J Pharmacol Sci **93**, 80 – 86 (2003)

Journal of Pharmacological Sciences

Full Paper

Effects of 17β-Estradiol and the Japanese Herbal Medicine *Keishi-bukuryo-gan* on the Release and Synthesis of Calcitonin Gene-Related Peptide in Ovariectomized Rats

Masamichi Noguchi*, Yasushi Ikarashi, Mitsutoshi Yuzurihara, Yoshio Kase, Shuichi Takeda, and Masaki Aburada

Tsumura Research Institute Medical Evaluation Laboratory, 3586 Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki 300-1192, Japan

Received April 17, 2003; Accepted July 7, 2003

Abstract. The purpose of this study is to clarify the effects of 17β-estradiol and the Japanese herbal medicine *Keishi-bukuryo-gan* on the release and synthesis of calcitonin gene-related peptide (CGRP) in ovariectomized (OVX) rats. The effect of ovariectomy on the release or synthesis was evaluated by measuring CGRP concentration in plasma after capsaicin (1.0 mg /kg, i.p.) injection or by measuring CGRP concentration and its mRNA expression in dorsal root ganglia in OVX rats. Ovariectomy attenuated the capsaicin-evoked increase in plasma concentration of CGRP, which was restored by treatment with 17β-estradiol (0.010 mg/kg, s.c.) or *Keishi-bukuryo-gan* (1,000 mg/kg, p.o.) for 7 days after ovariectomy. However, no significant differences were observed in the CGRP concentration and the mRNA expression of dorsal root ganglia by treating the rats with ovariectomy, 17β-estradiol, and *Keishi-bukuryo-gan*. These results suggest not only that estrogen deficiency attenuates CGRP release but also that 17β-estradiol or *Keishi-bukuryo-gan* normalizes the attenuated release process.

Keywords: calcitonin gene-related peptide, 17β-estradiol, *Keishi-bukuryo-gan*, ovariectomized rat, hot flash

Introduction

Hot flashes, which are characterized by transient episode of flushing, sweating and a sensation of heat, occur because of estrogen deficiency in menopausal women (1). Although many neuroendocrine substances, such as gonadotropin releasing hormone, noradrenaline, and opioid peptides, have been suggested as possible triggers for the occurrence of hot flashes (1, 2), the underlying mechanism has not been clarified. Recently, the plasma level of calcitonin gene-related peptide (CGRP), which is a potent vasodilator neuropeptide (3 – 5), was reported to increase rapidly during hot flashes in menopausal women (6 – 8), suggesting that CGRP is closely related to the occurrence of menopausal hot flashes. Furthermore, we previously demonstrated that ovariectomy not only potentiated CGRP-induced elevation of skin temperature and arterial vasorelaxation but also induced a lower concentration of endogenous CGRP in plasma and subsequent up-regulation of arterial CGRP receptors (9). From these results, we suggested that the low concentration of plasma CGRP due to ovarian hormone deficiency may induce the increase in the number of CGRP receptors due to up-regulation to maintain vascular adaptation and consequently amplify the stimulatory effects of CGRP to elevate skin temperature. Estrogen replacement and *Keishi-bukuryo-gan*, which is a Japanese herbal medicine that has been approved by the Ministry of Health, Labor, and Welfare of Japan for the remedy of menopausal symptoms, restored a series of CGRP-related responses observed in ovariectomized (OVX) rats by normalizing the plasma CGRP level (10). However, the effects of both medicines on circulating CGRP level have not been fully investigated yet. Endogenous CGRP is synthesized primarily in dorsal root ganglia, and it is transported to the perivascular CGRPergic nerve

*Corresponding author. FAX: +81-298-89-2158
E-mail: noguchi_masamichi@mail.tsumura.co.jp

endings and released into the circulation (11, 12). In addition, the CGRP synthesized in dorsal root ganglia is centrally transported to the spinal cord (11, 13). Recently, Tanaka et al. (14) suggested that antidromic release of CGRP into the peripheral blood is also induced by stimulation of the spinal cord. Therefore, we assume that the restorative effects of the plasma CGRP by estrogen and *Keishi-bukuryo-gan* might involve the synthesis and/or release processes of CGRP.

To clarify our hypothesis in OVX rats, the effects of estrogen and *Keishi-bukuryo-gan* on the synthesis of CGRP were examined by measuring CGRP concentrations in dorsal root ganglia and the spinal cord and, in particular, the CGRP mRNA level in the dorsal root ganglia, which is a prominent site of CGRP synthesis. The CGRPergic nerves have been shown to be sensory nerves that are sensitive to capsaicin (13, 15). Therefore, the effects of both medicines on the CGRP release process were examined by measuring changes in plasma CGRP levels evoked by exogenous capsaicin.

Materials and Methods

Animals

Ten-week-old female Sprague-Dawley rats weighing 200 – 250 g were purchased from Charles River Laboratories (Yokohama). The animals were allowed free access to water and standard laboratory food; and they were housed in stainless steel cages at a temperature of 23 ± 2°C, relative humidity of 55 ± 10%, and a 12-h light/dark cycle, with lights on from 0700 to 1900 h daily.

All experimental procedures were performed according to the "Guidelines for the Care and Use of Laboratory Animals" approved by the Laboratory Animal Committee of Tsumura & Co.

Drugs and reagents

The *Keishi-bukuryo-gan* (TJ-25) used in the present study, composed of equal parts of *Cinnamomum cortex*, *Paeoniae radix*, *Moutan cortex*, *Hoelen*, and *Persicae semen*, was obtained from Tsumura Co., Ltd (Tokyo) in the form of a dried powder extract.

Sodium pentobarbital was purchased from Dinabot Laboratories (North Chicago, IL, USA). Aprotinin, 17β-estradiol, capsaicin, and salmon sperm DNA were purchased from Wako Pure Chemical Industries (Osaka). [α^{32}P]dCTP (3000 Ci/mmol) and a DNA labeling kit for a [α^{32}P]dCTP-labeled cDNA probe were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe was generated using a first strand cDNA synthesis kit and reverse transcription-polymerase chain reaction (RT-PCR) kit (Toyobo Co., Osaka). A rat CGRP cDNA probe was generated by digestion with *Sau3AI* and *TaqI* and subsequently subcloned into pT7T3 following amplification of a rat CGRP cDNA fragment (nucleotides 142 – 607) by RT-PCR. Other reagents used for analysis were the highest purity commercially available.

Treatment of OVX rats with 17β-estradiol and Keishi-bukuryo-gan

Rats were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and bilaterally ovariectomized or sham-operated as controls. 17β-Estradiol (0.010 mg/kg, s.c.) dissolved in olive oil or *Keishi-bukuryo-gan* (1,000 mg/kg, p.o.) suspended in distilled water was administered for 7 days (once a day) from the second week after ovariectomy. Distilled water (10 ml/kg, p.o.) as a control was administered to sham-operated and OVX rats according to the same schedule. These rats were used for the following experiments to measure CGRP concentrations in plasma, the spinal cord, and dorsal root ganglia and CGRP mRNA levels in dorsal root ganglia 3 weeks after surgery, i.e., on the day following the final administration.

Measurement of plasma CGRP levels following injection of capsaicin in 17β-estradiol- or Keishi-bukuryo-gan-treated OVX rats

First, in order to examine the effect of ovariectomy on the release of CGRP into the circulation, 1.0 mg/kg of capsaicin dissolved in saline containing 10% Tween 80 and 10% ethanol or the same volume of the vehicle as a control was injected i.p. into the OVX ($n = 10$) and sham-operated rats ($n = 10$).

In another set of experiments, the effects of 17β-estradiol and *Keishi-bukuryo-gan* on the CGRP release into the circulation were examined in the OVX rats: capsaicin (1.0 mg/kg, i.p.) was injected into 17β-estradiol ($n = 8$)-, *Keishi-bukuryo-gan* ($n = 8$)-, or distilled water-treated OVX rats ($n = 8$). The same amount of capsaicin was injected into distilled water-treated sham-operated rats ($n = 8$). All rats were decapitated 7.5 min after the injection of capsaicin, and blood (approximately 6 ml) was collected in a polypropylene tube containing 6.0 mg of EDTA-2Na and 3,000 kIU of aprotinin and centrifuged at $1,500 \times g$ and 4°C for 15 min. Plasma (2 ml) was acidified with 160 μl of a mixture consisting of 5.0% formic acid, 1.0% trifluoroacetic acid (TFA), 80% 1 M HCl, and 1.0% NaCl. The acidified plasma was centrifuged at $7,000 \times g$ and 4°C for 20 min. The supernatant was put into an activated C-18 Sep-Pak disposable cartridge column (Amersham Pharmacia Biotech) and eluted with a mixture (2 ml) of

methanol/water/TFA (90:9:1). The eluate was vacuum-dried and stored at −80°C until assay. On the day of the assay, the frozen sample was thawed in a small amount (<20 μl) of 0.1% TFA at 4°C over a 30-min period. The CGRP level in each sample was measured in duplicate using a rat CGRP Radioimmunoassay Kit (Peninsula Laboratories Inc., San Carlos, CA, USA).

Measurements of CGRP levels in spinal cord and dorsal root ganglia in 17β-estradiol- or Keishi-bukuryo-gan-treated OVX rats

17β-Estradiol (n = 7)-, *Keishi-bukuryo-gan* (n = 7)-, or distilled water (n = 7)-treated OVX rats were decapitated. Distilled water-treated sham-operated rats (n = 7) were also decapitated as controls. A 1.5-cm length of the lumbar enlargement (intumescentia lumbalis) of the spinal cord and 6 pairs of dorsal root ganglia derived from L1 – L6 lumbar nerves in each rat were removed. The dorsal root ganglia of each rat were pooled as one sample. CGRP in the spinal cord or the pooled dorsal root ganglia was extracted by a modification of the method of Wimalawansa et al. (16). In brief, the tissue was homogenized in 2 ml of ice-cold 15% TFA buffer containing 1 M HCl, 1 M NaCl, 5% formic acid, and 100 kIU/ml aprotinin. The homogenate was centrifuged at 7,000 ×*g* and 4°C for 20 min. The pellet was homogenized in the same volume of the TFA buffer and centrifuged again. The supernatant was put into an activated C-18 Sep-Pak disposable cartridge column (Amersham Pharmacia Biotech) and eluted with a mixture (2 ml) of methanol/water/TFA (90:9:1). The eluate was vacuum-dried and stored at −80°C until assay. On the day of the assay, the frozen sample was thawed in a small amount (<20 μl) of 0.1% TFA at 4°C over a 30-min period. The CGRP level in each sample was measured in duplicate using a rat CGRP Radioimmunoassay Kit (Peninsula Laboratories Inc.). The total protein content in each sample was determined by the Bradford method (Nippon Bio-Rad Laboratories, Tokyo).

Measurement of CGRP mRNA levels in dorsal root ganglia in 17β-estradiol- or Keishi-bukuryo-gan-treated OVX rats

17β-Estradiol (n = 5)-, *Keishi-bukuryo-gan* (n = 5)-, or distilled water (n = 5)-treated OVX rats and distilled water-treated sham-operated rats (n = 5) were decapitated, and 6 pairs of dorsal root ganglia derived from L1 – L6 lumbar nerves in each rat were removed and pooled as one sample.

Total RNA in the dorsal root ganglia was extracted using a RNA extraction kit (Nippon Gene, Toyama) according to the manufacturer's protocol. In brief, each pooled sample was homogenized in 1 ml of RNA-extract reagent using a Teflon homogenizer (Iuchi, Tokyo). The homogenate that was left to stand for 5 min at room temperature, and the 0.2 ml of chloroform was added to it and mixed. After the mixture was centrifuged at 12,000 ×*g* and 4°C for 15 min, the aqueous phase was collected and 0.5 ml of isopropanol was added to it. The mixture was left to stand for 10 min at room temperature and centrifuged again. The RNA pellet obtained by removing the aqueous phase was washed with 1 ml of 70% ethanol and then dissolved in an equal amount of 10 mM tris(hydroxymethyl)aminomethane buffer, pH 8.0, containing 1 mmol EDTA-2Na. The concentration of total RNA in the isolated solution was determined by spectrophotometry (Japan Spectroscopic, Tokyo) at a wavelength of 260 nm. An aliquot of this RNA solution was used for Northern blot analysis to determine CGRP mRNA.

For Northern blot analysis, the isolated total RNA (12 μg per lane) was electrophoresed on a 1.3% agarose gel containing formaldehyde and transferred onto a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech). The transferred RNA on the membrane was fixed by UV cross-linking (Funakoshi, Tokyo). As prehybridization, this membrane was incubated in a hybridization buffer (Sigma-Aldrich, Tokyo) containing heat-denatured salmon sperm DNA (100 μg/ml) at 42°C for 2 h. The membrane was then hybridized at 42°C overnight with a rat CGRP cDNA probe or GAPDH cDNA probe labeled with [α^{32}P]dCTP using a DNA labeling kit. After hybridization, the membrane was washed with saline-sodium citrate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate. The radioactivities of CGRP mRNA and GAPDH mRNA on the membrane were quantified by using a BAS2000 bioimaging analyzer (Fuji Photo Film Co., Tokyo). For each sample, the signal of CGRP mRNA was normalized with respect to the corresponding signal for the GAPDH mRNA used as an internal control.

Statistical analyses

All values were represented as the mean ± S.E.M. The statistical significance was evaluated by a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test or Student's *t*-test. The significance level was accepted at $P<0.05$.

Results

Effect of ovariectomy on the release of CGRP into the circulation

In order to examine the effect of ovariectomy on the release of CGRP into the circulation, the plasma concen-

tration of CGRP was determined after capsaicin (1.0 mg/kg) or the vehicle (1.0 ml/kg) was injected i.p. into the OVX and sham-operated rats. CGRP concentration in plasma measured 7.5 min after injection of vehicle ($CGRP_{control}$) was 6.56 ± 0.34 pmol/L in sham-operated rats or 5.08 ± 0.34 pmol/L in OVX rats, respectively. CGRP concentration in plasma measured 7.5 min after injection of capsaicin ($CGRP_{capsaicin}$) was 17.86 ± 1.37 pmol/L in sham-operated rats or 8.68 ± 0.76 pmol/L in OVX rats, respectively. As shown in Fig. 1, increase of plasma CGRP concentration induced by injecting capsaicin was significantly lower ($P<0.01$) in OVX rats than those in sham-operated rats.

Effects of 17β-estradiol and Keishi-bukuryo-gan on capsaicin-induced elevation of plasma CGRP level in OVX rats

In another set of experiments, the effects of 17β-estradiol and *Keishi-bukuryo-gan* on the release of CGRP into the circulation were examined by measuring the plasma concentration of CGRP following injection of capsaicin (1.0 mg/kg) or the vehicle (1.0 ml/kg) in 17β-estradiol- and *Keishi-bukuryo-gan*-treated OVX rats. $CGRP_{control}$ was 9.90 ± 0.38 pmol/L in distilled water (10 ml/kg, p.o.)-treated sham-operated rats or 8.30 ± 0.41 pmol/L in distilled water (10 ml/kg, p.o.)-treated OVX rats, 9.90 ± 0.49 pmol/L in 17β-estradiol (0.010 mg/kg, s.c.)-treated OVX rats, and 9.50 ± 0.37 pmol/L in *Keishi-bukuryo-gan* (1,000 mg/kg, p.o.)-treated OVX rats, respectively. $CGRP_{capsaicin}$ was 15.32 ± 0.69 pmol/L in distilled water-treated sham-operated rat or 8.38 ± 1.19 pmol/L in distilled water-treated OVX rats, 13.65 ± 0.99 pmol/L in 17β-estradiol-treated OVX rats, and 14.69 ± 1.00 pmol/L in *Keishi-bukuryo-gan*-treated OVX rats, respectively. As shown in Fig. 2, capsaicin-induced elevation of plasma CGRP concentration in distilled water-treated OVX rats was significantly lower ($P<0.01$) than in distilled water-treated sham-operated rats. The lowered CGRP level was restored by treating with estrogen (17β-estradiol: 0.010 mg/kg, s.c.) or *Keishi-bukuryo-gan* (1,000 mg/kg, p.o.) for 7 days (once a day) from the second week after ovariectomy.

Effects of 17β-estradiol and Keishi-bukuryo-gan on the expression of CGRP mRNA in dorsal root ganglia of OVX rats

The results of Northern blot analysis of CGRP mRNA in dorsal root ganglia are shown in Fig. 3. No significant differences were observed in the expression of CGRP mRNA levels between OVX and sham-operated rats. In addition, treatment with 17β-estradiol or *Keishi-bukuryo-gan* did not affect the expression levels of CGRP mRNA in OVX rats.

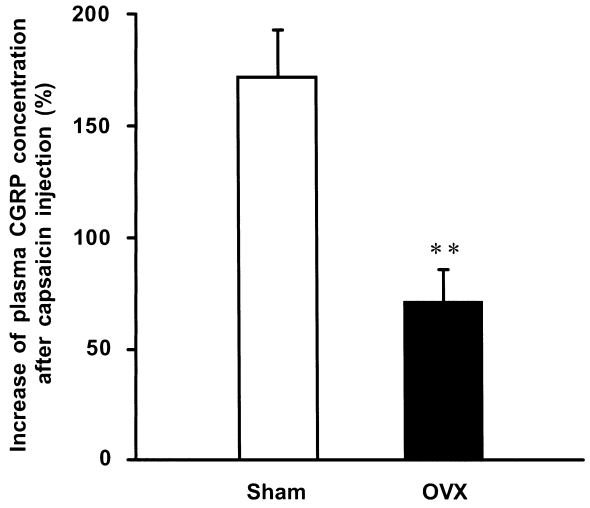

Fig. 1. Effect of ovariectomy on capsaicin-induced CGRP release into the circulation. Increase of CGRP concentration in plasma induced by injecting capsaicin was calculated as follows: increase of CGRP concentration in plasma induced by capsaicin (%) = ($CGRP_{capsaicin}$ / $CGRP_{control}$ − 1) × 100. Each value is expressed as the mean ± S.E.M. (n = 10). Statistical significance (**$P<0.01$) was compared between sham-operated and OVX groups using Student's *t*-test.

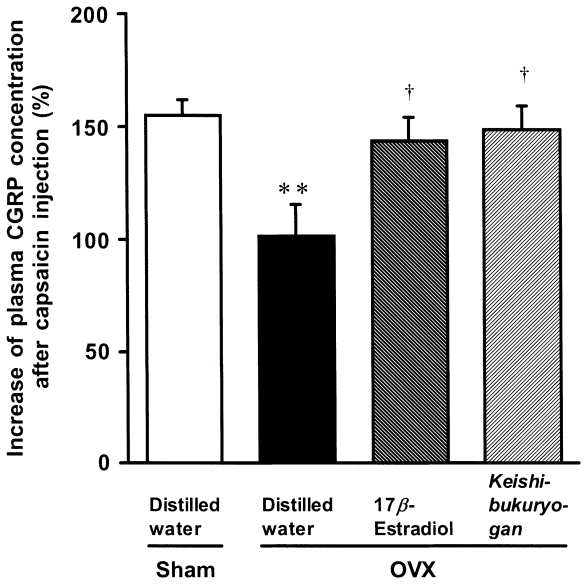

Fig. 2. Effects of 17β-estradiol and *Keishi-bukuryo-gan* on capsaicin-induced CGRP release into the circulation in OVX rats. Increase of CGRP concentration in plasma induced by injecting capsaicin was calculated as follows: increase of CGRP concentration in plasma induced by capsaicin (%) = ($CGRP_{capsaicin}$ / $CGRP_{control}$ − 1) × 100. Each value is expressed as the mean ± S.E.M. (n = 8). Significance with Dunnett's *t*-test following a one-way ANOVA [$F(3, 28) = 5.108$, $P<0.01$] is indicated as **$P<0.01$ *vs* distilled water-treated sham-operated group or $^{\dagger}P<0.05$ *vs* distilled water-treated OVX group.

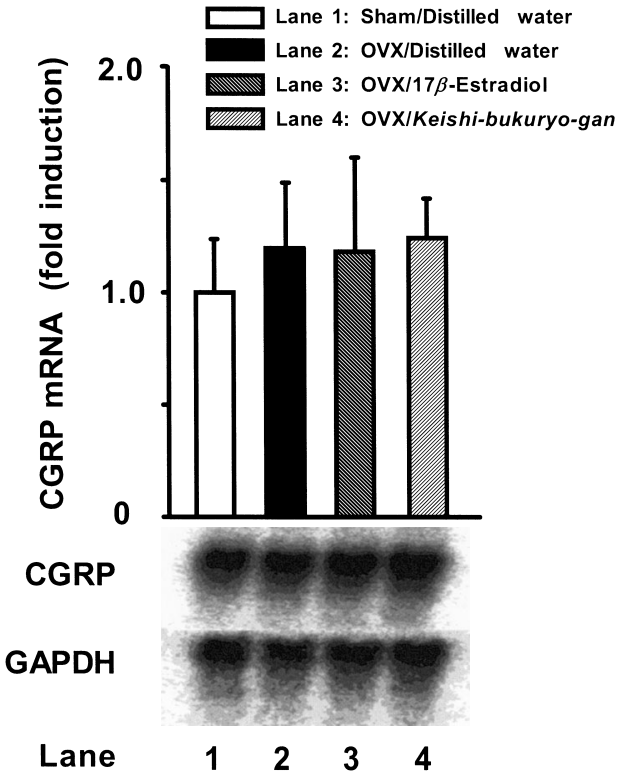

Fig. 3. Effects of 17β-estradiol (0.010 mg/kg, s.c.) and *Keishi-bukuryo-gan* (1,000 mg/kg, p.o.) on the expression of CGRP mRNA in dorsal root ganglia of OVX rats. To normalize the signal for CGRP mRNA, GAPDH mRNA was compared as an internal control. The CGRP mRNA/GAPDH mRNA ratios are shown as fold increase over the distilled water-treated sham-operated group. Each value is expressed as the mean ± S.E.M. (n = 3).

Effects of 17β-estradiol and Keishi-bukuryo-gan on CGRP concentrations in dorsal root ganglia and spinal cord in OVX rats

CGRP concentrations in dorsal root ganglia (A) and spinal cord (B) of OVX rats treated with 17β-estradiol or *Keishi-bukuryo-gan* are shown in Fig. 4. No significant differences were observed in CGRP levels of either dorsal root ganglia or spinal cord between OVX and sham-operated rats. Neither 17β-estradiol nor *Keishi-bukuryo-gan* affected CGRP levels in the dorsal root ganglia and spinal cord in OVX rats.

Discussion

We previously reported that 1) CGRP-induced elevation of skin temperature and vasorelaxation were greater in OVX rats than in sham-operated rats, 2) plasma concentration of endogenous CGRP was significantly lower in OVX rats than in sham-operated rats, and 3) the number of arterial CGRP receptors significantly increased in OVX rats (9). These CGRP-related responses in OVX rats were restored by hormone replacement of 17β-estradiol in OVX rats (10). From these results, we have suggested that the low concentration of endogenous CGRP in plasma due to ovarian hormone deficiency may induce up-regulation of arterial CGRP receptors and consequently amplify CGRP-induced elevation of skin temperature. In the present study, we confirmed that CGRP concentration in plasma significantly decreased in OVX rats. This result was the same

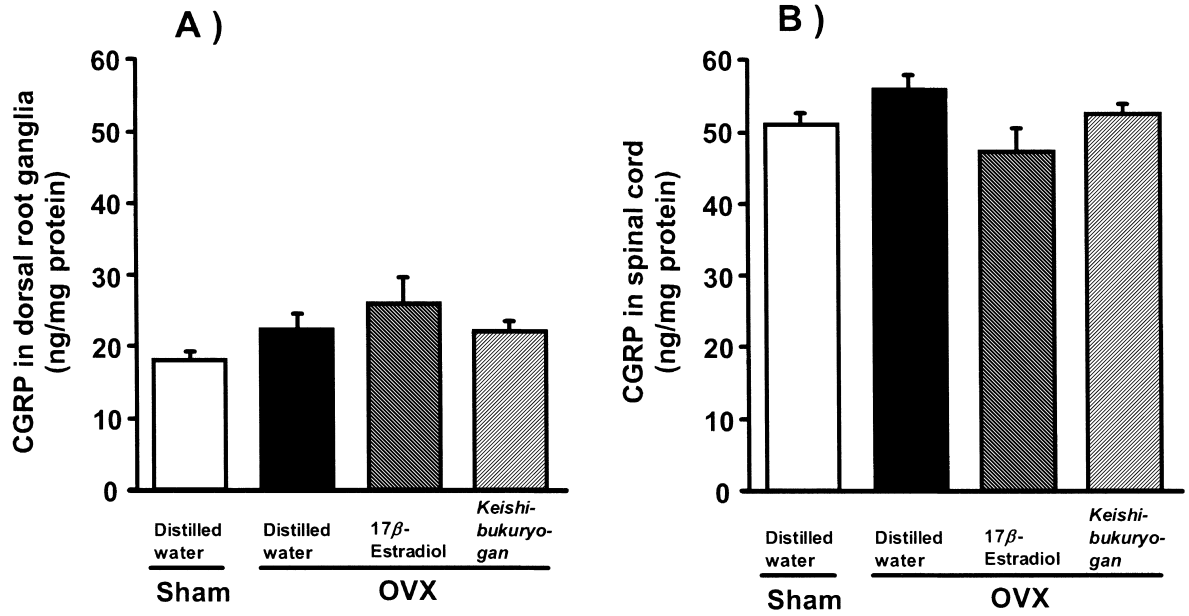

Fig. 4. Effects of 17β-estradiol (0.010 mg/kg, s.c.) and *Keishi-bukuryo-gan* (1,000 mg/kg, p.o.) on CGRP concentrations in dorsal root ganglia (A) and spinal cord (B) of OVX rats. Each value is expressed as the mean ± S.E.M. (n = 5 – 7).

as our previously reported data (9). This result also agrees with the results in a human study reported by Valentini et al. (7) demonstrating that plasma CGRP levels in postmenopausal women are lower than in fertile women.

Circulating CGRP is derived from the perivascular CGRPergic sensory nerve endings transported mainly from dorsal root ganglia, which is a prominent site of CGRP synthesis. It is known that the CGRP synthesized in dorsal root ganglia is also centrally transported to the spinal cord (11, 13). Recently, Tanaka et al. (14) reported that antidromic release of CGRP into the circulation via sensory nerves is induced by stimulation of the spinal cord, suggesting that the spinal cord is one of the factors regulating the plasma concentration of CGRP. The CGRPergic sensory nerves have been demonstrated to be sensitive to capsaicin; exogenously applied capsaicin induces CGRP release (17, 18). Zaidi et al. (12) reported that the plasma concentration of CGRP reaches a maximum 7.5 min following injection of capsaicin and returns to basal levels within 2 h. In the present study, we confirmed the increase in plasma concentration of CGRP 7.5 min after injection of capsaicin according to their procedure. In addition, the CGRP levels were significantly lower in OVX rats than in sham-operated rats, suggesting that the CGRP release is attenuated by estrogen deficiency. This suggestion was strongly supported by restoring the plasma level with estrogen replacement in OVX rats.

Sasamura and Kuraishi (17) reported that CGRP release due to capsaicin is induced by the influx of Ca^{2+} cations into capsaicin-sensitive sensory nerve terminals through vanilloid receptor subtype 1 (VR-1), which is a capsaicin receptor. Winston et al. (19) showed that nerve growth factor (NGF) treatment increased both VR-1 mRNA expression and capsaicin-evoked release of CGRP. They further showed that the effects are inhibited by treatment with k252a, an inhibitor of the tyrosine kinase A (trkA), which has a high affinity for the NGF receptor, suggesting that the release of CGRP from the nerve endings is influenced by NGF. Furthermore, it has been reported that estrogen up-regulates expression of trkA mRNA in sensory neurons, where estrogen receptors and NGF receptor are co-localized (20, 21). Together, these findings suggest that the number of VR-1 receptors may be decreased by attenuating the endogenous effect of NGF due to decrease in the activity of the trkA domain in the NGF receptor following estrogen deficiency with ovariectomy.

On the other hand, regarding the involvement of estrogen on CGRP synthesis in dorsal root ganglia, it is suggested that CGRP mRNA expression is up-regulated (20) or contrarily down-regulated (22) by estrogen through its receptors, although estrogen receptors obviously exist in the dorsal root ganglia (22). In addition, our present result indicated that CGRP concentrations in dorsal root ganglia and the spinal cord and the mRNA expression in the dorsal root ganglia were not affected by estrogen deficiency and estrogen replacement in OVX rats. Thus, at present, there is no consensus regarding the regulation of peptide synthesis by estrogen.

Keishi-bukuryo-gan restored the attenuation of capsaicin-induced CGRP release into the circulation without affecting CGRP synthesis in the dorsal root ganglia as well as did supplemental 17β-estradiol in OVX rats, suggesting that *Keishi-bukuryo-gan* also restores the lowered CGRP level in plasma by restoring its release from the terminals of capsaicin-sensitive CGRPergic sensory neurons. In addition, we previously demonstrated that 17β-estradiol and *Keishi-bukuryo-gan* restored CGRP-induced elevation of skin temperature, up-regulation of arterial CGRP receptors, and low concentration of plasma CGRP in OVX rats. Supplemental 17β-estradiol to OVX rats restored them by increasing the low concentration of plasma estrogen. However, *Keishi-bukuryo-gan* treatment to OVX rats restored them without increasing the low estrogen level. These results suggest that the restorative effects differ between estrogen and *Keishi-bukuryo-gan*: those of 17β-estradiol are dependent on changing the plasma estrogen concentration, but those of *Keishi-bukuryo-gan* are not (10). Recently, phytoestrogens such as isoflavones (23) or selective estrogen receptor modulators (SERM) such as raloxifene (24) are reported to produce estrogen-like effects without restoring decreased plasma estrogen level. We infer that *Keishi-bukuryo-gan* may contain phytoestrogen- or SERM-like substances. Although the detailed mechanism including active compounds and the site of action should be clarified in future studies, the effect of *Keishi-bukuryo-gan*, which does not confer estrogen activity on plasma, may be useful for women experiencing hot flashes with specific contraindications, such as a history of estrogen-dependent cancer (25).

In conclusion, we demonstrated that a low concentration of CGRP in plasma, which is suggested to be a trigger for elevation of skin temperature, was due to the inhibition of the releasing process in CGRPergic nerve terminals. In addition, it is suggested that 17β-estradiol and *Keishi-bukuryo-gan* restore the CGRP-release dysfunction due to estrogen deficiency in the nerve terminals.

Acknowledgments

We thank Dr. Kazuhiro Shiizaki, Dr. Jui-Tung Chen, and Dr. Atsushi Ishige for their useful advice.

References

1 Freedman RR. Physiology of hot flashes. Am J Human Biol. 2001;13:453–464.
2 Lomax P, Schönbaum E. Postmenopausal hot flashes and their management. Pharmacol Ther. 1993;57:347–358.
3 Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I. Calcitonin gene-related peptide is a potent vasodilator. Nature. 1985;313:54–56.
4 Wanaka A, Matsuyama T, Yoneda S, et al. Distribution patterns of calcitonin gene-related peptide-containing fibers in the wall of the three different arteries: an immunohistochemical study. Cell Mol Biol. 1987;33:201–209.
5 Kawasaki H, Tsuru H. Pharmacology and physiology of perivascular nerves regulating vascular function. Jpn J Pharmacol. 2002;88:7–8.
6 Chen JT, Hirai Y, Seimiya Y, Hasumi K, Shiraki M. Menopausal flushes and calcitonin-gene related peptide. Lancet. 1993;342:49.
7 Valentini A, Petraglia F, De Vita D, et al. Changes of plasma calcitonin gene-related peptide levels in postmenopausal women. Am J Obstet Gynecol. 1996;175:638–642.
8 Wyon YA, Spetz AC, Theodorsson GE, Hammar ML. Concentrations of calcitonin gene-related peptide and neuropeptide Y in plasma increase during flushes in postmenopausal women. Menopause. 2000;7:25–30.
9 Noguchi M, Ikarashi Y, Yuzurihara M, et al. Up-regulation of calcitonin gene-related peptide receptors underlying elevation of skin temperature in ovariectomized rats. J Endocrinol. 2002; 175:177–183.
10 Noguchi M, Ikarashi Y, Yuzurihara M, et al. Effects of the Japanese herbal medicine *Keishi-bukuryo-gan* and 17β-estradiol on calcitonin gene-related peptide-induced elevation of skin temperature in ovariectomized rats. J Endocrinol. 2003;176: 359–366.
11 Holzer P. Peptidergic sensory neurons in the control of vascular function: mechanism and significance in the cutaneous and splanchnic vascular beds. Rev Physiol Biochem Parmacol. 1992;121:49–146.
12 Zaidi M, Bevis PJR, Lynch C, Stevenson, MacIntyre I. Circulating CGRP comes from the perivascular nerves. Eur J Pharmacol. 1985;117:283–284.
13 Maggi CA. Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. Prog Neurobiol. 1995;45:1–98.
14 Tanaka S, Barron KW, Chandler MJ, Linderoth B, Foreman RD. Low intensity spinal cord stimulation may induce cutaneous vasodilation via CGRP release. Brain Res. 2001;896:183–187.
15 Kawao N, Shimada C, Itoh H, Kuroda R, Kawabata A. Capsazepine inhibits termal hyperalgesia but not nociception triggered by protease-activated receptor-2 in rats. Jpn J Pharmacol. 2002;89:184–187.
16 Wimalawansa SJ, Emson PC, MacIntyre I. Regional distribution of calcitonin gene-related peptide and its specific binding sites in rats with particular reference to the nervous system. Neuroendocrinology. 1987;46:131–136.
17 Sasamura T, Kuraishi Y. Peripheral and central actions of capsaicin and VR1 receptor. Jpn J Pharmacol. 1999:80:275–280.
18 Szallasi A, Blumberg PM. Vanilloid (capsaicin) receptors and mechanisms. Pharmacol Rev. 1999;51:159–211.
19 Winston J, Toma H, Shenoy M, Pasricha PJ. Nerve growth factor regulates VR-1 mRNA levels in cultures of adult dorsal root ganglion neurons. Pain. 2001;89:181–186.
20 Gangula PRR, Lanlua P, Wimalawansa S, Supowit S, DiPette D, Yallampalli C. Regulation of calcitonin gene-related peptide expression in dorsal root ganglia of rats by female sex steroid hormones. Biol Reprod. 2000;62:1033–1039.
21 Bjorling DE, Beckman M, Clayton MK, Wang ZY. Modulation of nerve growth factor in peripheral organs by estrogen and progesterone. Neuroscience. 2002;110:155–167.
22 Yang Y, Ozawa H, Lu H, et al. Immunocytochemical analysis of sex differences in calcitonin gene-related peptide in the rat dorsal root ganglion, with special reference to estrogen and its receptor. Brain Res. 1998;791:35–42.
23 Morito K, Hirose T, Kinjo J, et al. Interaction of phytoestrogens with estrogen receptors alpha and beta. Biol Pharm Bull. 2001;24:351–356.
24 Jisa A, Dornstauder E, Ogawa S, Inoue S, Muramatsu M, Jungbauer A. Transcriptional activities of estrogen receptor alpha and beta in yeast properties of raloxifen. Biochem Pharmacol. 2001;62:953–961.
25 Loprinzi CL, Barton DL, Rhodes D. Management of hot flashes in breast-cancer survivors. Lancet Oncol. 2001;2:199–204.