Effects of Tokishakuyakusan on hypothalamic pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP type I receptor (PAC1) expression in hypophysectomized and ovariectomized rats

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The traditional Chinese medicine, Tokishakuyakusan (TS; Dang-Gui-Shao-Yao-San in Chinese), is frequently applied in obstetrics and gynecology departments in Japanese hospitals. The novel neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP) that is transiently induced by the gonadotropin surge, plays an important role in the synthesis of estradiol and progesterone in ovarian granulosa cells. Here, we investigated the effect of TS on PACAP in hypophysectomized (HPX) or ovariectomized (OVX) female rats. The weight of the hypothalamus from HPX rats administered with oral TS for one week did not appreciably change. In contrast, TS decreased the weight of the hypothalamus that was increased by ovariectomy. Moreover, TS promoted the expression of PACAP and PACAP type I receptor (PAC1) mRNA and protein in the hypothalamus of HPX and OVX rats. However, bone metabolism that was increased by removing the pituitary or ovaries was not suppressed. These findings suggest that TS promotes the secretion of gonadotropic hormone through the expression of hypothalamic PACAP, PAC1 mRNA and PAC1 protein.

Key words Tokishakuyakusan, PACAP, PAC1, hypothalamus.

Introduction

Tokishakuyakusan (TS; Dang-Gui-Shao-Yao-San in Chinese) is a traditional Chinese medicine that is frequently applied in the obstetrics and gynecology departments of Japanese hospitals as it improves ovarian dysfunction such as luteal insufficiency[1] and amenorrhea, promotes the luteotropic release of estrogen and progesterone in the ovary, and stimulates cAMP accumulation.[2-9] The overall effects of TS reportedly supersede those of the individual herbs.[10]

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from the ovine hypothalamus based on its effect on pituitary adenylate cyclase. It belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP) family, and interacts with known hypothalamic releasing factors to modulate pituitary hormone secretion.[11-15] PACAP functions as a hypophysiotropic factor, regulates the synthesis and secretion of gonadotropin through the hypothalamo-hypophysial portal system[6] and plays an important role in estradiol and progesterone synthesis in ovarian granulosa cells by binding to PAC1 protein and activating intracellular cAMP production.[17-20] Moreover, PACAP and luteinizing hormone-releasing hormone synergistically affect the release of luteinizing hormone and follicle stimulating hormone in static rat pituitary cell culture.[21] Two biologically active C-terminal amidated forms, PACAP-38 and PACAP-27, comprise PACAP[11,13] and exert their actions by binding to cognate receptors; Type I sites (PAC1) that prefer PACAP-38 and PACAP-27 over VIP and Types II and III sites that have almost equally high affinity for PACAP-38, PACAP-27 and VIP.[11]

The effects of TS on hypothalamic PACAP and PAC1 expression in vivo have not yet been clarified. Here, we describe changes in PACAP and PAC1 mRNA, and PAC1 protein expression in the hypothalamus of HPX or OVX female rats after oral TS administration.

Since particular illness restricted to females, such as menopausal disorders, is caused by the collapse of the balance of the hypothalamus-pituitary-ovary system, the HPX or OVX female rat model demonstrated to be useful for clarifying the effects of TS.

Materials and Methods

Animals. Nine-week-old female Wistar rats (Japan SLC Inc., Shizuoka, Japan) were hypophysectomized (HPX), bilaterally ovariectomized (OVX), or sham-operated (Sham) at 8 weeks of age. The rats were maintained in a light, temperature and humidity controlled environment (lights on, 07:00-19:00 h; 22 ± 2°C and 50 ± 5%, respectively) with access to food and water ad libitum. The animals were handled in accordance with the Guide for Animal Experiments of the University of Toyama. Sham rats (control group, n = 4) were given water (10 ml/kg, p.o.) once each day for 7 days and HPX or OVX rats were divided into 3 groups of 3 animals each and administered with drugs once per day for

7 days as follows (1) water (10 ml/kg p.o.); (2) TS dissolved in water (1000 mg/kg p.o.) and (3) 17β-estradiol dissolved in 0.9% sodium chloride containing 1% Tween 20 (10 μg/kg, s.c.).

On day 7, urine samples were collected from metabolic cages to measure the bone resorption maker deoxypyridinoline (DPD).[22]

Reagents and Drugs. Six medicinal plants (Uchida Wakanyaku Co., Ltd., Tokyo, Japan) comprise TS (Table 1). A mixture of these plants TS (72 g) was extracted with 1000 ml of distilled-water at 100°C for 1 h. The decoction was filtered, concentrated under reduced pressure, and lyophilized. The powdered extract was dissolved in water immediately before oral administration. We purchased 17β-estradiol (Sigma-Aldrich Japan Co., Tokyo, Japan) and ISOGEN (Nippon Gene, Toyama, Japan), and the PCR primers were synthesized at Nippon EGT Co., Ltd. (Toyama, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). After 8 days, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), decapitated and then hypothalami were excised, immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from hypothalami using ISOGEN and dissolved in DEPC-water. The RNA concentration was measured by spectrophotometry at 260 nm, and quality was verified using a water-length scan. First standard cDNA was synthesized using 200 units of Super Script™III reverse trancriptase (Invitrogen) from 2.0 μg of total RNA and 5.0 μM oligo (dT) primer in a 20 μl mixture. Polymerase chain reactions proceeded in 10 μl volumes containing 1.0 μl first standard cDNA, 0.5 μM of sense and antisense primers (Table 2), 250 μM dNTPs and 2 units of Taq DNA polymerase (Promega, Madison, WI, USA) in reaction buffer containing 2.5 mM MgCl$_2$. Reactions proceeded in a thermal cycler (Astec, Fukuoka, Japan). The PCR products were resolved by electrophoresis on 6% polyacrylamide gels, stained with ethidium bromide, and quantified using ATTO densitograph 4.0 software.[23,24]

Immunohistochemistry. In another set of experiments, rats were perfused transcardially with 0.9% saline and 4% paraformaldehyde, then the brains were cryoprotected in 30% sucrose in 4% paraformaldehyde and frozen sections (10 μm) were cut using a microtome (Leica, Wetzlar, Germany). The sections were dipped in 0.3% hydrogen peroxide/phosphate buffer (PB) for 15 min, rinsed in PB three times, incubated in blocking solution (3% goat serum and 0.3% TritonX-100/PB) for 1 h at room temperature, and then incubated overnight at 4°C with a polyclonal antibody against PAC$_1$ (1:200, Gene Tex, Inc., TX, USA). On the following day, the sections were rinsed in PB three times, antigen was visualized after a 2 h incubation at room temperature using secondary goat anti-rabbit IgG (Alexa Fluor 594, Molecular Probes, OR, USA) and then the sections were rinsed three times in PB. Stained sections were mounted, stained with 4',6-diamidino-2-phenylindole (DAPI) and coverslipped with Vectashield (Vector Laboratories, CA, USA). Double immunolabelling was detected in the anterior periventricular nucleus of the hypothalamus using a fluorescence microscope (AX-70; Olympus, Tokyo, Japan).

Statistics. All values are represented as means ± S.E.M. Significant differences between Sham and HPX/OVX water-treated groups were analyzed by Student's *t*-test. Statistical significance between the HPX/OVX water-treated group, and the TS and 17β-estradiol-treated groups were evaluated by one-way ANOVA, followed by Dunnett's test (Prism; GraphPad, San Diego, CA, USA).

Results

Effects of TS on hypothalamus weight of HPX and OVX rats. One week after hypophysectomy, the ratio (%) of the weight of the hypothalamus to the body weight of the HPX group was significantly decreased compared with the sham group and the administration of TS and E2 did not change this ratio (Fig. 1A). One week after ovariectomy, this ratio was significantly increased compared with the sham group (Fig. 1B). The administration of both TS and E2 significantly decreased this ratio compared with the sham group (Fig. 2B). However, the appreciable effects of TS on hypothalamus weight of normal rats were not observed (data not shown).

Effects of TS on PACAP and PAC$_1$ mRNA expression in the hypothalamus of HPX rats. The expression of PACAP mRNA was slightly reduced one week after HPX group compared with the sham group (Fig. 2A). However,

Table 1. Component of Tokishakuyakusan

Herbal Name	Amount (g)	Lot Number
Angelicae Radix	9.0	271003
Paeoniae Radix	18.0	011203
Atractylodis Rhizoma	12.0	031203
Alismatis Rhizoma	12.0	101103
Hoelen	12.0	101003
Cnidii Rhizoma	9.0	181103

These 6 herbs were mixed and extracted with 1000 ml distillated-water at 100°C. The decoction was freeze-dried to obtain an extract in powder form.

Table 2. Details of primers employed and expected size of the PCR amplified cDNA

Molecule	Sense primer(5'-3')	Anti-sense primer(5'-3')	Product Size (bp)	Accession No.
PACAP	CTTTGCCCGCCGTCCTATTTA	GTGAAGATGCCGTCCGAGTGG	453	NM_001009776
PAC$_1$	TTTCATCGGCATCATCATCATCCTT	CCTTCCAGCTCCTCCATTTCCTCTT	374	NM_007407
β-actin	AACGGTCTCACGTCAGTGTA	GTGACAGCATTGCTTCTGTG	222	V01217

A

(10⁻³ %)
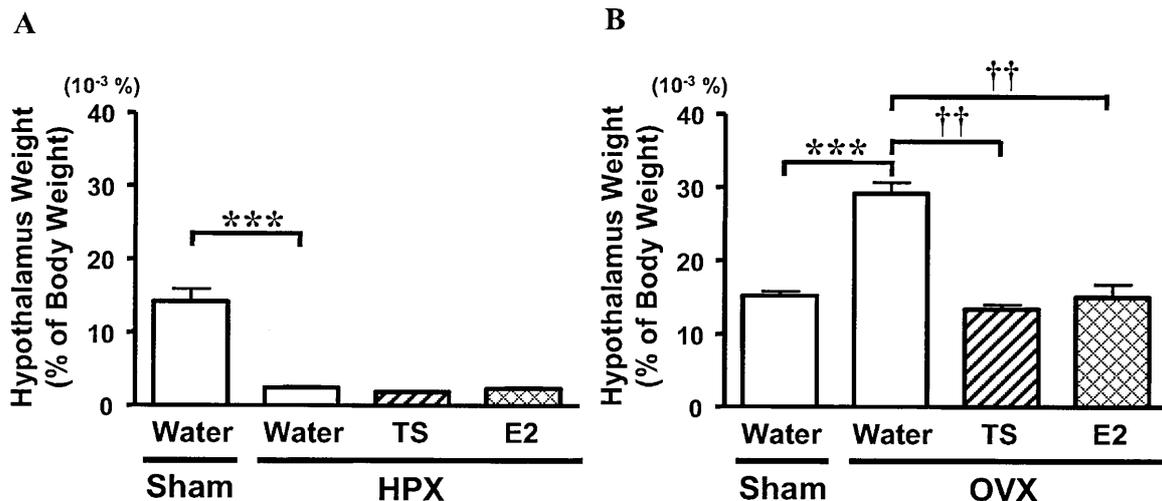

B

(10⁻³ %)

Fig. 1. Effects of TS on weight of hypothalamus from HPX and OVX rats.
    Sham, sham-operated rats; TS, Tokishakuyakusan; E2, 17β-estradiol. Statistical significance was examined by Student's t-test: ***, $p < 0.001$ compared with sham; one-way ANOVA followed by Dunnett's test: ††, $p < 0.01$ compared with HPX rats treated with water.
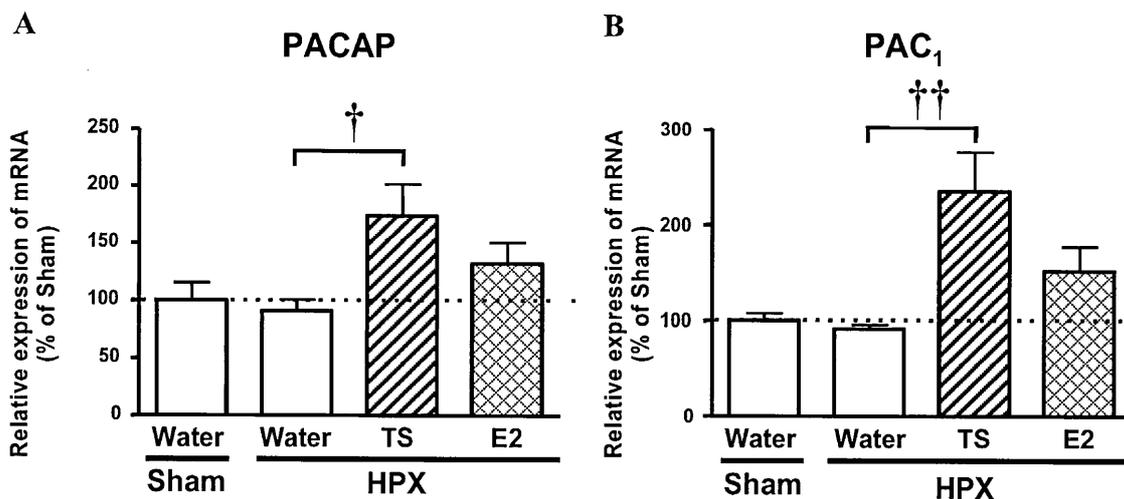
A

PACAP

B

PAC₁

Fig. 2. Effects of TS on PACAP and PAC₁ mRNA expression in hypothalamus of HPX rats.
    Sham, sham-operated rats; TS, Tokishakuyakusan; E2, 17β-estradiol. Statistical significance was examined by one-way ANOVA followed by Dunnett's test: †, $p < 0.05$; ††, $p < 0.01$ compared with HPX rats treated with water.

TS significantly increased PACAP mRNA expression when compared with the HPX group that was given water. The administration of E2 also increased PACAP mRNA expression, but not significantly. In addition, 1 week after hypophysectomy, PAC₁ mRNA expression did not change compared with the sham group (Fig. 2B). However, the administration of TS obviously increased PAC₁ mRNA expression compared with the HPX group given water, and that of E2 tended to increase PAC₁ mRNA expression, but not significantly.

Effects of TS on PACAP and PAC₁ mRNA expression in the hypothalamus of OVX rats. One week after ovariectomy, PACAP mRNA expression was significantly reduced for the OVX group given water compared with the

sham group (Fig. 3A). However, TS administration significantly recovered PACAP mRNA expression compared with the OVX group given water. The administration of E2 also increased PACAP mRNA expression, but not significantly. Additionally, 1 week after ovariectomy, PAC₁ mRNA expression in the OVX group given water was appreciably reduced compared with that in the sham group (Fig. 3B). However, TS administration significantly recovered PAC₁ mRNA expression compared with the OVX group given water, and E2 administration hardly affected PAC₁ mRNA expression.

Effect of TS on PAC₁ protein expression in the anterior periventricular nucleus of HPX rat hypothalami. One week after hypophysectomy, PAC₁ protein expression
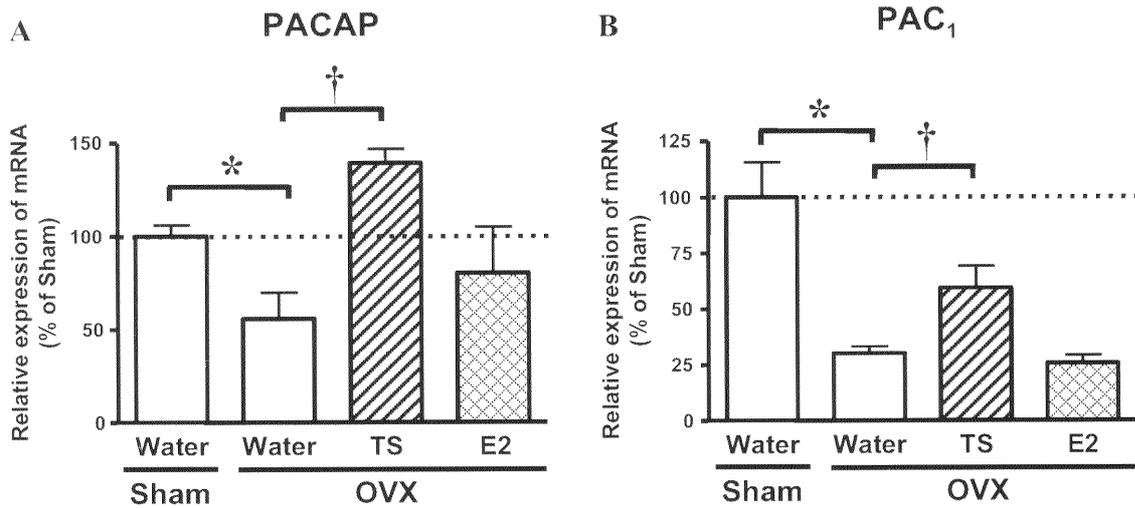
Fig. 3. Effects of TS on PACAP and PAC₁ mRNA expression in hypothalamus of OVX rats.
Sham, sham-operated rats; TS, Tokishakuyakusan; E2, 17β-estradiol. Statistical significance was examined by Student's t-test: *, $p < 0.05$ compared with sham; one-way ANOVA followed by Dunnett's test: †, $p < 0.05$ compared with HPX rats given water.
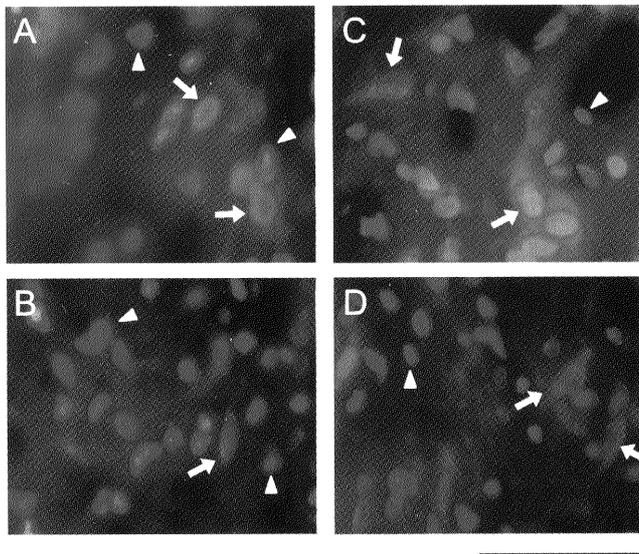

Fig. 4. Effect of TS on PAC₁ protein expression in hypothalamus of HPX rats.
Double-label immunofluorescence expression of PAC₁ protein (Red, arrows) and nucleus (Blue, arrowheads) in hypothalamus sections from sham-operated (A), HPX water- (B), HPX TS- (C) and HPX E2- (D) treated rats. Scale bar, 50μm.
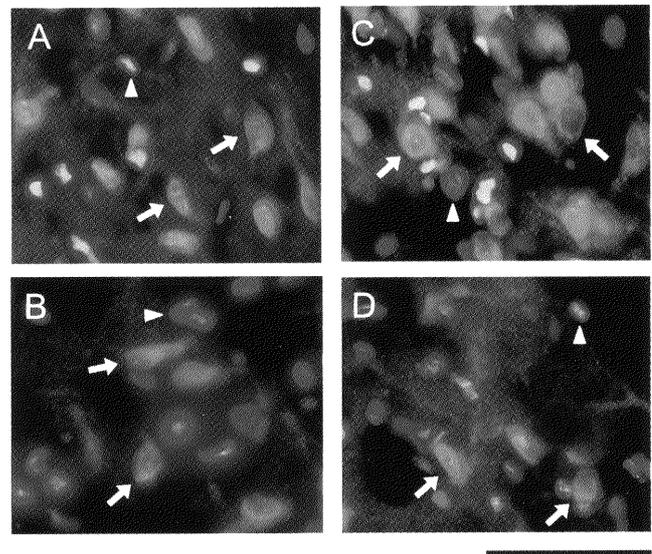
Fig. 5. Effect of TS on PAC₁ protein expression in hypothalamus of OVX rats.
Double-label immunofluorescence expression of PAC₁ protein (Red, arrows) and nucleus (Blue, arrowheads) in hypothalamus sections from sham-operated (A), OVX water- (B), OVX TS- (C), and OVX E2- (D) treated rats. Scale bar, 50μm.

in the anterior periventricular nucleus (PVa) of the hypothalamus was decreased in the HPX group compared with the sham group (Fig. 4A and B). However, TS administration significantly recovered PAC₁ protein expression in the PVa compared with that of the HPX group given water (Fig. 4B and C). The administration of E2 also recovered PAC₁ protein expression in the PVa, but to a lesser extent than TS (Fig. 4C and D).

Effect of TS on PAC₁ protein expression in the anterior periventricular nucleus of OVX rat hypothalami. One week after ovariectomy, PAC₁ protein expression in

the PVa of the OVX group was decreased compared with the sham group (Fig. 5A and B). The administration of TS significantly recovered PAC₁ protein expression in the PVa of the OVX group compared with the OVX group given water (Fig. 5B and C). The administration of E2 did not recover PAC₁ protein expression in the PVa and the expression level was lower than that after TS administration (Fig. 5C and D).

Effects of TS on deoxypyridinoline (DPD) in HPX and OVX rat urine. One week after hypophysectomy, urinary DPD was obviously increased (Fig. 6A) and neither TS nor
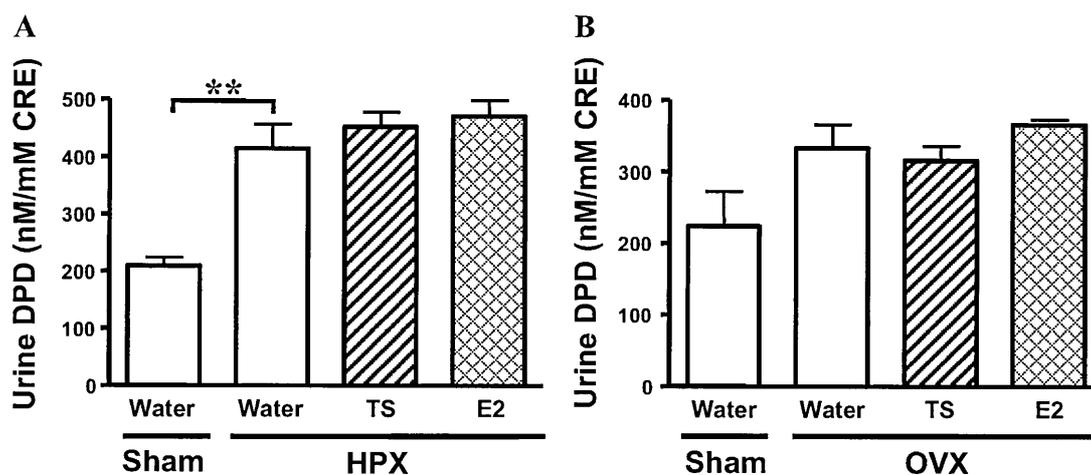
Fig. 6. Effects of TS on deoxypyridinoline (DPD) in urine of HPX and OVX rats.
Sham, sham- rats; TS, Tokishakuyakusan; E2, 17β-estradiol. CRE, creatinine. Statistical significance was examined by Student's *t*-test: ** $p<0.01$ compared with sham operated rats.

E2 affected the level (Fig. 6A). One week after ovariectomy, urinary DPD in the OVX group was not significantly changed (Fig. 6B) and likewise, neither TS nor E2 appreciably affected the level.

Discussion

We investigated the effects of TS on pituitary adenylate cyclase-activating polypeptide (PACAP) using hypophysectomized (HPX) or ovariectomized (OVX) female rats. We initially examined the effect of TS on the expression of hypothalamic PACAP and PACAP type I receptor (PAC_1) in the HPX or OVX rats. Shuto *et al.* measured hypothalamic PACAP mRNA levels 1 or 2 after HPX in male rats.[25] They found that one week after surgery, the hypothalamic PACAP mRNA level had decreased 70.3 ± 10.5% compared with controls. Our results were similar in that one week after HPX, the PACAP mRNA level decreased (Fig. 2A). The similar findings of the two studies suggest that the animal models are valid, but discrepancies associated with gender differences cannot be ruled out. After HPX in male rats, a combination of injected growth hormone, prolactin, thyroxine, corticosterone and testosterone as replacement therapy, recovered less PACAP mRNA compared with the relative recovery in the hypothalamus.[24] However, the administration of TS, but not of E2, increased the levels of hypothalamic PACAP mRNA in female HPX rats (Fig. 2A), suggesting that TS potently affects PACAP expression and might have similar effects to that of therapy with a hormone combination.

By binding to PAC_1 and activating intracellular cAMP production, PACAP plays an important role in the synthesis of estradiol and progesterone in ovarian granulosa cells.[17-19] Furthermore, TS has a luteotropic effect that includes the release of estrogen and progesterone in the ovary and stimulates cAMP accumulation.[2-8] We found here that TS administration to HPX rats increased the level of PAC_1

mRNA in the hypothalamus (Fig. 2B). Therefore, TS might stimulate the release of gonadal hormones via hypothalamic PACAP and PAC_1 stimulation. However, since TS directly affects progesterone secretion via the corpus luteum *in vitro*,[3,4,8,26] expression levels of PACAP and PAC_1 mRNA in the ovaries of HPX rats should be measured.

Progesterone either alone or in combination with estradiol recovers hypothalamic PACAP and PAC_1 mRNA levels in OVX rats [27] whereas 17β-estradiol does not.[26] We found that E2 did not alter the expression of PACAP and PAC_1 mRNA in the OVX group compared with that of the OVX given only water (Fig. 3A and B). However, TS administration increased the expression of hypothalamic PACAP and PAC_1 mRNA in the OVX rats. These data indicated that TS had a similar effect to progesterone combined with estradiol on hypothalamic PACAP and PAC_1 mRNA expression.

Immunohistochemical studies have shown that PACAP-immunoreactive fibers are widely distributed in the hypothalamus,[28] with cell bodies being localized to the paraventricular nuclei.[28-30] PACAP appears to be secreted from the median eminence into hypophyseal portal blood as levels are higher in rat portal blood than in peripheral plasma.[31] PACAP receptors are present in the anterior pituitary[32] and in the hypothalamus, particularly in the arcuate nucleus, anteroventral periventricular nucleus, median preoptic nucleus, suprachiasmatic preoptic nucleus, anterior periventricular nucleus (PVa), intermediate periventricular nucleus, posterior periventricular nucleus and suprachiasmatic nucleus.[33] The administration of TS, but not of E2, recovered the expression of PACAP and PAC_1 mRNA in the hypothalamus and of PAC_1 protein in PVa from HPX and OVX rats (Figs. 2, 3, 4 and 5), indicating that TS evokes PACAP secretion into hypophyseal portal blood. Furthermore, recent studies of the role of PACAP in reproductive physiology support the notion that PACAP functions in the timing of reproduction[34-41] and a Japanese clinical study has shown

that treating women with TS improves the likelihood of becoming pregnant.[42] Thus, TS might evoke the secretion of gonadotropic hormone via stimulated hypothalamic PACAP and PAC_1.

Ovariectomy-induced bone loss in rats and postmenopausal bone loss in women share many similar characteristics, and the skeletal response to therapy with E2 is similar.[43,44] However, levels of the urinary bone resorption marker, DPD, in E2 treated OVX rats did not decrease compared with those of sham operated rats (Fig. 6B). This finding indicated that the administered dose of E2 (10 μg/kg body weight) was low. Gaumet et al., reported that administering this dose to OVX rats does not decrease the urinary DPD level compared with that of sham operated rats.[45] A more recent report has suggested that follicle stimulating hormone (FSH; hypogonadal hormone), and not E2, directly regulates osteoclastic bone resorption and bone mass.[46] Hypophysectomy induced a significant increase in urine DPD, and TS did not decrease this value (Fig. 6A), indicating that TS does not have an FSH-like effect.

In summary, we demonstrated the effects of TS on hypothalamic PACAP and PAC_1 mRNA expression and PAC_1 protein expression in HPX and OVX rats. The secretion of gonadotropic hormone might be promoted by TS through hypothalamic expression of PACAP and PAC_1 mRNA and PAC_1 protein.

Acknowledgments

References

1) Usuki, S., Nakauchi, T., Higa, S., Someya, K.: The improvement of luteal insufficiency in fecund women by tokishakuyakusan treatment. Am. J. Chin. Med., 30, 327-338, 2002.

2) Usuki, S.: Effects of hachimijiogan, tokishakuyakusan and keishibukuryogan on estrogen and progesterone secretions by rat preovulatory follicles. Am. J. Chin. Med., 14, 161-170, 1986.

3) Usuki, S.: Effects of hachimijiogan, tokishakuyakusan and keishibukuryogan on progesterone secretions by corpus luteum. Am. J. Chin. Med., 15, 109-115, 1987.

4) Usuki, S.: Effects of herbal components of tokishakuyakusan on progesterone secretion by corpus luteum in vitro. Am. J. Chin. Med., 19, 57-60, 1991.

5) Usuki, S.: Effects of hachimijiogan, tokishakuyakusan, keishibukuryogan, ninjinto and unkeito on estrogen and progesterone secretion in preovulatory follicles incubated in vitro. Am. J. Chin. Med., 19, 65-71, 1991.

6) Usuki, S.: Tokishakuyakusan stimulates progesterone and estradiol-17 beta production by rat granulosa cells and progesterone, testosterone and estradiol-17 beta by the residual portion of the follicle in vitro. Am. J. Chin. Med., 19, 155-161, 1991.

7) Usuki, S.: The direct luteotropic effect of tokishakuyakusan in rats. Am. J. Chin. Med., 19, 163-169, 1991.

8) Usuki, S.: Tokishakuyakusan stimulates cyclic adenosine 3',5'-monophosphate accumulation and progestin production by corpora lutea. Am. J. Chin. Med., 16, 21-28, 1988.

9) Hagino, N.: Kampo medicine and neuroendocrine -From here to molecular biology. J. Trad. Med., 13, 105-117, 1996.

10) Usuki, S.: Blended effects of herbal components of tokishakuyakusan on rat corpus luteum function in vivo. Am. J. Chin. Med., 16, 107-116, 1988.

11) Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H.: Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem. Biophys. Res. Commun., 164, 567-574, 1989.

12) Arimura, A., Shioda, S.: Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. Front. Neuroendocrinol., 16, 53-88, 1995.

13) Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R.R., Stibbs, H.H., Arimura, A., Fujino, M.: A novel peptide which stimulates adenylate cyclase: molecular cloning and characterization of the ovine and human cDNAs. Biochem. Biophys. Res. Commun., 166, 81-89, 1990.

14) Christophe, J.: Type I receptors for PACAP (a neuropeptide even more important than VIP?). Biochem. Biophys. Acta., 1154, 183-199, 1993.

15) Arimura, A.: Receptors for pituitary adenylate cyclase-activating polypeptide Comparison with vasoactive intestinal peptide receptors. Trends. Endocrinol. Metab., 3, 288-294, 1992.

16) Rawlings, S.R., Hezareh, M.: Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. Endocr. Rev., 17, 4-29, 1996.

17) Gras, S., Hannibal, J., Fahrenkrug, J.: Pituitary adenylate cyclase-activating polypeptide is an auto/paracrine stimulator of acute progesterone accumulation and subsequent luteinization in cultured periovulatory granulosa/lutein cells. Endocrinology, 140, 2199-2205, 1999.

18) Heindel, J.J., Sneeden, J., Powell, C.J., Davis, B., Culler, B.D.: A novel hypothalamic peptide, pituitary adenylate cyclase-activating peptide, regulates the function of rat granulosa cells in vitro. Biol. Reprod., 54, 523-530, 1996.

19) Kotani, E., Usuki, S., Kubo, T.: Effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on progestin biosynthesis in cultured granulosa cells from rat ovary and expression of mRNA encoding PACAP type IA receptor. J. Reprod. Fertil., 112, 107-114, 1998.

20) Usuki, S., Kontani, E.: Effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on progestin biosynthesis in cultured luteal cells from rat ovary. Gynecol. Endocrinol., 15, 184-191, 2001.

21) Culler, M.D., Paschall, C.S.: Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone-releasing hormone. Endocrinology, 129, 2260-2262, 1991.

22) Ahn, E.-M., Nakamura, N., Akao, T., Nishihara, T., Hattori, M.: Estrogenic and antiestrogenic activities of the roots of Moghania philippinensis and their constituents. Biol. Pharm. Bull., 27, 548-553, 2004.

23) Tohda, M., Suwanakitch, P., Jeenapongsa, R., Hayashi, H., Watanabe, H., Matsumoto, K.: Expression changes of the mRNA of Alzheimer's disease related factors in the permanent ischemic rat brain. Biol. Pharm. Bull., 27, 2021-2023, 2004.

24) Hayashi, H., Tohda, M., Watanabe, H., Murakami, Y., Matsumoto, K.: The effects of Choto-san on the mRNA expression of Alzheimer's disease related factors in the permanent ischemic rat brain. Biol. Pharm. Bull., 28, 744-746, 2005.

25) Shuto, Y., Somogyvari-Vigh, A., Onda, H., Arimura, A.: Effect of hypophysectomy on pituitary adenylate cyclase activating polypeptide gene expression in the rat hypothalamus. Peptides, 16, 407-413, 1995.

26) Usuki, S.: Effects of Tokishakuyakusan and its components on rat corpus luteum function in vitro. Am. J. Chin. Med., 16, 11-19, 1988.

27) Ha, C.M., Kang, J.H., Choi, E.J., Kim, M.S., Park, J-W., Kim, Y., Choi, W.S., Chun, S.Y., Kwon, H.B., Lee, B.J.: Progesterone increases mRNA levels of pituitary adenylate cyclase-activating

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polypeptide (PACAP) and type I PACAP receptor (PAC$_1$) in the rat hypothalamus. *Mol. Brain Res.,* **78**, 59-68, 2000.

28) Koves, K., Arimura, A., Somogyvari-Vigh, A., Vigh, A., Miller, J.: Immunohistochemical demonstration of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, in the ovine hypothalamus. *Endocrinology,* **127**, 264-271, 1990.

29) Vigh, S., Arimura, A., Koves, K., Somogyvari-Vigh, A., Vigh, A., Sitton, J., Fermin, C.D.: Immunohistochemical localization of the neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), in human and primate hypothalamus. *Peptides,* **12**, 313-318, 1991.

30) Tamada, Y., Tanaka, M., Ichitani Y., Okamura, H., Yanaihara, N., Ibata, Y.: Pituitary adenylate cyclase-activating polypeptide (PACAP)-like immunoreactive neuronal elements in rat hypothalamus and median eminence with special reference to morphological background of its effect on anterior pituitary − light and electron microscopic immunocytochemistry. *Neurosci. Lett.,* **180**, 105-108, 1994.

31) Dow, R.C., Bennie, J., Fink, G.: Pituitary adenylate cyclase-activating peptide-38 (PACAP)-38 is released into hypophysial portal blood in the normal male and female rat. *J. Endocrinol.,* **142**, Rl-R4, 1994.

32) Gottschall, P.E., Tatsuno, I., Miyata, A., Arimura, A.: Characterization and distribution of binding sites for hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide. *Endocrinology,* **127**, 272-277, 1990.

33) Joo, K.M., Chung, Y.H., Kim, M.K., Nam, R.H., Lee, B.L., Lee, K.H., Cha, C.I.: Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptors (VPAC1, VPAC2, and PAC$_1$ receptor) in the rat brain. *J. Comp. Neurol.,* **476**, 388-413, 2004.

34) Ko, C., In, Y.-H., Park-Sarge, O.K.: Role of progesterone receptor activation in pituitary adenylate activating polypeptide gene expression in rat ovary. *Endocrinology,* **140**, 5185-5194, 1999.

35) Ko, C., Park-Sarge, O.K.: Progesterone receptor activation mediates LH-induced type- I pituitary adenylate cyclase activating polypeptide receptor (PAC(1)) gene expression in rat granulosa cells. *Biochem. Biophys. Res. Commun.,* **277**, 270-279, 2000.

36) Gras, S., Hedetoft, C., Pedersen, S.H., Fahrenkrug, J.: Pituitary adenylate cyclase activating peptide stimulates acute progesterone production in rat granulose/lutein cells via two receptor subtypes. *Biol. Reprod.,* **63**, 206-212, 2000.

37) Culler, M.D., Paschall, C.S.: Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone-releasing hormone. *Endocrinology,* **129**, 2260-2262, 1991.

38) Ortmann, O., Asmus, W., Diedrich, K., Schulz, K.-D., Emons, G.: Interactions of ovarian steroids with pituitary adenylate cyclase-activating polypeptide and GnRH in anterior pituitary cells. *Eur. J. Endocrinol.,* **140**, 207-214, 1999.

39) Szabo, E., Nemeskeri, A., Heinzlmann, A., Suzuki, N., Arimura, A., Koves, K.: Cell immunoblot assay study demonstrating the release of PACAP from individual anterior pituitary cells of rats and the effect of PACAP on LH release. *Regul. Pept.,* **109**, 75-81, 2002.

40) Koves, K., Kantor, O., Molnar, J., Heinzlmann, A., Szabo, E., Szabo, F., Nemeskeri, A., Horvath, J., Arimura, A..: The role of PACAP in gonadotropic hormone secretion at hypothalamic and pituitary levels. *J. Mol. Neurosci.,* **20**, 141-152, 2003.

41) Apostolakis, E.M., Lanz, R., O'Malley, B.W.: Pituitary adenylate cyclase-activating peptide: A pivotal modulator of steroid-induced reproductive behavior in female rodents. *Mol. Endocrinol.,* **18**, 173-183, 2004.

42) Murata T.: [Medical treatment of the irregular period by Kampo.], *Sanpuchiryou,* **45**, 330-336, 1982. Japanese.

43) Kalu, D.N.: The ovariectomized rat model of postmenopausal bone loss. *Bone Miner.,* **15**, 175-192, 1991.

44) Kalu, D.N., Liu, C.C., Salemo, E., Hollin, B., Echon, R., Ray, M.: Skeletal response of ovariectomized rats to low and high doses of 17 β-estradiol. *Bone Miner.,* **14**, 175-187, 1991.

45) Gaumet, N., Seibel, M.J., Braillon, P., Giry, J., Lebecaue, P., Davicco, M.J., Coxam, V., Rouffet, J., Delmas, P.D., Barlet, J.P.: Influence of ovariectomy on bone metabolism in very old rats. *Calcif. Tissue. Int.,* **58**, 256-262, 1996.

46) Sun, L., Peng, Y., Sharrow, A.C., Iqbal, J., Zhang, A., Papachristou, D.J., Zaidi, S., Zhu, L.-L., Yaroslavskiy, B.B., Zhou, H., Zallone, A., Sairam, M.R., Kumar, T.R., Bo, W., Braun, J., Cardoso-Landa, L., Schaffler, M.B., Moonga, B.S., Blair, H.C., Zaidi, M.: FSH directly regulates bone mass. *Cell,* **125**, 247-260, 2006.

Japanese abstract

当帰芍薬散は古くから婦人科領域で使われている代表漢方方剤の１つである。また，下垂体アデニル酸シクラーゼ活性化ポリペプチド（PACAP）は，視床下部において性腺刺激ホルモンの分泌促進，卵巣の顆粒膜細胞において女性ホルモン（エストラジオールとプロゲステロン）の生合成に重要な役割を持つことが知られている。しかしながら，当帰芍薬散の PACAP に対する影響は明らかにされていない。そこで我々は，下垂体摘出ラットと卵巣摘出ラットを用い，当帰芍薬散経口投与後の視床下部の解析を行った。両モデルラットにおいて，当帰芍薬散は PACAP の mRNA 発現を促進した。また PACAP 受容体（PAC$_1$）の mRNA，タンパク発現も促進した。しかしながら下垂体や卵巣の摘出により上昇した骨代謝を抑えることは出来なかった。以上の結果より，当帰芍薬散は視床下部における PACAP，PAC$_1$ を経由して性腺刺激ホルモンの分泌促進を行っている可能性が示唆された。

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