Effects of the Japanese herbal medicine *Keishi-bukuryo-gan* and 17 β -estradiol on calcitonin gene-related peptide-induced elevation of skin temperature in ovariectomized rats

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Abstract

The effects of a Japanese herbal medicine, *Keishi-bukuryo-gan*, and 17 β -estradiol on calcitonin gene-related peptide (CGRP)-induced elevation of skin temperature were investigated in ovariectomized (OVX) rats. 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 up-regulation of arterial CGRP receptors, suggesting that lowered CGRP in plasma due to ovarian hormone deficiency increases the number of CGRP receptors and consequently amplifies the stimulatory effects of CGRP to elevate skin temperature. Oral *Keishi-bukuryo-gan* (100–1000 mg/kg, once a day for 7 days) restored a series of

CGRP-related responses observed in OVX rats by normalizing plasma CGRP levels in a dose-dependent manner as effectively as s.c. injection. 17 β -estradiol (0.010 mg/kg, once a day for 7 days). However, *Keishi-bukuryo-gan* did not affect the lower concentration of plasma estradiol and the decreased uterine weight due to ovariectomy, although the hormone replacement of 17 β -estradiol restored them. These results suggest that *Keishi-bukuryo-gan*, which does not confer estrogen activity on plasma, may be useful for the treatment of hot flashes in patients for whom estrogen replacement therapy is contraindicated, as well as menopausal women.

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Introduction

Hot flashes are the most common symptom of the climacteric and are reported by the vast majority of menopausal women (Reber & Spitzer 1987). The symptom is subjectively experienced as a sensation of internal heat, spreading upward from the chest to the neck and face, and profuse sweating often occurs in these areas (Freedman 2001). Although hot flashes accompany the estrogen withdrawal that occurs at menopause, the mechanism underlying the symptom has not been clarified. Recently, it was reported that the plasma level of calcitonin gene-related peptide (CGRP), a vasodilator neuropeptide (Brain *et al.* 1985, Wanaka *et al.* 1987), in menopausal women rapidly increases during hot flashes (Chen *et al.* 1993, Valentini *et al.* 1996, Wyon *et al.* 2000). This is supported by our recent finding that i.v. injection of CGRP elevates skin temperature in ovariectomized (OVX) rats more than in sham-operated (Noguchi *et al.* 2002). In addition, an increase in blood flow caused by CGRP-induced vasodilation and an up-regulation of CGRP receptors following the lower concentration of plasma CGRP are observed in OVX rats. On the basis of these results, we speculated

that ovarian hormone deficiency increases the number of CGRP receptors and consequently amplifies the stimulatory effects of CGRP to elevate skin temperature (Noguchi *et al.* 2002). If the series of the CGRP-related responses described above were restored by estrogen replacement, our hypothesis would be strongly supported. Therefore, the first objective of the present study was to investigate the effect of 17 β -estradiol, which is a potent estrogen, on the CGRP-related responses in OVX rats.

Keishi-bukuryo-gan is a traditional herbal medicine called 'Kampo medicine' in Japan. This remedy for menopausal symptoms has been approved by the Ministry of Health, Labor and Welfare in Japan. To date, there are several studies demonstrating that *Keishi-bukuryo-gan* ameliorates menopausal hot flashes (Mochimaru *et al.* 1984, Tanaka 2001). However, whether the CGRP-related mechanism is involved or not in the anti-hot flashes effect of *Keishi-bukuryo-gan* has not been investigated. Therefore, the second objective of the present study was to investigate the effects of *Keishi-bukuryo-gan* on CGRP-induced elevation of skin temperature and a series of the CGRP-related responses in OVX rats.

Materials and Methods

Animals

Ten-week-old female Sprague–Dawley rats weighing 200–250 g were purchased from Charles River Laboratories (Yokohama, Japan). The animals were allowed free access to water and standard laboratory food, and housed in stainless steel cages at a temperature of $23 \pm 2^\circ\text{C}$, relative humidity $55 \pm 10\%$ and a 12 h light:12 h darkness cycle, with lights on from 0700 to 1900 h daily.

The rats were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg) and bilaterally ovariectomized or sham-operated as controls.

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, Japan), in the form of a dried powder extract.

Rat αCGRP was purchased from Peptide Institute (Osaka, Japan). ^{125}I -CGRP (specific activity 2000 Ci/mmol) was purchased from Peninsula Laboratories (San Carlos, CA, USA). Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$), urethane, α -chloralose and bacitracin were purchased from Sigma Chemical (St Louis, MO, USA). 17β -Estradiol and aprotinin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium pentobarbital was purchased from Dinabot Laboratories (North Chicago, IL, USA). Other reagents used for analysis were the highest purity commercially available.

Measurement of CGRP-induced elevation of skin temperature

Keishi-bukuryo-gan (100, 300 or 1000 mg/10 ml per kg, p.o., $n=7$ or 8 in each group) suspended in distilled water or 17β -estradiol (0.010 mg/ml per kg, s.c., $n=7$) dissolved in olive oil was administered for 7 days (once a day) from the 2nd week after ovariectomy. Distilled water (10 mg/kg, p.o., $n=8$) as control or *Keishi-bukuryo-gan* (1000 mg/10 ml per kg, p.o., $n=8$) was administered to sham-operated rats for 7 days according to the same schedule. Measurement of skin temperature was carried out on the day following the final administration according to a procedure described previously (Noguchi *et al.* 2002). In brief, rats were anesthetized with i.p. co-injection of urethane (0.75 g/kg) and α -chloralose (0.06 g/kg), and then two thermistor probes (SXN-54; Technol Seven Co., Yokohama, Japan) were taped to the plantar faces of both hind feet. Forty minutes later, the mean temperature

was automatically measured at 5 min intervals throughout the experiment. Data were recorded by a K932 recording device (Technol Seven Co.). αCGRP (10 $\mu\text{g/kg}$, i.v.) dissolved in saline was injected after the basal temperature was stable. The area under the temperature curve (AUC) following the injection of αCGRP was calculated using PAG-CP (Pharmacokinetic Analysis and Graphics for Clinical Pharmacology) analysis (Medical Research AS Medica, Osaka, Japan).

Measurement of CGRP-induced relaxation of $\text{PGF}_{2\alpha}$ -induced vasoconstriction in isolated mesenteric vascular beds

In another set of experiments, distilled water (10 ml/kg, p.o., $n=11$), *Keishi-bukuryo-gan* (1000 mg/10 ml per kg, p.o., $n=13$) or 17β -estradiol (0.010 mg/ml per kg, s.c., $n=12$) was administered to OVX rats, and distilled water (10 ml/kg, p.o., $n=9$) to sham-operated rats according to the schedule described in the section 'Measurement of CGRP-induced elevation of skin temperature'. Changes in the resistance of the mesenteric vascular bed obtained from drug-treated rats were evaluated on the day following the final administration according to a procedure described previously (Noguchi *et al.* 2002). In brief, all rats were anesthetized with pentobarbital (50 mg/kg, i.p.). A vascular bed including small arteries was isolated, placed in a water-jacketed organ bath maintained at 37°C and perfused at a constant rate of 5 ml/min using a peristaltic pump (AC-2110; Atto Co., Tokyo, Japan) with a modified Krebs solution gassed with 5% CO_2 in O_2 . The modified Krebs solution was composed of (in mM): NaCl 120.0, KCl 5.0, MgSO_4 1.2, NaHCO_3 25.0, CaCl_2 2.4 and glucose 5.0. Changes in the perfusion pressure were measured with a pressure transducer (San-ei Co., Tokyo, Japan) and recorded on a polygraph (Rika Denki Co., Tokyo, Japan). After the basal pressure was stable, 10 μM $\text{PGF}_{2\alpha}$ was continuously perfused to elevate the pressure by vasoconstriction of the vascular bed. After the $\text{PGF}_{2\alpha}$ -induced vasoconstriction reached a plateau, αCGRP (30 pmol/10.0 μl) dissolved in saline was added to the perfusate. The relaxation rate induced by CGRP was expressed as a percentage of the maximum pressure obtained by the $\text{PGF}_{2\alpha}$ -induced constriction.

CGRP receptor-binding assay in isolated mesenteric vascular beds

In another set of experiments, *Keishi-bukuryo-gan* (1000 mg/10 ml per kg, p.o., $n=8$) or 17β -estradiol (0.010 mg/ml per kg, s.c., $n=8$) was administered to OVX rats according to the schedule described in the section 'Measurement of CGRP-induced elevation of skin temperature'. Distilled water (10 ml/kg, p.o.) was also administered to sham-operated rats ($n=8$) and OVX rats ($n=8$) in the same schedule. Plasma membranes of mesenteric arteries from all rats were prepared on the day

following the final administration according to a procedure described previously (Noguchi *et al.* 2002). The pooled mesenteric vascular beds of two rats in each group were used for the determination. In brief, the two mesenteric vascular beds isolated from decapitated rats were placed in cold 0.25 M sucrose. The mesenteric arteries obtained by removing fatty tissue, lymph nodes and veins from the vascular beds were homogenized in 0.25 M sucrose with a Polytron homogenizer (Kinematica Inc., Cincinnati, OH, USA) at setting 7 for 30 s. The crude membrane fraction (pellet) was obtained by centrifugation at 100 000 *g* at 4 °C for 30 min. The fraction was suspended in 5.0 ml 0.25 M sucrose and layered on 5.0 ml 29% sucrose solution. After centrifugation at 100 000 *g* for 120 min, the plasma membrane fraction (the middle layer) was collected. The membrane fraction was washed with 0.25 M sucrose and centrifuged at 100 000 *g* for 30 min. The sediment was suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂ and 0.25 M sucrose. The concentration of membrane protein was determined by the method of Lowry *et al.* (1951). Finally, the concentration of the membrane suspension was adjusted to 1.0 mg/ml with the buffer.

The CGRP receptor-binding assay was performed as described previously (Noguchi *et al.* 2002). In brief, the membrane suspension (20–25 µg protein) was incubated with ¹²⁵I-CGRP (final concentration 50 pM) in 100 µl 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂, 2 mM EGTA, 1.0% BSA, 2 mM bacitracin, 200 kIU/ml aprotinin, 50 mM NaCl and 0.25 M sucrose. After incubation at 23 °C for 1 h, the incubation medium was passed through a GF/B filter (Whatman International Ltd, Maidstone, Kent, UK) that had been pre-soaked in polyethylenimine for 1 h. Receptor-ligand complexes on the filter were rinsed three times with 3 ml ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂ and 0.25 M sucrose. Radioactivity on the filter was counted by a COBRA II autogamma counting system (Packard, Meriden, CT, USA). Non-specific binding was determined by adding 0.5 µM unlabeled CGRP instead of the labeled peptide.

Measurement of plasma concentration of CGRP

To determine the plasma CGRP level, *Keishi-bukuryo-gan* (1000 mg/10 ml per kg, p.o., *n*=10) or 17β-estradiol (0.010 mg/ml per kg, s.c., *n*=9) was administered to OVX rats according to the schedule described in the section 'Measurement of CGRP-induced elevation of skin temperature'. Distilled water (10 ml/kg, p.o.) was also administered to sham-operated rats (*n*=9) and OVX rats (*n*=10) in the same schedule. On the day following the final administration, all rats were decapitated, and blood (approximately 6 ml) was collected in a polypropylene tube containing 6.0 mg EDTA-2Na and 3000 kIU of aprotinin and centrifuged at 1500 *g* at 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 7000 *g* at 4 °C for 20 min. The supernatant was put into an activated C-18 Sep-Pak disposable cartridge column (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) 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 RIA kit (Peninsula Laboratories).

Measurements of plasma concentration of estradiol and uterine weight

In another set of experiments to determine its effect on plasma estradiol level and uterine weight, *Keishi-bukuryo-gan* (1000 mg/10 ml per kg, p.o., *n*=7) or 17β-estradiol (0.010 mg/ml per kg, s.c., *n*=7) was administered to OVX rats according to the schedule described in the section 'Measurement of CGRP-induced elevation of skin temperature'. Distilled water (10 ml/kg, p.o.) was also administered to sham-operated rats (*n*=7) and OVX rats (*n*=8) on the same schedule. On the day following the final administration, all rats were anesthetized with diethyl ether, and then blood (approximately 6 ml) was collected from the abdominal aorta in a polypropylene tube containing 6.0 mg EDTA-2Na. The blood was centrifuged at 1500 *g* at 4 °C for 15 min. The plasma obtained was stored at -80 °C until estradiol assay. Plasma concentration of estradiol was measured using a ¹²⁵I-estradiol RIA kit (Diagnostic Products Co., Los Angeles, CA, USA).

The uterus was removed and weighed after exsanguination on the day of experiment.

Statistical analysis

All values are represented as the mean ± S.E.M. The statistical significance was evaluated by a one-way ANOVA followed by Dunnett's test. The significance level was accepted at *P*<0.05.

Results

Effects of Keishi-bukuryo-gan and 17β-estradiol on CGRP-induced elevation of skin temperature

In all groups, skin temperature was maximally elevated 40–50 min after the injection of CGRP (10 µg/kg, i.v.), and thereafter it recovered to the basal levels by 120 min. The degree of changes in skin temperature elevated by CGRP was calculated as AUC in each group. Figure 1 shows the changes in each group as a percentage of AUC

Figure 1 Effects of *Keishi-bukuryo-gan* and 17 β -estradiol on CGRP-induced elevation of skin temperature in OVX rats. Each value is expressed as the mean $\pm$ S.E.M. ($n=7$ or 8). The AUC in each group is expressed as a percentage of the AUC in the water-treated control group of sham-operated rats. Significance with Dunnett's test following a one-way ANOVA ($F(6,47)=7.028$, $P<0.001$) is indicated as ** $P<0.01$ vs the sham-operated control group, or † $P<0.05$ and †† $P<0.01$ vs the water-treated group of OVX rats.

in the control group; when the AUC of the water-treated control group of the sham-operated rats was expressed as 100%, ovariectomy significantly enhanced CGRP-induced elevation of skin temperature (210% of control group, $P<0.01$). In OVX rats, *Keishi-bukuryo-gan* (100–1000 mg/kg, p.o.) inhibited the elevation of skin temperature in a dose-dependent manner. A significant inhibition was observed at a dose of 1000 mg/kg *Keishi-bukuryo-gan* ($P<0.05$). 17 β -Estradiol (0.010 mg/kg, s.c.) significantly inhibited the CGRP-induced elevation of skin temperature as effectively as the 1000 mg/kg dose of *Keishi-bukuryo-gan*.

On the other hand, *Keishi-bukuryo-gan* (1000 mg/kg, p.o.) did not affect CGRP-induced elevation of skin temperature in sham-operated rats.

Effects of Keishi-bukuryo-gan and 17 β -estradiol on CGRP-induced relaxation of PGF_{2 α} -induced vasoconstriction in isolated mesenteric vascular beds

Continuous perfusion of PGF_{2 α} (10 μ M) constricted the isolated mesenteric vascular beds obtained from sham-operated and OVX rats, and the constriction reached a plateau. No significant differences were observed in the degree of PGF_{2 α} -induced constriction in vascular beds between sham-operated and OVX rats. As shown in

Fig. 2, CGRP (30 pmol) relaxed the PGF_{2 α} -induced constriction in the vascular bed isolated from sham-operated rats. The vasorelaxation effect, however, was significantly greater in OVX rats than in sham-operated rats ($P<0.01$). In OVX rats, *Keishi-bukuryo-gan* (1000 mg/kg, p.o.) and 17 β -estradiol (0.010 mg/kg, s.c.) significantly inhibited the CGRP-induced vasorelaxation ($P<0.01$).

Effects of Keishi-bukuryo-gan and 17 β -estradiol on ¹²⁵I-CGRP binding in isolated mesenteric arteries

The results of specific binding of ¹²⁵I-CGRP to CGRP receptors in mesenteric arteries of OVX and sham-operated rats are shown in Fig. 3. The receptor binding (2.28 fmol/mg protein) significantly ($P<0.01$) increased in OVX rats over that (0.83 fmol/mg protein) in sham-operated rats. *Keishi-bukuryo-gan* (1000 mg/kg, p.o., $P<0.05$) and 17 β -estradiol (0.010 mg/kg, s.c., $P<0.01$) significantly inhibited the ovariectomy-induced increase in the receptor binding.

Effects of Keishi-bukuryo-gan and 17 β -estradiol on plasma concentration of CGRP

Effects of *Keishi-bukuryo-gan* and 17 β -estradiol on plasma concentration of CGRP in plasma of OVX rats are

Figure 2 Inhibitory effects of *Keishi-bukuryo-gan* and 17β-estradiol on CGRP-induced relaxation of PGF_{2α}-induced vasoconstriction in mesenteric vascular beds isolated from OVX rats. Data calculated as percentage relaxation of the maximum pressure obtained by PGF_{2α}-induced contraction are expressed as the mean ± S.E.M. ($n=9-13$). Significance with Dunnett's test following a one-way ANOVA ($F(3,41)=11.218$, $P<0.001$) is indicated as ** $P<0.01$ vs the sham-operated group, or †† $P<0.01$ vs the water-treated group of OVX rats.

shown in Fig. 4. The CGRP level (6.38 ± 0.39 pmol/l) in OVX rats was significantly lower ($P<0.05$) than that (13.34 ± 1.41 pmol/l) in sham-operated rats. The ovariectomy-induced lower level was restored ($P<0.05$) to sham-operated level by treating with *Keishi-bukuryo-gan* (1000 mg/kg, p.o.) or 17β-estradiol (0.010 mg/kg, s.c.).

Effects of *Keishi-bukuryo-gan* and 17β-estradiol on plasma concentration of estradiol and uterine weight

The plasma concentration of estradiol and the uterine weight in OVX and sham-operated rats are shown in Table 1. The estradiol level in OVX rats was significantly lower ($P<0.05$) than that in sham-operated rats. The lower level of estradiol was significantly ($P<0.01$) increased by supplying 17β-estradiol (0.010 mg/kg, s.c.), but it was not affected by treating with *Keishi-bukuryo-gan* (1000 mg/kg, p.o.).

Uterine weight also significantly ($P<0.01$) decreased in OVX rats over that in sham-operated rats. The decreased tissue weight was recovered ($P<0.01$) by supplying 17β-estradiol, but it was not affected by *Keishi-bukuryo-gan*.

Discussion

We have demonstrated in a previous study (Noguchi *et al.* 2002) that CGRP-induced elevation of skin temperature

Figure 3 Effects of *Keishi-bukuryo-gan* and 17β-estradiol on ¹²⁵I-CGRP specific binding in plasma membrane of mesenteric arteries isolated from OVX rats. *Keishi-bukuryo-gan* (1000 mg/kg, p.o.) or 17β-estradiol (0.010 mg/kg, s.c.) was administered for 7 days (once a day) from the 2nd week after ovariectomy. Distilled water was administered to sham-operated and OVX rats for 7 days according to the same schedule. Measurement was carried out in duplicate using the pooled plasma membrane isolated from two rats in each group on the day following the final administration. Each value is expressed as the mean ± S.E.M. ($n=4$). Significance with Dunnett's test following a one-way ANOVA ($F(3,12)=9.983$, $P<0.01$) is indicated as ** $P<0.01$ vs the sham-operated group, or † $P<0.05$ and †† $P<0.01$ vs the water-treated group of OVX rats.

is antagonized by pretreatment with CGRP₁ receptor antagonist CGRP₈₋₃₇, suggesting that the elevation is due to the response through CGRP₁ receptors. On the other hand, it has been demonstrated that the mesenteric vascular bed is densely innervated by CGRP-containing nerves (Ishida-Yamamoto & Tohyama 1989) and the main CGRP receptor type mediating relaxation in the mesenteric arteries is also a CGRP₁ receptor (Han *et al.* 1990, Lei *et al.* 1994). These suggest that mesenteric vascular beds including small arteries, for which preparation is comparatively easy, are useful materials to examine the changes in CGRP₁ receptors and the mediated responses, in place of skin arteries for which preparation is rather difficult. Therefore, we investigated the changes in CGRP-induced relaxation and the number of CGRP receptors by using mesenteric arteries.

In the present study, we demonstrated that ovariectomy potentiated not only CGRP-induced elevation of skin temperature but also CGRP-induced arterial relaxation, which agrees with the results of our previous study

Figure 4 Effects of *Keishi-bukuryo-gan* and 17β-estradiol on plasma concentration of CGRP in OVX rats. Each value is expressed as the mean ± S.E.M. ($n=9$ or 10). Significance with Dunnett's test following a one-way ANOVA ($F(3,34)=4.308$, $P<0.05$) is indicated as * $P<0.05$ vs the sham-operated group, or † $P<0.05$ vs the water-treated group of OVX rats.

(Noguchi *et al.* 2002). In addition, the ^{125}I -CGRP binding to mesenteric arterial CGRP receptors significantly increased in OVX rats. This also supports the results of a previous Scatchard analysis, which demonstrated that the maximal number of binding sites (B_{max}) of ^{125}I -CGRP to arterial CGRP receptors was higher in OVX rats than in sham-operated rats without altering the dissociation constant (K_d) value (Noguchi *et al.* 2002). The increase in the number of CGRP receptors suggested by the increases of ^{125}I -CGRP binding rate and B_{max} was always accompanied by lower levels (10.40 pg/ml, a quarter the amount of the sham-operated level) of endogenous CGRP in the plasma under the condition of estrogen deficiency in OVX rats. However, the plasma estradiol is not completely abolished by ovariectomy, because it may be synthesized from C_{19} steroids in a reaction catalyzed by aromatase in extraovular tissues, such as adipose tissue and skin fibroblasts, in the OVX condition (Simpson *et al.* 1994, Bulun *et al.* 2000). It has been reported that both estrogen and progesterone, which are ovarian hormones, stimulate CGRP peptide synthesis in dorsal root ganglia neurons by increasing CGRP mRNA (Gangula *et al.* 2000a). The neuronal CGRP is transported towards the perivascular nerve endings and released into the circulation (Zaidi *et al.* 1985, Holzer 1992). Circulating levels of CGRP in rats and humans have been reported to increase with pregnancy and decrease at postpartum (Stevenson *et al.* 1986,

Table 1 Effects of *Keishi-bukuryo-gan* and 17β-estradiol on concentration of plasma estradiol and uterine weight in OVX rats. *Keishi-bukuryo-gan* (1000 mg/kg, p.o., $n=7$) or 17β-estradiol (0.010 mg/kg, s.c., $n=7$) was administered for 7 days (once a day) from the 2nd week after ovariectomy. Distilled water was administered to sham-operated ($n=7$) and OVX ($n=8$) rats for 7 days according to the same schedule. Measurement was carried out on the day following the final administration

Group	Plasma estradiol (pg/ml)	Uterine weight (mg)
Sham-operation	43.59 ± 6.23	368 ± 19
OVX	10.40 ± 3.36*	91 ± 4**
OVX + <i>Keishi-bukuryo-gan</i>	9.02 ± 2.91*	92 ± 5**
OVX + 17β-estradiol	58.60 ± 14.53††	357 ± 14††

Each value is expressed as the mean ± S.E.M. Significance with Dunnett's test following a one-way ANOVA (plasma estradiol: $F(3,25)=9.556$, $P<0.001$ or uterine weight: $F(3,25)=60.529$, $P<0.001$) is indicated as * $P<0.05$ and ** $P<0.01$ vs sham-operated group, or †† $P<0.01$ vs OVX group.

Saggese *et al.* 1990, Gangula *et al.* 2000b). Valentini *et al.* (1996) have reported that plasma CGRP levels in post-menopausal women are lower than in fertile women. These findings also suggest that ovarian hormones regulate plasma CGRP levels. Taken together, the present results suggest that ovarian hormone deficiency may decrease the CGRP level in plasma and consequently may increase the number of CGRP receptors, i.e. up-regulation of the receptors to maintain vascular adaptation. Because skin temperature is regulated by regional blood flow (Rand *et al.* 1965, Kronenberg 1994), the elevation of skin temperature potentiated by CGRP in OVX rats is thought to be mediated by the blood flow increase following the vasodilation by this peptide of the up-regulated arterial CGRP receptors in the skin. This hypothesis is strongly supported by the results of the supply of 17β-estradiol, which is a potent estrogen, to OVX rats; the hormone replacement clearly inhibited ovariectomy-induced elevation of skin temperature with the decrease of plasma CGRP level, up-regulation of CGRP receptors and potentiation of vasorelaxation.

On the other hand, *Keishi-bukuryo-gan* inhibited not only CGRP-induced elevation of skin temperature but also a series of the CGRP-related responses in OVX rats without restoring the lowered plasma estrogen and the decreased uterine weight, as well as supplemental 17β-estradiol with restoring them, while treatment with both medicines commonly restored the lowered CGRP level in plasma and consequently inhibited up-regulation of CGRP receptors. These results suggest that *Keishi-bukuryo-gan* does not confer any estrogen activity on plasma and it does not potentiate estrogen-production in the extraovular tissues in OVX rats. It is known that some plants contain substances referred to as 'phytoestrogens', such as isoflavones (Morito *et al.* 2001) and coumestrol

(Kuiper *et al.* 1998, Diel *et al.* 2001), that activate estrogen-controlled functions by activating transcription via estrogen receptors (ERs). In addition, raloxifen, which is known as a synthetic selective ER modulator (SERM), exhibits anti-estrogenicity in the breast and uterus, but acts as an agonist in bone and liver (Jisa *et al.* 2001). Recently, a phytoestrogen genistein derivative 6-carboxymethyl genistein was reported to possess the same selectivity profile as raloxifen in tissues derived from OVX rats (Somjen *et al.* 2002). If such phytoestrogen-like or SERM-like substances are contained in *Keishi-bukuryo-gan*, the estrogen-like activity to release CGRP from the perivascular nerve endings into the circulation may be induced without restoring the decreased plasma estrogen level in OVX rats. To date, we have isolated and identified many ingredients including paeoniflorin, oxypaeoniflorin, benzoylpaeoniflorin, albiflorin, paeonol, amygdalin, cinnamic acid, cinnamic aldehyde and penta-*O*-gallonyl- β -D-glucopyranose from the extract of *Keishi-bukuryo-gan* (Usuki 1987). However, there have been no reports investigating potential phytoestrogen-like and SERM-like properties of each ingredient. Recently, we have reported that *Keishi-bukuryo-gan*, which is extracted from a blend of five galenicals, does not activate the transcription in estrogen-dependent human breast cancer cell line MCF-7, which expresses ER α abundantly (Shiizaki *et al.* 1999). This finding suggests that *Keishi-bukuryo-gan* may not have an ER α -like effect. However, we cannot rule out the possibility that *Keishi-bukuryo-gan* has phytoestrogen- and SERM-like effects, because ER β has been shown to have different tissue distribution and different ligand binding specificity from ER α (Kuiper *et al.* 1997, 1998, Watanabe *et al.* 1997). *Keishi-bukuryo-gan*, which did not affect plasma estrogen activity and uterine weight, may have tissue specificity compared with supplemental estrogen that is considered to affect all estrogen-target tissues including uterine with increasing plasma estrogen levels. Therefore, it is important to characterize the estrogenic properties of *Keishi-bukuryo-gan* in future studies. In addition, *Keishi-bukuryo-gan* did not affect CGRP-induced elevation of skin temperature in sham-operated rats. This fact suggests that this herbal medicine may act only under the condition of hormonal imbalance in ovariectomy. We also have to investigate the mechanism in future studies.

In conclusion, *Keishi-bukuryo-gan* and 17 β -estradiol inhibited the potentiation of CGRP-induced elevation of skin temperature in OVX rats. The reversible results due to estrogen replacement in a series of the CGRP-related responses strongly support our previous hypothesis that ovarian hormone deficiency induces up-regulation of CGRP receptors due to the lowered level of plasma estrogen and consequently amplifies the ability of CGRP to elevate skin temperature. The present study also suggests that estrogen might be useful as hormone replacement therapy for menopausal hot flashes. In addition, *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 (Loprinzi *et al.* 2001), as well as menopausal women (Reber & Spitzer 1987, Freedman 2001).

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