateral, –0.81 mm ventrodorsal) [22] and fixed on the skull with screws and dental cement. After surgery, the rats were caged individually. Rats with signs of infection, continued weight loss or incorrect placement of the cannula were excluded from the experiment. One week later, TRH was injected once daily into 3V (1 µl/min, 5 µl total) for 3 or 5 days with 25, 200, 300 or 500 ng TRH/kg (n = 4, for each treatment). Controls were similarly injected with saline vehicle (n = 4). The highest dose used in our work is many times lower than doses used by other authors under i.c.v. administration protocols [23 ,24].

Peripheral TRH Treatment

Rats were anesthetized with chloral hydrate (400 mg/kg) and a 10-cm length of PE-10 tubing was inserted into the jugular vein; the tubing was exteriorized over the back of the head through a small incision, sutured to the skin, and sealed with a 30 G stainless steel wire. The animals were treated 2 days after surgery; TRH (25 ng/kg) was administered for 3 days.

Cold Exposure

For each experimental period, the rats were divided into two matched weight groups. One group was maintained in a 4 °C cold room for 64 h. This condition has been previously shown to increase tyrosine hydroxylase activity in the adrenal and, in some cases, in the superior cervical ganglia [25]. This procedure has also been used to discriminate the changes in feeding behavior during prolonged cold exposure [26]. A second group was kept at 22 °C, and served as controls.

At the end of each experiment, rats were killed by decapitation, their celiac ganglia, adrenal glands and ovaries were rapidly removed, frozen and maintained at –80 °C until analysis of catecholamines, enzymatic activity and total RNA preparation; whole trunk blood was collected, and the plasma was recovered by centrifugation and stored at –20 °C for subsequent triiodothyronine (T_3) assays.

RNA Preparation and Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Celiac ganglia total RNA was extracted using Trizol Reagent® (Gibco BRL, Gaithersburg, Md., USA). T-OH mRNA was determined as previously described [27] with minor modifications. After we tested the best concentration of total RNA to be in the linear range of the reaction, we choose 200 ng to perform reverse transcription. Previously, RNA was heated to 75 °C during 5 min and chilled on ice. Transcription was performed at 37 °C during 1 h, using 0.5 m*M* NTPs, 10 m*M* DTT, 176 n*M* random hexamers (Invitrogen, Carlsbad, Calif., USA), 25 U RNAsin (Promega, Madison Wisc., USA), 300 U reverse transcriptase (Invitrogen) and first strand buffer, in a final volume of 30 µl. We used a multiplex PCR assay for T-OH and hypoxantine-phosphoribosyl-transferase (HPRT, a constitutive gene of neurogical tissue as described by Pernas-Alonso et al. [27]). To verify that the mRNA samples were not contaminated with genomic DNA, HPRT primers were designed from a codogenic sequence of the HPRT gene which spans an intronic sequence, and generates either a 370 bp fragment for cDNA or a longer fragment to show possible products amplified from contaminating genomic DNA as described [27]. 10 µl of the RT reaction were incubated with 2 U of

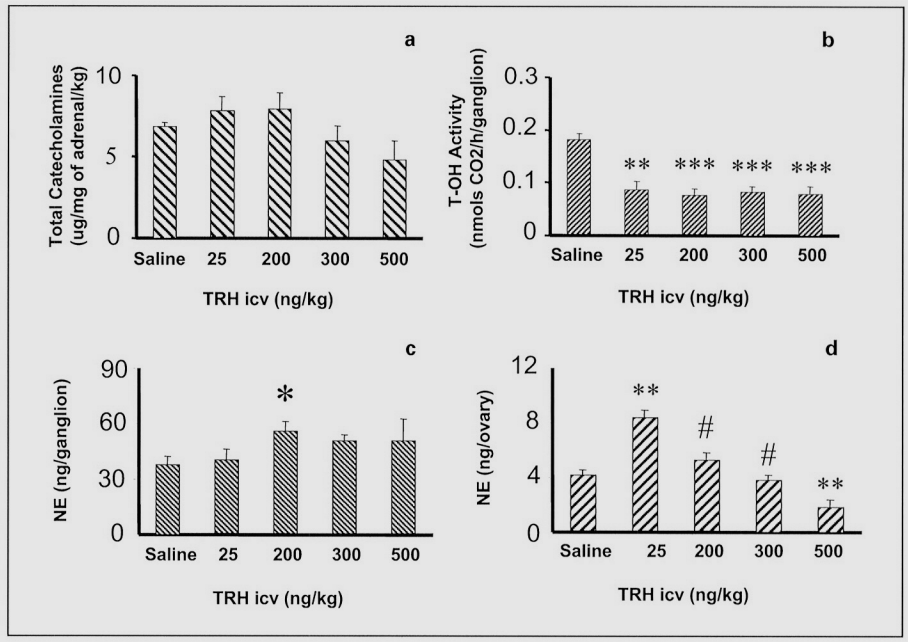

Fig. 1. The effect of TRH administration to the third ventricle on sympathetic nerve activity. A cannula was chronically implanted in the third ventricle and rats were injected once a day for 5 days with saline or different concentrations of TRH (1 µl/min, 5 µl total) as indicated in the figure. **a** Total concentration of adrenal catecholamines (CA), is expressed as µg of catecholamines/mg adrenal/kg body weight. **b** Inhibition of T-OH activity in celiac ganglia. T-OH activity is expressed as nmol of CO_2 formed per h/ganglia. **c** Changes in NE content in celiac ganglia. NE is expressed as ng of NE/ganglion. **d** Changes in NE content in ovary. NE is expressed as ng NE/ovary. Each bar represents the mean ± SEM of four independent observations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ vs. control; # $p < 0.05$ at doses of 200 and 300 ng/kg vs. 25 ng/kg.

DNA Taq polymerase (Promega), 0.2 m*M* dNTPs, 15 pmol of each primer in a final volume of 30 µl. The PCR was programmed for 26 cycles, and consisted of denaturation at 95 °C for 1 min, annealing at 57 °C for 45 s and extension at 72 °C for 1 min, using a DNA thermal cycler (MJ Research Inc.). The PCR oligonucleotide primers were previously published [27] and generate either a 370- or a 274-bp fragment for HPRT and T-OH, respectively. All RT-PCR and PCR reactions included the use of water instead of template as negative controls. RT-PCR products were electrophoresed in 2.0% agarose gels, stained with ethidium bromide and photographed. Band intensities were measured with the UN-SCAN-IT program (Silk Scientific, Orem, Utah, USA), and normalized to that of the corresponding HPRT bands. Because we did not find differences between the experimental groups, we only show the corresponding gel and not the bars of the relative densitometric analysis.

Tyrosine Hydroxylase Activity

The activity of tyrosine hydroxylase (T-OH) was determined by the method of Waymire et al. [28] as previously described [29]. We measured the $^{14}CO_2$ released from 1-^{14}C-tyrosine (spec. act. 52 mCi/mmol, New England Nuclear, Boston, Mass., USA) after hydroxylation by T-OH and subsequent decarboxylation induced by the addition of an extract of dopa decarboxylase (DDC) to the assay. The experimental procedure was performed with a saturating concentration of 1.0 m*M* 6-methyl-tetrahydrobiopterine (Sigma Chem. Co., St. Louis, Mo., USA) as a cofactor for T-OH; enzymatic activity is expressed as pmol CO_2 formed/30 min.

Determination of Norepinephrine and Total Adrenal Catecholamines

The ovaries and celiac ganglia were homogenized in 0.2 *M* $HClO_4$ and the suspensions were centrifuged (15,000 *g*, 10 min); catecholamines present in the supernatant were determined by a specific radioenzymatic method [30] as previously described [29, 31]. As there is a high catecholamine content in the adrenal we used a colorimetric method for total catecholamine determination in this tissue [18]. This method measures both norepinephrine and epinephrine by the formation of noradrenochrome and adrenochrome when the samples are oxidized with iodine at a pH higher than 6.

Determination of T_3

Triiodothyronine (T_3) was determined by a Microparticle Enzyme Immunoassay (MEIA) for the quantitative determination of total circulating T_3 in plasma as described [32].

Statistical Analyses

Differences between two groups were analyzed with Student's t test. Comparisons between several groups were performed by use of a one-way analysis of variance, followed by the Student-Newman-Keuls multiple comparison test for unequal replications.

Results

Effect of i.c.v.-Administered TRH on Sympathetic Activity

After 5 days of TRH treatment, all parameters studied were affected, except for total catecholamine content in adrenals (fig. 1a). T-OH activity in celiac ganglia (fig. 1b) was inhibited at all doses tested (25, 200, 300, and 500 ng of TRH). Mean inhibition was 55% compared to control values in saline-injected animals (controls vs. 25 ng/kg treated animals, $p < 0.01$, and $p < 0.005$ vs. 200, 300 and 500 ng/kg, n = 4). Although there was an increase in the

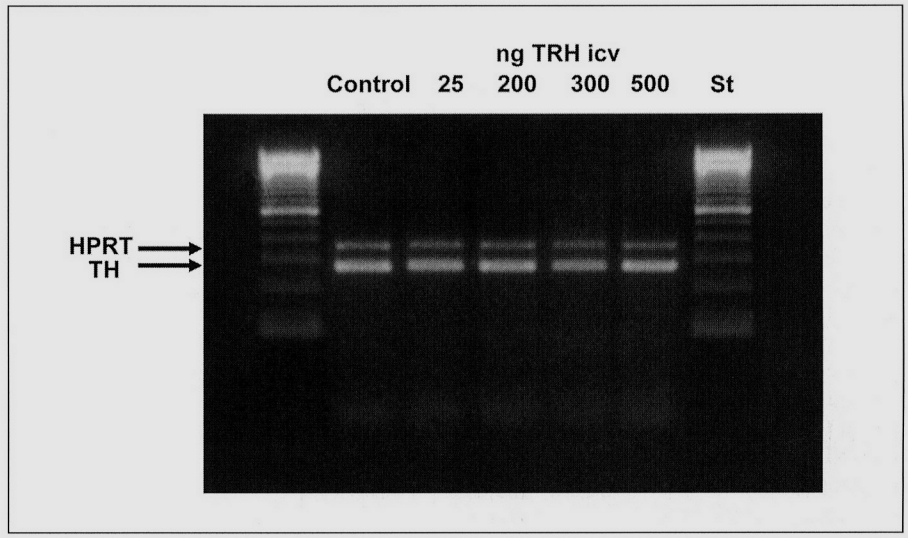

Fig. 2. Changes in the mRNAs for tyrosine hydroxylase (T-OH) and hypoxantine-phosphoribosyl-transferase (HPRT) after 5 days of a daily i.c.v. administration of TRH in doses of 25, 200, 300, and 500 ng. Figure shows a 2% agarose gel with both the T-OH HPRT signals for 200 ng of total RNA submitted to the PCR procedure according to methods. No changes in the intensity of the signal were found. Each lane is a representative sample for each of the different doses used in quintuplicate for each experimental condition.

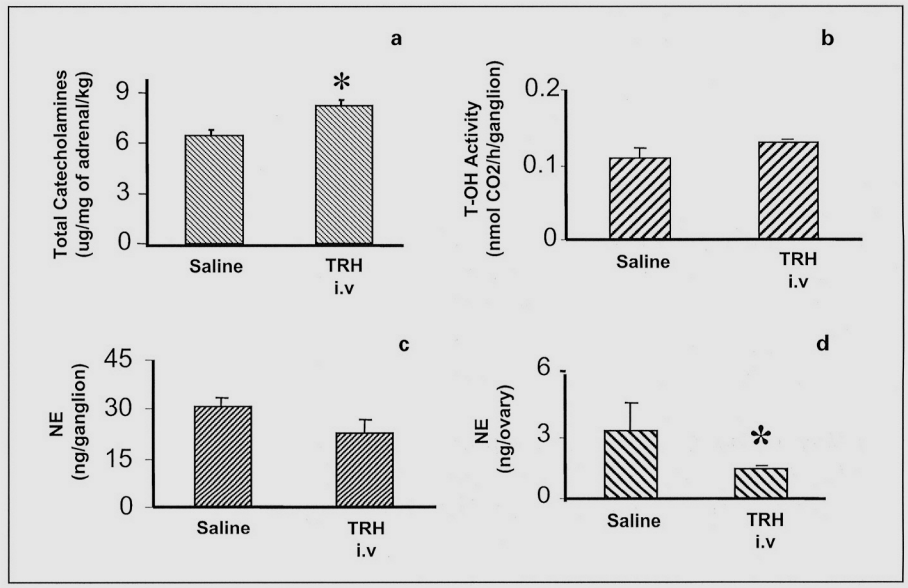

Fig. 3. The effect of systemic administration of TRH on sympathetic activity. Rats were injected once daily for 3 days, via a cannula implanted in the jugular vein, with saline or TRH 25 ng/kg body weight. **a** Increase in concentration of adrenal catecholamines (CA), is expressed as μg of catecholamines/mg adrenal/kg body weight. **b** T-OH activity in the celiac ganglia is expressed as nmol of CO_2 formed/h/ganglia. **c** NE content in the celiac ganglia is expressed as ng of NE/ganglia. **d** Decrease in the content of NE in the ovary. NE is expressed as ng of NE/ovary. Each bar represents the mean ± SEM of four independent observations. * $p < 0.05$ vs. control.

NA content of celiac ganglia at doses higher than 200 ng, it only reached significance for the latter dose (37.95 ± 4.3 vs. 56.17 ± 5.2 ng NA/ganglion, mean value ± SEM, $p < 0.05$, $n = 4$) (fig. 1c). Finally, the NA content of the ovary was significantly increased after a single dose of 25 ng/kg (controls: 0.14 ± 0.02 ng of NA/ovary, TRH 25 ng/kg: 0.28 ± 0.02 ng of NA/ovary), but at higher doses (200, 300 ng/kg) the response decreased and turned even into an inhibitory effect for the 500-ng dose (0.06 ± 0.02 ng of NA/ovary; mean value ± SEM, $p < 0.01$, $n = 4$). In order to analyze whether or not decreased T-OH activity resulted from inhibition of gene expression induced by the TRH administration at the central level, we studied changes in T-OH mRNA by semiquantitative PCR. As shown in figure 2, no change in T-OH mRNA levels were found at any i.c.v. TRH dose used.

Sympathetic Activity following i.v. Administration of TRH

Administration of 25 ng TRH (a dose able to affect the brain) [33, 34] intravenously once a day for 3 days resulted in a significant increase in adrenal catecholamines (6.45 ± 0.38 μg/mg tissue/kg in controls vs. 8.19 ± 0.36 μg/mg tissue/kg in i.v. TRH-treated rats, $n = 5$, $p < 0.05$; fig. 3a). In contrast, neither T-OH activity at the ganglia (fig. 3b) nor NA content in the celiac ganglia were

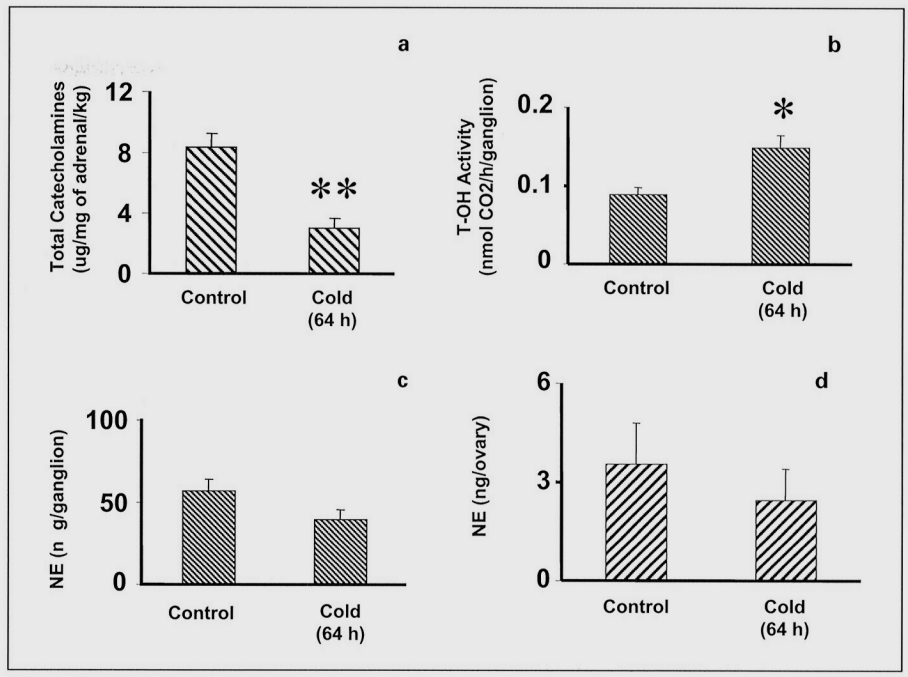

Fig. 4. The effect of cold exposure on sympathetic activity. Rats were maintained at 4°C for 64 h; controls were kept at 22°C. **a** Changes in the total content of adrenal catecholamines (CA) is expressed as μg/mg of adrenal/kg of body weight. **b** Changes in T-OH activity in the celiac ganglia. T-OH activity is expressed as nmol of CO_2 formed/h/ganglion. **c** NE content in celiac ganglia is expressed as ng of NE/ganglion. **d** NE content in the ovary is expressed as ng of NE/ovary. Each bar represents the mean ± SEM of four independent observations. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control.

affected (fig. 3c), but there was a significant decrease in ovarian NA (1.5 ± 0.03 ng NA/ovary vs. 3.3 ± 1.2 ng of NA/ovary in controls, mean value ± SEM, $p < 0.05$, $n = 4$; fig. 3d), suggesting a local action of TRH or of another thyrotropic component at the ovarian nerve terminals.

Effect of Cold Exposure on Sympathetic Activity

After 64 h cold exposure, a significant decrease in total adrenal catecholamines was observed (8.38 ± 0.89 μg/mg tissue/kg in controls vs. 3.02 ± 0.59 μg/mg tissue/kg in cold-stressed rats, $n = 4$, $p < 0.01$; fig. 4a). T-OH activity in celiac ganglia (fig. 4b) increased (0.09 ± 0.007 in controls vs. 0.15 ± 0.016 nmol CO_2/h/ganglion, mean value ± SEM, $p < 0.05$, $n = 4$) in animals subjected to cold exposure. In contrast, NA content was affected neither in the ganglia (fig. 4c) nor in the ovaries (fig. 4d).

Effect of TRH Administration and Cold Exposure on Body Weight

As shown in table 1, all experimental conditions applied induced a significant loss of body weight. Cold-stressed rats lost 7.15 ± 1.5% of their initial body weight ($p < 0.05$, mean value ± SEM with respect to controls), animals treated intravenously with TRH lost 0.8 ± 0.5% and rats treated centrally with TRH between 1.7 and 3.14% of their initial body weight ($p < 0.01$ with respect to controls).

Table 1. Effect of TRH administration or cold exposure on body weight

		% change in body weight
Control		2.44±0.5
Cold 64 h		−7.15±1.5**
TRH i.v.	25 ng/kg	−0.87±0.5*
TRH i.c.v.	25 ng/kg	−3.14±0.9*
	200 ng/kg	−2.05±1.6*
	300 ng/kg	−3.10±1.8*
	500 ng/kg	−1.67±1.7*

Changes in body weight were calculated as the difference between initial and final weights after each treatment and expressed as the percentage of change. Results are presented as mean ± SEM of four independent observations, except for the controls, which represents the mean of all controls rats ($n = 12$).

** $p < 0.01$ vs. control; * $p < 0.05$ vs. control.

Plasma Levels of T_3 after i.c.v. TRH Administration or Cold Exposure

To determine pituitary reactivity under the different experimental conditions involving TRH, plasma levels of T_3 were assessed. As shown in table 2, T_3 plasma levels increased in the group subjected to cold exposure (45.83 ± 1.56 vs. 55.07 ± 2.33 ng/dl, mean value ± SEM, $p <$

Table 2. Effect of TRH administration and cold exposure on plasma T_3 levels

		T_3, ng/dl
Control		42.38 ± 3.0
Cold 64 h		55.07 ± 2.33*
TRH i.c.v.	25 ng/kg	49.66 ± 1.5
	200 ng/kg	44.35 ± 6.6
	300 ng/kg	46.70 ± 12.3
	500 ng/kg	43.96 ± 2.9

Rats were killed by decapitation, whole trunk blood was taken, and the plasma was removed by centrifugation. T_3 levels in plasma are expressed as ng of T_3 per 100 ml of plasma. Each value represents the mean ± SEM of four independent observations, except the control value which represent the mean of all controls ± SEM.

* $p < 0.05$ vs. control.

0.05, n = 4), a paradigm known to activate endogenous TRH secretion. However, in animals receiving central i.c.v. TRH treatment, no change in T_3 plasma levels was observed, indicating that central administration of TRH was ineffective at the pituitary level.

Discussion

We have presented evidence implicating TRH as a putative neurotransmitter acting at an upstream level of sympathetic pathways which originate in the brain and affect ovarian function. Although our work did not precisely locate the central site of TRH actions involved here, a strong body of evidence points out the PVN as a major source of TRH neurons [20, 35]. These neurons project both directly and indirectly to preganglionic sympathetic neurons located in the spinal cord before reaching the ganglia [6, 36]. In order to analyze the effect of TRH on sympathetic neurons, we used intracerebroventricular administration of TRH through a cannula implanted in the third ventricle. Besides, to discriminate between central and peripheral effects of TRH, we also used intravenous administration of TRH and a cold stress protocol that involves both peripheral and central components.

Central Stimulation of Sympathetic Nerves by TRH

Intracerebroventricular administration of TRH (25 ng/kg body weight) induced a significant decrease in T-OH activity in celiac ganglia without affecting NA content. Celiac ganglia are the origin of the main sympathetic pathway to ovarian endocrine cells. Therefore, changes in the activity of the enzymes involved in the biosynthesis of NA in the ganglia are commonly used as indices of sympathetic activity affecting the ovaries [18, 29]. The decrease in T-OH activity in the ganglia could affect either decreased enzymatic capacity (linked or not to decreased biosynthesis of the enzyme), or increased outflow of enzyme molecules from the ganglion to ovarian nerve terminals. Since T-OH mRNA was not affected by any dose of TRH used, and given that the enzyme activity was measured under saturating concentrations of the substrate, the decrease in T-OH activity is more likely explained by an accelerated flow of the enzyme to nerve terminals. This conclusion is not completely speculative, since it is in agreement with physiological data previously reported by us during activation of ovarian sympathetic nerves in rats [37].

Increased expression of NA biosynthetic enzymes in the ganglia is likely to correspond to an increased need of the neurotransmitter at the ovary [37], responding to a trophic signal originating in the ovary and still effective many days later [38]. A similar mechanism could account for the change in NA found in the ovary and the ganglia of rats treated centrally with TRH. After 5 days of TRH treatment changes should affect availability of the presynthetized neurotransmitter and its enzymes rather than gene expression.

In summary, since TRH stimulates the firing rate of sympathetic neurons [39], we can hypothesize that an increased outflow of NA from the ganglion to ovarian nerve terminals is a consequence of increased sympathetic activity. It could be responsible for the decrease in ovarian NA content when the dose of TRH is increased from 25 to 500 ng/kg body weight. As discussed above, our protocol did not last long enough to allow development of a compensatory increase in T-OH expression and recovery of NA content in the ovary.

On the other hand, the adrenal gland, another peripheral sympathetic target, did not present changes in catecholamine content after i.c.v. administration of TRH, suggesting that TRH actions at the celiac ganglia are selective.

Peripheral Stimulation of Sympathetic Nerves by TRH

Since TRH administered centrally could theoretically diffuse not only in other brain areas, but also in the pituitary, we also tested the effects of peripheral administration of TRH. Using i.v. doses of TRH previously described as affecting the thyrotropic axis [33, 34], we did not observe any change in NA content or T-OH activity

at the celiac ganglia. The reduction in ovarian NA content after i.v. TRH suggests that, in that case, the peptide acts by the thyrotropic axis, which can itself affect the ovary. It has been demonstrated that T_3 acting on specific receptors stimulates the release of noradrenaline from mesenteric sympathetic ganglia [40]. Similarly, the fact that the ovary possesses T_3 receptors [41] is compatible with a local action of T_3 on the ovary, thus explaining an indirect effect of TRH administered i.v. on ovarian NA content.

In addition, peripheral actions of TRH also resulted in a clear increase in adrenal catecholamines, an effect previously shown to be mediated by glucocorticoid release from the gland [42]. Since TRH is also a potent prolactin-releasing factor, changes in adrenal catecholamines could be due to activation of PRL receptors which have been reported at this level [43].

In conclusion, although TRH applied peripherally was also able to modify ovarian sympathetic parameters, it did not affect the same parameters as i.c.v. TRH administration.

Cold Stress Stimulation of Sympathetic Nerves

Cold stress is an experimental paradigm that mimics, at least in part, effects of intravenous TRH. It has been demonstrated that plasma levels of TSH and of free thyroid hormones remain elevated 5 days after cold exposure [44]. Cold stress is known to activate TRH-dependent neurohormonal [16] as well as central pathways [12, 45]. The former leads to a thyrotropic activation and high plasma levels of TSH and thyroid hormones [13, 14]. In the present experiments, animals subjected to cold stress, had increased plasma levels of T_3 in contrast to those receiving central TRH treatment. Interestingly, decreased ovarian NA content was proportional to the magnitude of the increase in T_3 plasma levels, suggesting a direct effect of this hormone on sympathetic nerve activity, as demonstrated in the case of mesenteric ganglia [40].

Cold exposure may also activate the corticotropic axis [46], increasing adrenal glucocorticoid release [47] and limiting the response of catecholamine biosynthesis to cold stress [48] as well as release, uptake and metabolism of sympathetic nerves [49], including those supplying the ovary [50].

Interestingly, experimental conditions used here and involving TRH activation resulted in body weight loss. Depending on the route of TRH administration, activation of central [51] or peripheral [52] thermogenic mechanisms have been correlated with weight loss. Besides its peripheral actions, cold exposure could activate central non-neurohormonal thermogenic mechanisms, as does i.c.v. TRH.

Autonomous actions attributed to hypothalamic TRH [53] include stimulation of gastric acid secretion [20] and thermogenic effects [54]. The present results suggest that ovarian function should be added to the list of autonomous functions in which TRH controls NA levels. Stressful situations [55] such as cold exposure [16] or neural signals [56, 57] leading to TRH activation could affect synthesis and/or content of ovarian NA and behave as etiologic factors of ovarian pathologies involving participation of sympathetic nerves, as described in the polycystic ovary syndrome [18, 29].

In conclusion, our data suggest that TRH exerts a stimulatory effect of central origin on the autonomous nervous system that controls ovarian activity. Inhibition of T-OH activity accompanied by accumulation of NA at the ganglion site, as well as decreased ovarian NA, support the hypothesis that TRH can affect the firing rate of ovarian sympathetic neurons, probably at their site of origin in the central nervous system. TRH can thus be viewed as an additional neural signal involved in ovarian regulation.

Acknowledgments

The authors wish to thank Ms Diane F. Hill for editorial assistance of the paper and Dr. Carmen Romero from the Clinical Hospital of Universidad de Chile for T_3 determination.

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