Effects of forced swimming stress on thyroid function, pituitary thyroid-stimulating hormone and hypothalamus thyrotropin releasing hormone expression in adrenalectomy Wistar rats

QIUYAN SUN, AIHUA LIU, YANAN MA, ANYI WANG, XINHONG GUO, WEIPING TENG and YAQIU JIANG

Department of Endocrinology and Metabolism, Institute of Endocrinology, Liaoning Provincial Key Laboratory of Endocrine Diseases, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

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Abstract. In order to study the impact that is imposed on the hypothalamic-pituitary-thyroid (HPT) axis of adrenalectomy male Wistar rats by stress caused by swimming, the blood level of triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH), the expression of TSHβ mRNA at the pituitary and thyrotropin releasing hormone (TRH) expression at the paraventricular nucleus (PVN) were measured. A total of 50 male Wistar rats of 6-8 weeks of age and with an average weight of 190-210 grams were randomly divided into the following two groups: The surgical (without adrenal glands) and non-surgical (adrenalectomy) group. These two groups were then divided into the following five groups, according to the time delay of sacrifice following forced swim (10 min, 2 h, 12 h and 24 h) and control (not subjected to swimming) groups. A bilateral adrenalectomy animal model was established. Serum TSH in the blood was measurement by chemiluminescent immunoassay, and cerebrum tissue were excised for the measurement of TRH expression using an immunohistochemistry assay. In addition, pituitaries were excised for the extraction of total RNA. Finally, reverse transcription-quantitative polymerase chain reaction was performed for quantitation of TSHβ. Following swimming, the serum T3, T4 and TSH, the TSHβ mRNA expression levels in the pituitary and the TRH expression in the PVN of the surgical group were gradually increased. In the non-surgical group, no significant differences were observed in the serum T3, T4 and TSH levels compared with the control group. The TSHβ mRNA expression at the pituitary showed a similar result. Furthermore, the TRH expression at PVN was gradually increased and stress from swimming could increase the blood T4, T3 and TSH levels, TSHβ mRNA expression at the pituitary and TRH expression at the PVN in adrenalectomy Wistar rats. Moreover, the index in the surgical group changed significantly compared with the non-surgical group. In conclusion, the results suggest that there is a positive correlation between stress from forced swimming and the variation of the HPT axis.

Introduction

Thyrotropin releasing hormone (TRH) is produced in medial neurons of the paraventricular nucleus (PVN) of the hypothalamus. The secretion of TRH stimulates the release of thyroid stimulating hormone (TSH) from the pituitary gland, which travels to the thyroid via the blood where it stimulates the secretion of thyroid hormone (TH). TH then acts on the hypothalamus and pituitary to exert negative feedback. In addition to TRH, a number of hypothalamic proteins can regulate the secretion of TSH, including somatostatin (SST) (1). Additionally, deiodinase, which is an enzyme affecting the concentration of T3 (triiodothyronine) and T4 (thyroxine) tissue, is important in thyroid-mediated signal transduction (2).

Correspondence to: Professor Yaqiu Jiang, Department of Endocrinology and Metabolism, Institute of Endocrinology, Liaoning Pro vincial Key Laboratory of Endocrine Diseases, The First Affiliated Hospital of China Medical University, 155 North Nanjing Street, Shenyang, Liaoning 110001, P.R. China E-mail: jiangyaqiu@yeah.net

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that low levels of T3 are an independent risk factor for poor prognosis and mortality caused by heart disease (11). Furthermore, it has previously been demonstrated that combinations of T3 and antidepressants can significantly enhance the treatment of refractory depression (12). However, current investigations of the association between stress and thyroid hormone levels in the peripheral blood have generated mixed results, indicating a slight increase or no change in thyroid hormone blood levels in response to mild stress (psychological stress-slow-binding experiments) (13,14), or a decrease following high stress (such as electric shock stimulation) (6,15,16). Some researchers have concluded that such changes reflect the sensitivity of the HPT axis to the stress strength and intensity (17,18). Additionally, studies have revealed that TRH mRNA levels in the PVN change following stress (15,16,19), and thus stress responses may partially act through the regulation of TRH expression in the central nervous system.

In the classical model of stress, cortisol hormone levels exhibit significant increases, and a number of studies have revealed that increased cortisol can influence pituitary TSH release (20,21). However, there are studies suggesting that cortisol exerts effects on the hypothalamus (1,20). Furthermore, glucocorticoid receptors are discovered in the TRH neurons of the PVN, and glucocorticoid response elements have been identified on the TRH gene (22).

The present study aimed to establish bilateral adrenalectomy Wistar rats by artificially blocking the effect of cortisol, and sought to investigate the influence of acute stress on the HPT axis by measuring alterations of blood T4, T3, TSH and pituitary TSH subtypes mRNA levels, as well as TRH expression levels in the PVN.

Materials and methods

Animals. A total of 50 (6-8-week-old) specific-pathogen-free grade male Wistar rats, weighing 190-210 g were purchased from Vital River Company (Beijing, China) and housed under controlled temperature and light conditions (23±2˚C; 12-h light/dark cycle) with ad libitum access to standard rat chow and water. The present study conformed to the guidelines outlined by the Animal Care and Use Research Committee of China Medical University. Starting 1 week prior to the experiment, the animals were handled for 5 min every day to accustom the rats to the experimenters. Then, the rats were randomly divided into the following two groups: The surgical group (without adrenal glands) and the non-surgical group (with adrenal glands). These two groups were then divided into five groups, where each group consisted of 5 animals. Surgery was performed as previously described (23,24). Experiments were all performed between 8:00 and 12:00 am. Group 1 consisted of blank controls where the animals were decapitated without subjection to stress, and their blood samples were taken. Group 2 were forced to swim in a water bath set to 26˚C for 10 min and were then immediately decapitated and their blood samples collected. Group 3 were forced to swim in a water bath set at 26˚C for 10 min and after 2 h these animals were decapitated and their blood samples obtained. Group 4 were forced to swim in a water bath set at 26˚C for 10 min and after 12 h were subjected to decapitation and their blood samples collected. Group 5 were forced to swim in a water bath set at 26˚C for 10 min and then decapitated after 24 h and their blood samples obtained. The brains were quickly removed and frozen in -40˚C isopentane (Sangon Biotech, Shanghai, China) and stored at -70˚C. Blood samples were immediately centrifuged at 956 x g at 0˚C for 3-5 min and the plasma stored at -20˚C.

Serum hormone measurements. Peripheral serum T3, T4 and TSH levels were measured by chemiluminescence (Immulite; Siemens Healthcare, Ltd., Surrey, UK), according to the manufacturer's instructions.

Immunohistochemical staining of pituitary TSHβ. Pituitary tissue was removed from the -70˚C freezer, equilibrated for ≥30 min (to prevent a sudden change of the temperature, which would affect the structure of the organization), sliced into 10 μm sections, mounted on poly-lysine coated slides and transported on dry ice to be stored in the -70˚C freezer.

Immunohistochemistry sections were removed from the -70˚C freezer and fixed in 4% paraformaldehyde and phosphate-buffered saline (PBS) (pH=7.4) at room temperature for 1 h, rinsed three times in 0.01 M PBS for 3 min each time, soaked in 3% H2O2 for 10 min to block endogenous peroxidase and then rinsed again in three times of 0.01 M PBS for 3 min each time. The sections were then incubated with a rabbit anti-TRH primary antibody (1:100 dilution; bs-1053R; BIOSS, Beijing, China) at 37˚C for 90 min and then washed three times in 0.01 M PBS for 3 min each time. Sections were subsequently covered with drops of polymer helper reagent from the horseradish peroxidase-conjugated secondary antibody kit (PV9003 ready-to-use type; ZSBG-Bio, Beijing, China), incubated at 4˚C for 30 min and rinsed three times in 0.01 M PBS for 2 min each time. Following this, sections were incubated with poly-horseradish peroxidase anti-goat IgG from the PV9003 reagent box (read-to-use type) at 4˚C for 30 min and rinsed three times in 0.01 M PBS for 2 min each time. 3,3'-diaminobenzidine solution (BIOSS) was prepared according to the manufacturer's instructions. The sections were developed with chromogen (BIOSS) for 3 min and rinsed under running tap water in order to terminate the reaction. They were then stained with hematoxylin and eosin for 30 sec, rinsed in running tap water until the water was colorless, immersed twice in hydrochloric acid alcohol, incubated in blocking solution for 5 min and then mounted with 50% glycerogelatin. Staining results were quantitatively analyzed under an image analysis system (MetaMorph/DPI0/Bx41; Olympus Corporation, Tokyo, Japan) at a magnification of x400. The average optical density of positive staining of the pituitary tissues was calculated as follows: Five sections were selected from each group; from each slice three microscopic pictures were randomly captured at a magnification of x400. The average integrated optical density (IOD) of these 15 microscopic pictures was calculated as the measured value of this group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) measurement of pituitary TSHβ mRNA.

RNA extraction. Total RNA from the Wistar rat pituitary was extracted using TRIzol (TaKaRa Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Frozen rat pituitary tissues were removed from -70˚C and homogenized in 1 ml TRIzol reagent on ice. The homogenates were then pipetted...
Table I. Primer sequences.

| Protein | Genes | Genebank accession number | Primer | Amplification length |
|---------|-------|----------------------------|--------|----------------------|
| TSHβ   | Tshb  | NM_013116.1                | Sense GTGCCTACTGCGCCATCACA Anti-sense AGCAACATGCTGGTGACATC | 557 bp |
| GAPDH  | Gapdh | NC_005103.2                | Sense TGGTGAAGGTCGGTGACAC Anti-sense CCATGAGTTGAGCTCAATGAGG | 123 bp |

TSH, thyroid stimulating hormone.

20 times using 1 ml sterile syringes and transferred to a new 1.5 ml centrifuge tube. Homogenates were pipetted repeatedly until there were no apparent precipitate in the lysates, and were then incubated at room temperature for 5 min.

Purity and concentration of RNA. A total of 2 µl RNA solution was added to 198 µl DEPC-treated water and 260 and 280 nm optical density (OD) values were determined using a UV spectrophotometer. RNA samples with an OD260/OD280 ratio >1.8 were used in the studies. The RNA concentration was calculated as (µg/ml)=OD260 x 40 x dilution factor, and the solutions were divided into small aliquots and stored at -70°C until further use.

cDNA synthesis. Total RNA was diluted to 100 ng/µl and cDNA was synthesized by reverse transcription (RT) using PrimeScript RT Reagent kit (RR036A), according to the manufacturer’s instructions (TaKaRa Bio, Inc.). The RT kit contained the following reagents: 2.0 µl 5X primescript buffer; 0.5 µl oligo dT primer (50 µM); 0.5 µl random 6-mers (100 µM); 0.5 µl primescript RT enzyme mix; 1.5 µl RNA-free H2O; and 5.0 µl total RNA. The reagents were mixed and centrifuged at 3,824 x g for 3-5 sec at room temperature and RT was performed at 37°C for 15 min. Next, the inactivation of RT was performed at 85°C for 5 sec. The reaction tube was then placed on ice immediately after the end of the reaction, and the cDNA was stored at -70°C or directly used for the next step, qPCR. The volume of cDNA added did not exceed 1/10 of the total volume of the total qPCR reaction system (v/v).

Assay design. TSHβ and GAPDH sequences were obtained from the Gene Bank database and Primer version 5.0 (TaKaRa Bio, Inc.) was used to design the primers. Primers were synthesized and purified by TaKaRa Bio, Inc., and are shown in Table I. A reference gene is included as an internal standard to correct for sample to sample variations in RT-qPCR.

In the present study, GAPDH was selected as the endogenous control in the studies. The primers were dissolved in double-distilled water to a concentration of 100 µmol/µl and aliquots were stored at -70°C. Next, the primers were further diluted to 10 µmol/µl for the next step of the experiments. The preparation of the qPCR reaction was performed according to the SYBR Green qPCR protocol. The qPCR reaction was performed on a LightCycler 480 Real-Time PCR System (Roche Diagnostics GmbH, Basel, Switzerland) with the following programs: Program 1 (denaturation), 95°C/30 sec and 20°C/sec for 1 cycle; program 2 (PCR reaction), 95°C for 5 sec and 20°C/sec, 59°C for 30 sec and 20°C/sec for 40 cycles; and program 3 (melting curve), 95°C for 0 sec and 20°C/sec, 65°C for 15 sec and 20°C/sec, and 95°C for 0 sec and 0.1°C/sec.

Following the reaction, a qPCR amplification curve was established and the following requirements were met: The melting curve had a single peak and the standard curve had an R² value ≥0.98. Moreover, the amplification efficiency was E ≥2.0, and the negative control showed no fluorescence signal. In addition, the final result, or gene correction value, was equal to the quantitative results of the target gene/quantitative results of GAPDH. LightCycler 480 Rotor-Gene Real-Time Analysis Software 6.0 was used with the option set as ‘Advanced Relative Quantitation’ (senior relative quantification) to analyze the changes in mRNA expression levels.

Statistical analysis. Data processing and analysis was performed using SPSS version 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Data were evaluated by one-way analysis of variance and the independent-samples t test was performed within groups, and evaluated by factorial design. P<0.05 was considered to indicate a statistically significant difference.

Results

Serum levels of T3, T4 and TSH. Following the forced swimming in the surgical 10 min and 2 h groups, the serum T3 and T4 levels were gradually increased and significantly different compared with the control group (P<0.05). The serum T3 and T4 levels in the 10 min group reached a peak and gradually decreased over time. In the non-surgical group, the serum T3 and T4 showed no significant differences compared with the control group.

In the surgical group, the serum TSH was gradually increased in the 10 min, 2 h and 12 h groups and reached a peak in the 12 h group; a significant difference were observed compared with the control group (P<0.05). In the non-surgical group, the serum TSH was slight elevated but no significant difference was observed compared with the control group. Furthermore, the trend of the serum TSH was observed compared with the surgical group.

Expression levels of rat pituitary TSHβ mRNA. In order to determine whether forced swimming stress can affect expression levels of TSHβ mRNA in rat pituitary tissue, RT-qPCR was used to analyze the pituitary tissue TSHβ mRNA expression levels.
The option of Advanced Relative Quantitation was set in order to analyze TSHβ mRNA levels in rat pituitary tissue. The results revealed that after 10 min of forced swimming in the surgical and non-surgical groups, Wistar rat pituitary TSHβ mRNA expression levels increased in all of the groups, but these increases were no significantly different compared...
with the control group. Furthermore, the index in the surgical group changed significantly compared with the non-surgical group (Figs. 10-12).

**Hypothalamus TRH protein levels.** As demonstrated by immunohistochemical staining of frozen sections, 10 min of forced swimming immediately triggered increases in pituitary TRH expression levels (presented as IOD). TRH expression levels significantly increased 10 min after forced swimming in the surgical and the non-surgical groups. TRH expression levels significantly increased 10 min after forced swimming in the surgical group. In addition, there was no significant difference between the surgical and non-surgical groups (Figs. 13-17).

**Discussion**

Thyroid hormone is important in the endocrine system and serves a role in maintaining homeostasis and development. Furthermore, it is intimately involved in the body's stress response. In response to stress, hormone secretion and metabolic homeostasis imbalances may induce nervous system, circulatory system, endocrine system and mental disorders, as well as lead to physical and mental diseases, such as Graves' disease and depression (25,26). Through studies of thyroid function changes in response to stress, it is possible to predict the impact of stress on the human body and what can be expected from intervention, and elucidate the function of the thyroid hormone and the mechanisms by which the body adapts to environmental changes.

It has previously been demonstrated that 10 min of forced swimming can immediately increase glucocorticoid secretion significantly by the hypothalamic-pituitary-adrenal (HPA) axis (27). However, by 2 h after stress, the glucocorticoid levels return to normal. It has been indicated that a high-dose of glucocorticoids suppresses serum TSH in a healthy individual and was controlled at the level of the hypothalamus (28). The present study aimed to establish bilateral adrenalectomy Wistar rats in order to block the effect of cortisol. A forced swim stress was applied to rats to examine whether it had the same effects on the physiological function of the HPT axis as on the HPA axis. It was revealed that 10 min of swimming stress immediately elevated the peripheral blood levels of T3, T4 and TSH or pituitary TSH levels in the surgical group, and that these were markedly different compared with the non-surgical group. These data indicate that the response of the HPT axis to forced swimming is different to that of the HPA axis, as it is a prolonged reaction and can last for an extended period of time.

Typically, the effects of thyroid hormone are observed several days later and can last for a number of weeks. However, a recent study revealed that thyroid hormones may cause acute effects within hours. For example, significant changes in brain-derived neurotrophic factor mRNA in the hippocampus of rats intraperitoneally injected with T3 have been observed within 2 h (29). Additionally, food intake increases within 2 h of a subcutaneous injection with T3, regardless of the energy consumption status (30). In addition, the effects of T3 on hippocampal neurons have been demonstrated (31). The acute effects of thyroid hormones may function through cell receptors in order to control gene expression, but may act through other mechanisms (32).

In the present study, serum T3, T4 and TSH levels were concurrently elevated 10 min after forced swimming in the surgical group, suggesting that forced swimming stress can increase the blood levels of thyroid hormones. As mentioned
earlier, a recent study has revealed that moderate stressful stimuli (such as psychological and chronic restraints) can cause alterations in rat serum thyroid hormone levels (33), which is similar to the results of this experiment. In order to further address the mechanism of stress response, RT-qPCR was used to analyze the expression levels of pituitary TSHβ mRNA.

The increase in pituitary mRNA expression levels in the surgical group in the current study demonstrated that the effects on the HPT axis of stress are a possible source of TSH production. In addition, the level of TRH expression increased in the surgical group. The trend in the surgical group was more evident compared with that in the non-surgical group. This suggests that there are direct or indirect connections between the HPT and HPA axes, which perhaps organize the core elements, such as energy mobilization. These complex and intertwined axes regulate the body in order to adapt to complex and stressful situations (34). Glucocorticoids, as a product of the HPA axis, can inhibit the HPT axis at the level of the hypothalamus and pituitary. Furthermore, intravitreal corticosteroids inhibit the level of TRH mRNA expression on the hypothalamic PVN (35) and inhibit peripheral conversion of T4 to T3 (36). In addition, a number of in vitro studies have provided evidence that glucocorticoids stimulate the production of TSH (37). By contrast, HPT can affect the HPA axis. The aforementioned evidence has proves that there is a trend between the surgical and non-surgical groups. A study by Helmreich et al (34) revealed that chronic mild stress in the HPA axis and the hypothalamic arcuate nucleus agouti-related protein (AGRP) are most likely involved in the regulation of the HPT axis (34). However, with regard to acute stress, AGRP, which is involved in the HPT axis, has not yet been reported.

A positive correlation between stress and the increase of PVN TRH mRNA levels has previously been reported (38). Various types of stress will produce different effects on the HPT axis (39), thus indicating that besides TRH, there are other neurological proteins or feedback mechanisms that can
affect the HPT axis, and TRH activity change is not necessarily accompanied by changes in mRNA levels. Processing of the TRH molecule can change its activity (40), for example in the ependyma located at the base of the hypothalamus (41), the TRH-specific poly-l-proline type II can directly alter the biological activity of TRH (42). Further studies of these proteins as well as deiodinase and SST may further reveal their role.

The present study aimed to investigate the acute effects of forced swimming stress on the HPT axis of rats. It was revealed that peripheral T3, T4 and TSH levels increased, and an increased expression of pituitary TSH was observed in the 12-h surgical group. A previous study by Helmreich et al (34) reports that in mice that were given repeated and inescapable foot electrical stimulation for 14 days consecutively experienced a significant drop in their plasma T3 and T4 levels, whereas their TRH mRNA levels in the hypothalamic PVN did not change. In addition, they discovered that TRH mRNA levels were closely associated with plasma glucocorticoid levels, HPT composition and weight changes. Furthermore, it was identified that T3 are associated with AGRP mRNA levels, and that T4 are associated with plasma glucocorticoid levels and HPT composition change; the levels of T3 were significantly reduced 2 h later under inescapable stress conditions. These observations are in contrast with the results of the present study, but this discrepancy may be due to a difference in stress paradigms. In the study by Helmreich et al (34), an iterative chronic stress was used, and the impact of stress on the HPT axis was measured for a number of days or weeks. What the authors did not measure was the change in the HPT following acute stress, for example after the first inescapable foot HPT electrical stimulation. The present study had a stronger focus on acute stress and employed a physical and psychological double stress-forced swimming stress, with the effects of the stress measured in minutes to hours. Acute stress was shown to lead to an increase in thyroid hormones and TSH levels, and the response of the HPT axis to acute stress was delayed but lasted longer than that of the HPA axis.

As the majority of the previous literature has reported, most stresses caused by exogenous noxious stimuli can increase the thyroid hormone level in the short term, but in the long term they lead to a reduction (18,35,37). This is consistent with the staging of the stress response reported by Selye (43). The mechanism by which stress causes changes in thyroid function is unclear. However, a number of studies have reported that there are numerous stress factors, including extreme cold or heat, that can directly act through the central temperature sensors to control HPT, TRH and TSH release, leading to an increase in thyroid hormone secretion (32,44). Acute stress primarily acts through the locus coeruleus-sympathetic-adrenal medullary system to elicit a response, and thus excite the sympathetic nervous system, speed up adrenal medulla function and increase the concentration of plasma catecholamine to stimulate thyroid hormone secretion. It is suggested that thyroid hormone reduction, particularly T3, caused by long-term stress are closely related to HPA. Overall, the effects of chronic stress on the HPT axis function is an area that requires further investigation and research.

In conclusion, swim stress increases the blood levels of T3, T4, TSH and the TSHβ mRNA expression at the pituitary in adrenalectomized Wistar rats, and this is associated with the HPT axis. Furthermore, the significant difference between the index of surgery between the non-surgical and surgical groups suggests that the adrenal glands have a role in the correlation between stress induced by forced swimming and variation in HPT levels.

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