BIOCHEMICAL PHARMACOLOGY 72 (2006) 582–587

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Sodium tanshinone IIA sulfonate derived from Slavia miltiorrhiza Bunge up-regulate the expression of prolactin releasing peptide (PrRP) in the medulla oblongata in ovariectomized rats

Yao Xiaoa [a,b], *Wang Xiao Qing* [c], *Ma Shu Lan* [a], *Chen Bo Ying* [a,*]

[a] *Department of Neurobiology and Integrative Medicine, Shanghai Medical College, Fudan University, P.O. Box 291, 138 Yi Xue Yuan Road, Shanghai 200032, PR China*

[b] *Department of Endocrinology, Huashan Hospital, Fudan University, 12 Wulumuqi Middle Road, 200041 Shanghai, PR China*

[c] *Department of Orthopaedics, Ninth People's Hospital, Shanghai Jiaotong University, 200011 Shanghai, PR China*

ARTICLE INFO

Article history:
Received 6 March 2006
Accepted 19 May 2006

Keywords:
Slavia miltiorrhiza
Sodium tanshinone IIA sulfonate
Prolactin releasing peptide (PrRP)
Medulla oblongata
Ovariectomy
Rat

ABSTRACT

Sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA, is isolated from the root of Salvia miltiorrhiza known as "Danshen". Although injection of S. miltiorrhiza extract and STS is used successfully in clinics in China for treating postmenopausal syndrome, the exact mechanism for its therapeutic basis is poorly understood. The present study was undertaken to characterize the effect of STS on the expression of prolactin releasing peptide (PrRP) in the medulla oblongata in ovariectomized rats. In addition, estrogen (E2) levels were detected in OVX rats treated with STS. The results showed that STS might significantly increase the blood level of E2 and PrRP cell number in the medulla oblongata of ovariectomized rats. The number of PrRP immunoreactivity (ir) neurons was higher in the group ovariectomized with STS than that in the ovariectomized group. The numbers of PrRP-ir neurons in Sham and Sham + STS were not significantly different between the two groups. These results suggest that the mechanism that STS improved postmenopausal symptoms induced by ovariectomy in rats might be related to the modulation of the blood E2 level and the expression of PrRP in medulla oblongata of ovariectomized rats.

1. Introduction

In postmenopausal women, ovarian estrogen deficiency results in a series of postmenopausal symptoms such as hot flashes, insomnia, headaches, postmenopausal bone loss and associated bone fracture [1]. Estrogen replacement therapy has been used for preventing not only postmenopausal symptoms but also postmenopausal bone loss [1,2]. However, because of concern about the side effects of estrogen replacement therapy, many patients and their physicians are reluctant to begin or continue its use [3,4]. Therefore, many researchers have searched for naturally occurring substances that prevent postmenopausal symptoms.

Sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA, is isolated from the root of Salvia miltiorrhiza known as "Danshen", a traditional Chinese herbal medicine, which has been widely used in clinical practice for the prevention of cardiac diseases, arthritis and other inflammation-related disorders based on its pharmacological actions in multiple tissues [5]. It is reported that STS can reduce

* *Corresponding author*. Tel.: +86 21 54237693.
E-mail address: boyingchen@yahoo.com.cn (B.Y. Chen).

0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.05.014

postmenopausal symptoms and suppress bone resorption [6,7]. Also we have reported that STS enhanced the protein level of aromatase, a key enzyme that converts androgens to estrogens, in subcutaneous abdominal adipose and liver tissues in ovariectomized rats [8]. However, the mechanisms by which STS may protect against postmenopausal syndrome have not been elucidated.

Prolactin-releasing peptide (PrRP) is a neuropeptide isolated from the bovine hypothalamus as a ligand of an orphan G protein-coupled receptor [9]. The PrRP-immunoreactive (PrRP-ir) neurons were densely distributed in the nucleus of the solitary tract nucleus (NTS) and lateral reticular nucleus (LRN) [10,11]. PrRP shows specific prolactin (PRL)-releasing activity in vitro [9] and in vivo [12,13]. It has been shown that PrRP expression in the medulla oblongata was estrogen dependent and PrRP could stimulate luteinizing hormone (LH) and follicle stimulating hormone (FSH) via a hypothalamic mechanism involving the release of gonadotropin releasing hormone (GnRH) [14]. Our previous study showed that the synthesis and release of GnRH were decreased in the hypothalamus of ovariectomized rats [15]. These data suggest that PrRP may regulate the disorder of the hypothalamic–pituitary–ovary axis of ovariectomized rats.

These results led us to hypothesize that the expression of PrRP in the brain of ovariectomized rats might be affected with administration of STS. To test the hypothesis, we detected the expression of PrRP in medulla oblongata and blood estrogen level in the OVX rats that received STS.

2. Materials and methods

2.1. Reagents

STS was obtained from Institute of Materia Medica of Academia Sinica in Shanghai, China. The monoclonal antibody of PrRP was purchased from Phoenix Pharmaceuticals (USA). Biotinylated anti-rabbit IgG and avidin-biotinylated peroxidase complex were purchased from Vector Lab (CA). Estrogen RIA kits were purchased from the National Atomic Energy Research Institute (Beijing, China). Trizol reagent, oligo$(dT)_{18}$ primer, dNTP and M-MLV reverse transcriptase, RNase inhibitor were purchased from Invitrogen (CA). All other reagents made in China were of AR level.

2.2. Animals and treatments

Adult female Sprague–Dawley rats (weighing180–200 g), with regular 4-day estrus cycles were purchased from Medical Experimental Animals Center of Fudan University (Shanghai, China). Prior to experimental manipulation, rats were allowed to acclimate for 1 week and maintained under 12 h light:12 h dark conditions with free access to food and water. Vaginal smears were taken and inspected daily, and rats with normal estrous cycles were used for the study. All rats in the study were used strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rats were randomly divided into four groups (eight rats for each group): sham-operated control rats (Sham), sham-operated rats with STS (Sham + STS), ovariectomized rats (OVX) and ovariectomized rats with STS (OVX + STS). To perform ovariectomy, rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). A midline incision was made to expose the lower abdominal cavity and the ovaries were removed in total. Sham operations were performed in a similar fashion but the abdomen was closed without removing ovarian tissue. Four weeks after the operation, Sham + STS and OVX + STS were treated with STS dissolved in saline (30 mg/kg, i.p.) once daily for up to 7 days. Sham and OVX were treated with saline.

2.3. RIA of blood estrogen concentrations

At the time of sacrifice, the blood samples of the OVX, OVX + STS rats were collected (6 h after the last STS treatment) from left ventricle, respectively, those of the Sham and Sham + STS during the period of proestrus to avoid spontaneous estrogen surges that occur during estrus. The plasma was separated by centrifugation and stored at −70 °C until assayed. Concentrations of blood estrogen were determined by double-antibody RIA kit. The samples were assayed in duplicate, and all the subjects' samples were assayed together. The sensitivity of the kit was 1.4 pg/ml; the intra- and inter-assay coefficients of variation were 6.5% and 8.3%.

2.4. Immunohistochemistry

After the final drug administration, Rats were anesthetized with 50 mg/kg sodium pentobarbital and perfused through the aorta with 150 ml normal saline (0.9% NaCl, NS) followed by 200 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.4). After fixation, the brains were removed and immersed overnight in 0.1 M PBS containing 20% sucrose. Frozen coronal sections (35 μm thick) of the medulla oblongata were made with a freezing microtome.

Immunohistochemistry procedures used in this study were similar to those described earlier [11]. Free-floating sections were treated with 0.01 M PBS for 15 min. After extensive rinsing, the sections were quenched with 0.3% H_2O_2 in PBS for 10 min and blocked with 0.01 M PBS. Thereafter, sections were incubated with the rabbit anti-PrRP antiserum at a dilution of 1:500 in PBS containing 10% goat serum at 4 °C for 48 h, the sections were rinsed three times in PBS and incubated for 30 min with biotinylated anti-rabbit IgG at 1:50 dilution in PBS. The sections were rinsed and incubated with avidin-biotinylated peroxidase complex in PBS for 30 min. The peroxidase-substrate reaction was performed for 5 min using DAB. The sections were mounted on gelatin-coated glass slides and dried in air, then dehydrated with ethanol, cleared with xylene and covered with coverslips. Neural structures were identified according to the rat brain atlas [16].

PrRP positive neurons were quantified by a video camera and a computer-aided densiometric image-analysing software program. Sections were included for the statistical evaluation if the anatomical structure could easily be identified.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the medulla oblongata by using Trizol reagent according to the manufacture's instructions.

The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm followed by electrophoresis on 1.5% agarose gels. One microgram of RNA was reverse-transcribed into cDNA by using oligo(dT)$_{18}$ primers and M-MLV reverse transcriptase at 42 °C for 1 h in standard buffer. Sequence-specific primers for PrRP and the house-keeping gene β-actin were used to cDNA amplification. Primers used to amplify PrRP cDNA were 5′-GACGTGGCTTCTGTGCTTGCTGC-TG-3′ (sense) and 5′-GCAGCACTGTCTTCTCGAGCTG-3′ (anti-sense) and yielded a PCR product of 268 bp. The primers for β-actin were 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ (sense) and 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′ (antisense) and the size of PCR product was 285 bp. cDNA was amplified using the following parameters: 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, in a total of 30 cycles with a final extension step at 72 °C for 10 min. These PCR profiles were determined by the amplification for PrRP mRNA and β-actin to ensure that PCR was performed within the linear part of the amplification curves (see Section 3.2). PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated densitometrically using Quantity One-4.4.1 imaging software (Bio-Rad Laboratories). Levels of mRNA were expressed as the ratio of band intensity of PrRP relative to that for β-actin.

2.6. Statistical analysis

All data are presented as mean ± S.D. Statistical analysis was performed on raw data using one-way analysis of variance (ANOVA) and Bonferroni *t*-test for multiple comparisons. The value of $P < 0.05$ was considered significant.

3. Results

3.1. Effects of STS on the blood concentrations of estrogen

The ovariectomized rat is a classical animal model for postmenopausal syndrome by estrogen depletion. To investigate the effect of STS on estrogen levels of ovariectomized rats, we treated ovariectomized rats with STS and measured the blood levels of estrogen. The administration of STS (30 mg/kg) to ovariectomized rats caused a significant increase of blood estrogen in ovariectomized rats. However, treatment of Sham rats with STS has no effect on the levels of blood E2 (Fig. 1).

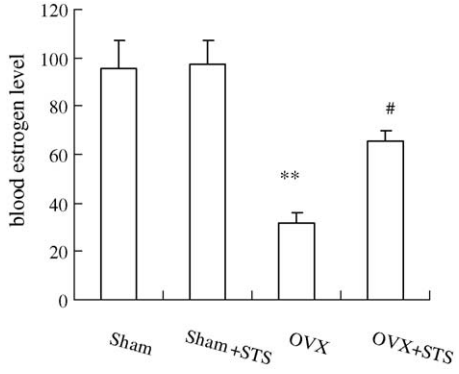

Fig. 1 – Effects of STS on the blood estrogen concentration in ovariectomized rats. The level of estrogen in blood was measured by RIA kit. Values are expressed as the mean ± S.D. $^{**}P < 0.01$ compared with Sham; $^{\#}P < 0.05$ compared with OVX.

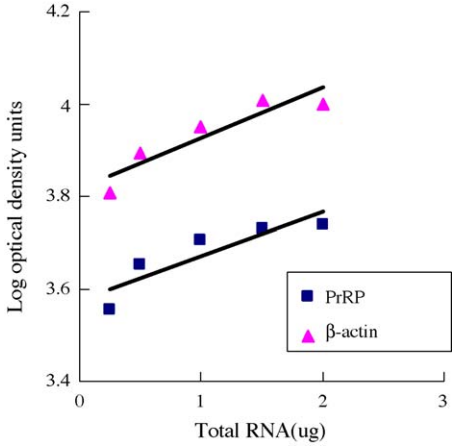

Fig. 2 – RT-PCR analysis showing the amplification of PrRP mRNA and β-actin mRNA with increasing amount of total RNA. The results showed a linear relationship between log DNA product and RNA amount for both PrRP mRNA and β-actin mRNA when total RNA amount was below 1.5 μg.

3.2. Effect of STS on the expression of PrRP mRNA of medulla oblongata in ovariectomized rats

To confirm that RT-PCR yields were not affected by the efficiency of the RT reaction, different amounts of total RNA from the medulla oblongata were reverse transcribed and amplified by PCR. When the amount of total RNA was increased from 0.25 to 2 μg, there was an exponential increase in yields for both PrRP mRNA and β-actin mRNA, indicating that no saturation of the RT reaction has occurred (Fig. 2). Experiments with fixed total RNA and varied PCR cycles were also carried out to ensure that the PCR reaction was within the linear part of the amplification curve. As illustrated in Fig. 3, amplification of cDNA from β-actin and PrRP gene were linear up to approximately 35 cycles. Therefore, amplifications of cDNA from β-actin and PrRP mRNA were carried out using 30 cycles.

RT-PCR analysis showed a decreased expression of PrRP mRNA in the medulla oblongata from ovariectomized rats compared to Sham and Sham + STS rats that was confirmed by densitometry. PrRP mRNA levels in OVX rats were significantly lower than those in Sham rats. STS treatment increased PrRP mRNA levels in OVX rats. However, there were no disparities between Sham and Sham + STS rats (Fig. 4).

3.3. The effects of STS on the number of PrRP-ir neurons in NTS and LRN

Immunostaining of the medulla oblongata sections revealed that PrRP-ir neurons were present exclusively in a small region in the caudal part of the solitary tract nucleus (NTS) and lateral reticular nucleus (LRN). The number of PrRP cells at NTS and

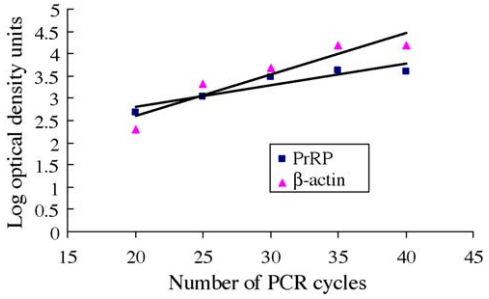

Fig. 3 – RT-PCR analysis showing the amplification of PrRP mRNA and β-actin mRNA with increasing PCR cycles. The results demonstrated linear PCR amplification when the number of cycles was up to 35 for β-actin mRNA and PrRP mRNA.

LRN was calculated. At NTS, The number of PrRP neurons was less in OVX group than in Sham and Sham + STS groups. There were no disparities between Sham and Sham + STS groups. However, STS administration can increase the number of PrRP neurons of OVX compared with OVX group. At LRN, there was no significant difference among the four groups (Fig. 5).

4. Discussion

The interesting finding in the present study is that STS significantly increases the blood E2 level in OVX. The biosynthesis of estrogens from androgens is catalyzed by the key enzyme aromatase. In our previous work, we found the aromatase expression in adipose and liver tissues increased significantly after given the treatment of STS in ovariectomized rats [8]. The effects of STS on the extragonadal aromatization may contribute to promote the blood concentrations of estrogen in the ovariectomized rats. Above all, we hypothesize that the sources of the high E2 levels produced in ovariectomized rats treated with STS may be the adipose and liver tissues that convert androgens mainly from adrenal cortex to estrogens.

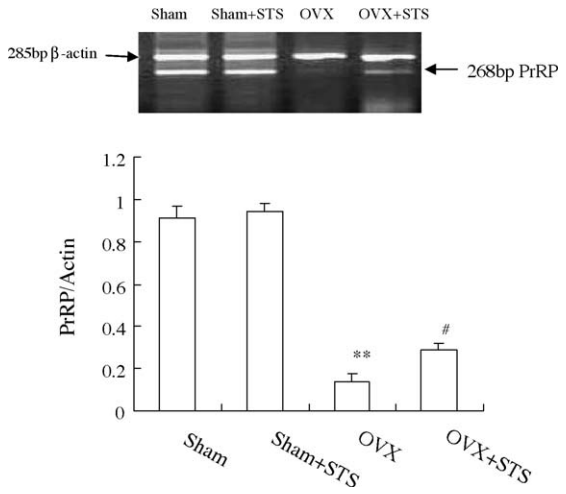

Fig. 4 – Effects of STS on the expression of PrRP mRNA in medulla oblongata from Sham, Sham + STS, OVX and OVX + STS rats. Upper panel: ethidium bromide staining gels of RT-PCR products for PrRP mRNA and β-actin mRNA from medulla oblongata of Sham, Sham + STS, OVX, OVX + STS groups. Arrowheads indicate the lengths of RT-PCR products. Lower panel: relative optical density of PrRP mRNA normalized against β-actin mRNA from medulla oblongata of Sham, Sham + STS, OVX, OVX + STS groups. $^{**}P < 0.01$ compared with Sham; $^{\#}P < 0.05$ compared with OVX.

The significant decrease of PrRP in the medulla oblongata induced by estrogen depletion in OVX rats shown in the current study was consistent with previous studies [12,13]. We showed that the number of PrRP-ir neurons in the NTS of the medulla oblongata of OVX rats was most decreased compared with that of Sham rats. STS administration increased the number of PrRP-ir neurons in the NTS and the expression of PrRP mRNA of medulla oblongata of OVX rats. Because the results obtained by immunohistochemistry can only show the quantity of certain immunoreactive substances in situ, we also detected the release of PrRP. Our unpublished data on the release of PrRP from medial preoptic area (MPOA) by push–pull perfusion suggest that the level of PrRP release in OVX + STS is higher than that in OVX. Therefore, we hypothesize that STS may enhance both the release and synthesis of PrRP in ovariectomized rats.

Our findings raise the question as to how STS increase the expression of PrRP in medulla oblongata of OVX rats. Kataoka found that PrRP gene expression is regulated by gonadal steroid hormones in the medulla oblongata, and parts of PrRP synthesizing neurons are considered to be directly influenced by estrogen in the NTS [12]. The increase of PrRP expression in OVX + STS may be due to the increased level of blood E2 induced by STS. It is reported that administration of estrogen after ovariectomy induced an increase in PrRP mRNA expression both in NTS and LRN. But STS had no effect on the number of PrRP in LRN in the present study. Differences in the results may be attributable to species, treatments and degree of deprived estrogen, as each of these potentially confounding variables may influence the feedback of estrogen on PrRP.

The largest concentration of PrRP peptide is found in the medulla oblongata where it is associated with noradrenergic neurons that project to and activate GnRH neurons in the hypothalamus [17]. Within the hypothalamus PrRP-ir neurons are found in the BST [11], in close proximity to the preoptic area that contains the cell bodies of the GnRH neurons [17]. It is reported that PrRP following intracerebroventricular (ICV) injection stimulates LH and FSH via a hypothalamic mechanism [14]. The results of push–pull perfusion technique also suggest that exogenous PrRP may play a role in mediating the steroid-induced LH surge by activating GnRH neurons in the medial preoptic area (MPOA) [18]. All these above data suggested that PrRP was involved in the regulation of the reproductive endocrine system. STS may regulate the dysfunctional hypothalamic–pituitary–ovarian axis of ovariectomized rats by increasing the expression of PrRP in medulla oblongata.

Taken together with the above results, STS may have a stimulatory effect on the expression of PrRP and the level of blood E2 in ovariectomized rats. The present study evaluated

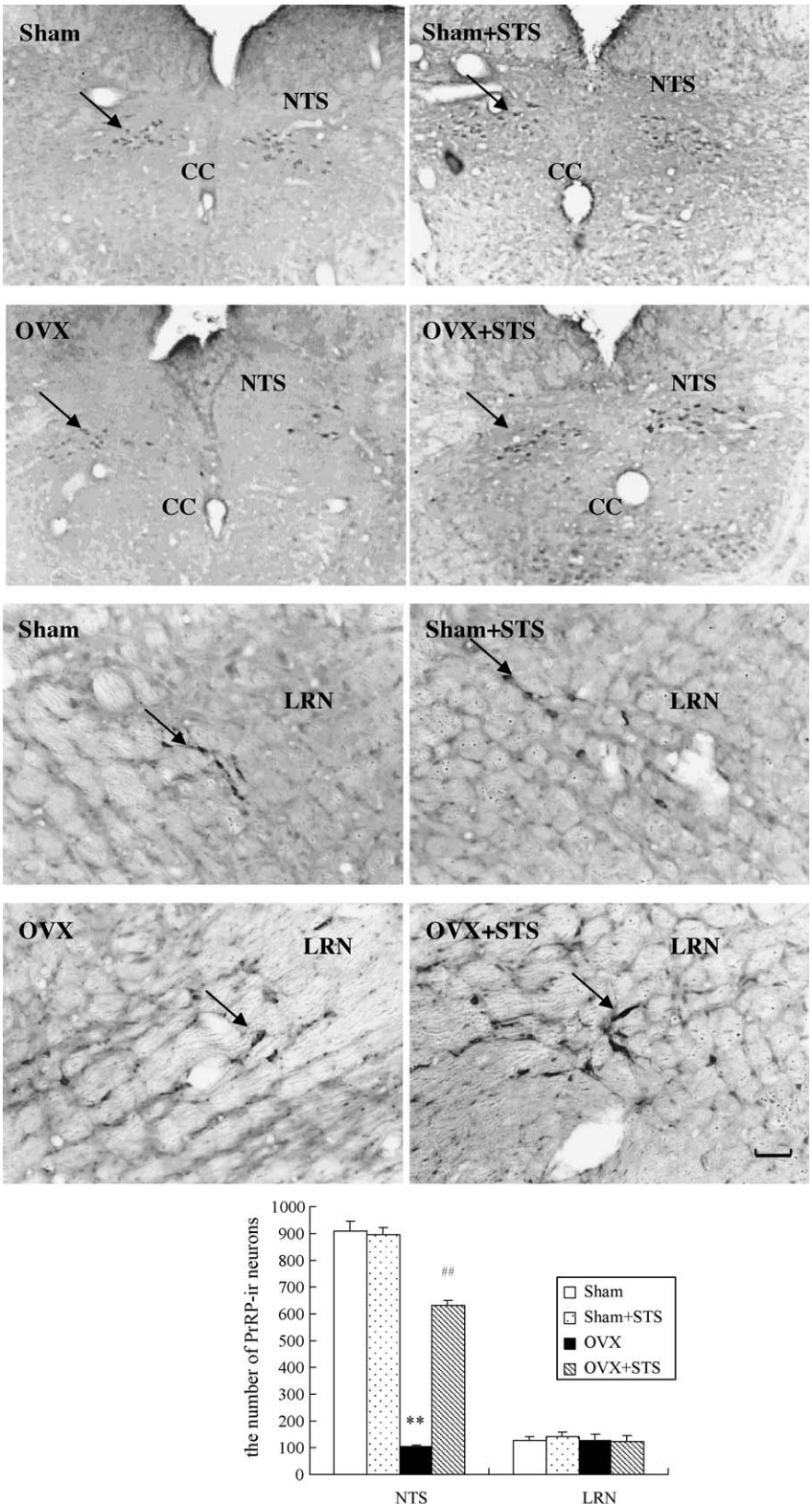

Fig. 5 – Effects of STS on the number of PrRP positive neurons in NTS and LRN of Sham, Sham + STS, OVX and OVX + STS groups. Upper panel: PrRP-ir neurons in NTS and LRN. Positive signals are indicated by arrows. Lower panel: the number of PrRP immunoreactive neurons in NTS and LRN. Comparison among Sham (open bars), Sham + STS (stippled bars), OVX (solid bars) and OVX + STS (streaked bars) groups. The measurements of PrRP-ir positive neurons are described in Section 2. Values are presented as the mean ± S.D. $^{**}P < 0.01$ compared with Sham; $^{\#\#}P < 0.01$ compared with OVX group. NTS, solitary tract nucleus; LRN, lateral reticular nucleus; CC, central canal. Scale bar = 500 μm.

the effect of STS on the expression of PrRP in ovariectomized rats, which is valuable in research for the mechanism of STS on reproductive disorders.

Acknowledgement

This project was financed by the National Natural Science Foundation of China (No. 39870914).

REFERENCES

[1] Genant HK, Baylink DJ, Gallaghter JC. Estrogens in the prevention of osteoporosis in postmenopausal women. Am J Obstet Gynecol 1989;161:1842–6.
[2] Lindsay R, Hart DM, Clark DM. The minimum effective dose of estrogen for prevention of postmenopausal bone loss. Obstet Gynecol 1984;63:759–63.
[3] Mishell DR. Estrogen replacement therapy: an overview. Am J Obstet Gynecol 1989;161:1825–7.
[4] Hahn RG. Compliance considerations with estrogen replacement: withdrawal bleeding and other factors. Am J Obstet Gynecol 1989;161:1854–8.
[5] Gao YG, Tan JX. Pharmacology of tanshinone. Acta Pharmaceutic Sinica 1979;14:75.
[6] Li LM, Zhou Y, Shao XS, Liu YM, Wang R, Wang DY. Ketosalvia miltorrhiza preventing postmenopausal osteoporosis. Chin J Osteo 1996;2(1):78–80.
[7] Kim HH, Kim JH, Kwak HB, Huang H, Han SH, Ha H, et al. Inhibition of osteoclast differentiation and bone resorption by tanshinone IIA isolated from Salvia miltiorrhiza Bunge. Biochem Pharmacol 2004;67(9):1647–56.
[8] Han XB, Ma SL, Wang CX, Chen BY. Tanshinone strengthens the regulative effect of electroacupuncture on the dysfunction of hypothalamic–pituitary–ovarian is in ovariectomized rats. Shanghai J Acupuncture Moxibustion 2004;23(10):36–8.
[9] Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, et al. A prolactin-releasing peptide in the brain. Nature 1998;393:272–6.
[10] Chen C, Dun SL, Dun JH, Chang JK. Prolactin-releasing peptide-immunoreactivity in A1 and A2 noradrenergic neurons of the rat medulla. Brain Res 1999;822:276–9.
[11] Maruyama M, Matsumoto H, Fujiwara K, Kitada C, Hinuma S, Onda H, et al. Immunocytochemical localization of prolactin-releasing peptide in the rat brain. Endocrinology 1999;140:326–2333.
[12] Tokita R, Nakata T, Katsumata H, Konishi S, Onodera H, Imaki J, et al. Prolactin secretion in response to prolactin-releasing peptide and the expression of the prolactin-releasing peptide gene in the medulla oblongata are estrogen dependent in rats. Neurosci Lett 1999;276:103–6.
[13] Watanobe H. In vivo release of prolactin-releasing peptide in rat hypothalamus in association with luteinizing hormone and prolactin surges. Neuroendocrinology 2001;74:359–66.
[14] Seal LJ, Small CJ, Kim MS, Stanley SA, Taheri S, et al. Prolactin releasing peptide (PrRP) stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) via a hypothalamic mechanism in male rats. Endocrinology 2000;141:1909–12.
[15] Zhao H, Tian ZZH, Chen BY. Molecular mechanism of electroacupuncture regulating hypothalamic GnRH system in ovariectomized rats. Shanghai J Acupuncture Moxibustion 2003;22(1):3–6.
[16] Paxinos G, Watson C. The rat brain in stereotaxic coordinates, 4th ed., Academic Press; 1998.
[17] Gibson MJ. Role of the preoptic area in the neuroendocrine regulation of reproduction: an analysis of functional preoptic homografts. Ann NY Acad Sci 1986;474:53–63.
[18] Wright DE, Jennes L. Origin of noradrenergic projections to GnRH Perikarya-containing areas in the medial septum-diagonal band and preoptic area. Brain Res 1993;621:272–8.