Up-regulation of calcitonin gene-related peptide receptors underlying elevation of skin temperature in ovariectomized rats

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Abstract

We investigated the mechanism for the augmentation of the calcitonin gene-related peptide (CGRP)-induced elevation of skin temperature in ovariectomized (OVX) rats. I.v. injection of αCGRP (10 µg/kg) elevated skin temperature of the hind paws. The elevation was significantly greater in OVX rats than in sham-operated rats and was inhibited by pretreatment with human CGRP₈₋₃₇ (100–1000 µg/kg i.v.), a CGRP receptor antagonist, in a dose-dependent manner. In addition, ovariectomy not only potentiated vasorelaxation due to αCGRP but increased the number of CGRP receptors in mesenteric arteries. Further, the plasma concentration of endogenous CGRP was significantly lower in OVX rats. These results suggest 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. Therefore, the increased number of CGRP receptors may be responsible for potentiation of exogenous αCGRP-induced elevation of skin temperature in OVX rats. The mechanism underlying the hot flashes observed in menopausal women may also involve, in part, the up-regulation of CGRP receptors following ovarian hormone deficiency.

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Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid regulatory neuropeptide derived from alternative splicing of the calcitonin/CGRP gene (Rosenfeld et al. 1983). It exists in the central nervous system and peripheral nerves of the vascular wall (Lee et al. 1985, Wanaka et al. 1987). In humans (Gennari & Fischer 1985, McEwan et al. 1988) and rats (Kobayashi et al. 1995, Shen et al. 2001), administration of CGRP not only decreased arterial blood pressure and resistance of the peripheral vascular bed but also elevated skin temperature by increasing blood flow. These studies have shown CGRP to be a potent vasodilator that plays an important role in hemodynamic changes related to regulation of skin temperature.

A hot flash, which often occurs in menopausal women, generally begins with a sudden outpouring of sweat and an increase in heart rate and peripheral blood flow, causing a drastic increase in skin temperature as blood flows to the skin (Kronenberg 1994, Kobayashi et al. 1995). Chen et al. (1993) clinically demonstrated a positive correlation between plasma CGRP levels and the frequency of hot flashes in menopausal women and also an increase in plasma CGRP levels during hot flashes in the same patient. Kobayashi et al. (1995) reported that CGRP-induced elevation of skin temperature was significantly greater in ovariectomized (OVX) rats. These suggest that CGRP plays an important role in the occurrence of hot flashes due to ovarian hormone deficiency in women during the menopause. However, the mechanism underlying hot flashes has not been clarified. In the present study, therefore, we investigated the mechanism for elevation of skin temperature induced by CGRP 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 ± 2 °C, relative humidity of 55 ± 10% and a 12 h light:12 h darkness cycle, with lights on from 0700 to 1900 h daily.

The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and bilaterally ovariectomized or sham-operated as controls. These animals were used 3 weeks
after the surgery. A surgical technique for ovariectomy that has been established by monitoring decreased estradiol levels in plasma and decreased tissue weight of the uterus was used in the present study.

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.

Reagents
Rat αCGRP and human CGRP<sub>8–37</sub> were purchased from Peptide Institute Inc. (Osaka, Japan). <sup>125</sup>I-CGRP (specific activity 2000 Ci/mmol) was purchased from Peninsula Laboratories Inc. (San Carlos, CA, USA). Prostaglandin (PG) F<sub>2α</sub>, urethane, α-chloralose and bacitracin were purchased from Sigma Chemical (St Louis, MO, USA). Aprotinin was purchased from Wako Pure Chemical Industries Ltd (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 skin temperature
OVX (<i>n</i>=33) or sham-operated (<i>n</i>=34) 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. Ltd, Yokohama, Japan) were taped to the plantar face of both hind feet to measure the skin temperature. 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. Ltd). Human CGRP<sub>8–37</sub> (100, 500 or 1000 µg/kg) dissolved in saline was injected i.v. into the tail vein after basal temperature was stable, and αCGRP (10 µg/kg i.v.) dissolved in saline was injected 10 min later.

The area under the temperature curve (AUC) following incubation at 23 °C for 1 h, the incubation medium was passed through a GF/B filter (Whatman International Ltd, Tokyo, Japan) with a modified Krebs solution gassed with 5% CO<sub>2</sub> in O<sub>2</sub>. The modified Krebs solution was composed of (in mM): NaCl 120·0, KCl 5·0, MgSO<sub>4</sub> 1·2, NaHCO<sub>3</sub> 25·0, CaCl<sub>2</sub> 2·4 and glucose 5·0. Changes in the perfusion pressure were measured with a pressure transducer (San-ei Co. Ltd, Tokyo, Japan) and recorded on a polygraph (Rika Denki Co. Ltd, Tokyo, Japan). After the basal pressure was stable, 10 µM PGF<sub>2α</sub> was continuously perfused to elevate the pressure by vasocontraction of the vascular bed. After the PGF<sub>2α</sub>-induced vasocontraction reached a plateau, various concentrations of αCGRP (<i>10</i><sup>-11</sup>–<i>10</i><sup>-9</sup> mol/10·0 µl) were added to the perfusate to evaluate the vasorelaxation. The relaxation rate was expressed as a percentage of the maximum pressure obtained by the PGF<sub>2α</sub>-induced contraction.

CGRP receptor binding assay
Plasma membrane from rat mesenteric arteries was prepared according to the method described by Wei et al. (1976). Mesenteric vascular beds isolated from decapitated OVX (<i>n</i>=20) or sham-operated (<i>n</i>=20) 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 resuspended 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 resuspended in 50 mM Tris–HCl buffer, pH 7·4, containing 5 mM MgCl<sub>2</sub> 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.

CGRP receptor binding assay was performed by a modification of the method of Wimalawansa et al. (1987). In brief, the membrane suspension (20–25 µg protein) was incubated with various concentrations of <sup>125</sup>I-CGRP in 100 µl 50 mM Tris–HCl buffer, pH 7·4, containing 5 mM MgCl<sub>2</sub>, 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 polyethyleneamine 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 MgCl<sub>2</sub> 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. The maximal number of binding sites (B_max) and the dissociation constant (K_d) were calculated by Scatchard analysis.

\[ \text{RIA of plasma CGRP} \]

The blood (approximately 6 ml) from decapitated OVX (n=6) or sham-operated (n=5) rats was collected in polypropylene tubes containing 6·0 mg EDTA and 3000 kIU 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 Inc., San Carlos, CA, USA).

\[ \text{Statistical analysis} \]

All values were represented as means ± S.E.M. The statistical significance of skin temperature or vasorelaxation data was evaluated by a two-way or one-way ANOVA followed by Dunnett’s t-test. Student’s t-test was used for comparison of plasma CGRP levels in OVX and sham-operated rats. For all tests the significance level was accepted at P<0·05.

\[ \text{Results} \]

\[ \alpha \text{CGRP-induced elevation of skin temperature and antagonism with the CGRP receptor antagonist human CGRP}_{8-37} \text{ in OVX rats} \]

Changes in skin temperature following injection of \( \alpha \text{CGRP} \) (10 µg/kg i.v.) in sham-operated or OVX rats are shown in Fig. 1. The skin temperature was maximally elevated 40–50 min after the injection of \( \alpha \text{CGRP} \) in both operated rats, and thereafter it recovered basal levels by 120 min. The AUC data shown in Fig. 1C indicated that the elevation of skin temperature in the control group (P<0·01) and CGRP\(_{8-38}\) (500 µg/kg i.p.)-treated group (P<0·05) was significantly greater in OVX rats than in sham-operated rats. The CGRP-induced elevations of skin temperature in both operated rats were inhibited by pretreatment with human CGRP\(_{8-37}\) (100–1000 µg/kg i.p.) in a dose-dependent manner.

\[ \text{Vasorelaxation effect of } \alpha \text{CGRP on } \text{PGF}_{2\alpha}-\text{induced contraction of mesenteric vascular beds isolated from OVX rats} \]

Changes in the resistance of the peripheral vascular bed as an index of vasoconstriction or vasorelaxation were examined in OVX and sham-operated rats. Continuous perfusion of PGF\(_{2\alpha}\) (10 µM) contracted the isolated mesenteric vascular beds, and the contraction reached a plateau. No significant differences were observed in the degree of PGF\(_{2\alpha}\)-induced contraction between vascular beds obtained from OVX or sham-operated rats in a dose-dependent manner. The vasorelaxation effect, however, was significantly greater in OVX rats than in sham-operated rats (F(1,108) = 29·812, P<0·001).

\[ \text{Changes in } ^{125}\text{I-CGRP binding in isolated mesenteric arteries of OVX rats} \]

The effect of ovariectomy on \(^{125}\text{I-CGRP} \) binding was investigated in isolated mesenteric arteries. The Scatchard plot is shown in Fig. 3. The correlation between the bound/free ratio and specific \(^{125}\text{I-CGRP} \) binding was linear with a K_d of 0·333 nM and a B_max of 5·76 fmol/mg protein in sham-operated rats or with a K_d of 0·387 nM and a B_max of 20·18 fmol/mg protein in OVX rats; the B_max value was approximately 3·5 times higher in OVX rats than in sham-operated rats, although no differences were observed in the K_d values between the two groups.

\[ \text{Changes in plasma concentration of endogenous CGRP in OVX rats} \]

The effect of ovariectomy on plasma concentration of endogenous CGRP was examined 3 weeks after ovariectomy or sham operation. The CGRP level in OVX rats (7·14 ± 0·53 pmol/l) was significantly lower (P<0·01) than that in sham-operated rats (13·46 ± 1·56 pmol/l).

\[ \text{Discussion} \]

We first of all demonstrated in the present study that i.v. injection of \( \alpha \text{CGRP} \) caused an elevation of skin temperature that was significantly greater in OVX rats than in sham-operated rats. This agrees with the results of a previous study reported by Kobayashi et al. (1995). In addition, we demonstrated that the \( \alpha \text{CGRP} \)-induced elevation of skin temperature was inhibited by human CGRP\(_{8-37}\) in both OVX and sham-operated rats. Human
CGRP$_{8-37}$ is the most potent CGRP antagonist available to date and appears to differentiate between the two CGRP receptors called CGRP$_1$ and CGRP$_2$. CGRP$_1$ receptors are characterized by their sensitivity to the antagonistic actions of CGRP$_{8-37}$, whereas CGRP$_2$ receptors are resistant to CGRP$_{8-37}$ (Dennis et al. 1990, Mimeault et al. 1991, Holzer 1992). It has been demonstrated that CGRP$_1$ receptor antagonist human CGRP$_{8-37}$ exhibits higher affinity for the CGRP receptors in rat left atrium, when human CGRP and rat CGRP are used as an agonist; human CGRP$_{8-37}$ blocks human CGRP- and rat CGRP-induced responses with $pA_2$ values of 7.17 and 7.35 respectively (Juaneda et al. 2000, Wu et al. 2000). In addition, human CGRP$_{8-37}$ inhibits rat CGRP-induced vasorelaxation in isolated rat mesenteric vascular beds (Han et al. 1990). These suggest that human CGRP$_{8-37}$ is an effective antagonist against rat and human CGRP$_1$ receptors. Therefore, it is suggested that the CGRP$_{8-37}$-induced elevation of skin temperature in the present study may be due to the response through CGRP$_1$ receptors.

The mesenteric vascular bed is densely innervated by CGRP-containing sensory nerves (Uddman et al. 1986, Ishida-Yamamoto & Tohyama 1989), and in vitro electrical field stimulation indicated that activation of these nerves induces vasodilation (Kawasaki et al. 1988, Fujimori et al. 1989, 1990). Lei et al. (1994) suggested that the main CGRP receptor type mediating relaxation in rat mesenteric small arteries is the CGRP$_1$ subtype. The $^{125}$I-CGRP binding assay in the present study indicated that the specific binding was linear in the arteries isolated from OVX and sham-operated rats and that no significant difference in the $K_d$ values was observed between the differently treated rats, suggesting a single class of high-affinity specific binding sites for CGRP in the mesenteric arteries. These suggest that mesenteric vascular beds including small arteries are useful materials to examine the changes in CGRP$_1$ receptor and CGRP-mediated responses, in place of skin arteries. To clarify the mechanism underlying the potentiation of αCGRP (CGRP$_{1}$...

![Figure 1](image-url) Effects of the CGRP antagonist human CGRP$_{8-37}$ on the skin temperature response to αCGRP in sham-operated (A) and OVX rats (B). AUC data following injection of αCGRP (10 µg/kg i.v.) in each treatment are shown in (C). αCGRP was administered 10 min after pretreatment with saline (C) or human CGRP$_{8-37}$ (100 (▲), 500 (■) or 1000 (●) µg/kg i.v.). The number of animals in each group (n=6–10) is indicated in (C). Each value is expressed as the mean ± S.E.M. The AUC in each treatment group is expressed as a percentage of AUC in the sham-operated control group. In the AUC data, statistical significance of skin temperature in sham-operated or OVX rats was analysed by a one-way or two-way ANOVA followed by Dunnett’s test. Factorial two-way ANOVA analysis revealed that there were significant effects of group (F(1,59)=15.693, P<0.001) and CGRP$_{8-37}$ dose (F(3,59)=19.259, P<0.001). In this Figure, significance with post hoc analyses (Dunnett’s t-test) following the ANOVA analyses are indicated; *P<0.01 compared with the corresponding control. The effect of ovariectomy on αCGRP-induced elevation of skin temperature was compared between sham-operated control and OVX control using Student’s t-test; †P<0.05, ††P<0.01.
activator)-induced elevation of skin temperature in OVX rats, therefore, we investigated the changes in αCGRP-induced relaxation and the number of CGRP receptors using mesenteric arteries isolated from OVX rats. We demonstrated that infusion of αCGRP induced vasodilation (or vasorelaxation) of isolated arteries contracted by PGF$_{2\alpha}$. It has been demonstrated that CGRP is a potent vasodilator and causes a regional blood flow increase in rats (Marshall et al. 1986, Kawasaki et al. 1988, Han et al. 1990, Kobayashi et al. 1995), and that skin temperature in rats is regulated by regional blood flow (Rand et al. 1965). Therefore, the elevation of skin temperature induced by αCGRP in the present study is thought to be mediated by the blood flow increase following the vasodilator action of this peptide in the skin. This finding in rats is consistent with the previous observation that CGRP increases regional blood flow in humans, especially in the skin (Jager et al. 1990, Jernbeck et al. 1990). We also demonstrated that αCGRP-induced vasorelaxation was significantly greater in OVX rats than in sham-operated rats, suggesting that ovarian hormone deficiency following ovariectomy causes the skin temperature increase through the hypervasodilation. The hypervasodilation is thought to be due to the increase in the number of CGRP receptors in arteries because the B$_{max}$ value indicating the number of CGRP receptors was higher in OVX rats.

Endogenous CGRP synthesized primarily in dorsal root ganglia neurons is transported towards the peripheral nerve endings and released into the circulation (Zaidi et al. 1985, Holzer 1992). In the present study, the endogenous CGRP level in the circulation was significantly lower in OVX rats than in sham-operated rats, suggesting that ovarian hormone deficiency following ovariectomy decreases the plasma CGRP level. It has been reported that both estrogen and progesterone, which are ovarian hormones, stimulate CGRP peptide synthesis in dorsal root ganglia neurons through increasing CGRP mRNA (Gangula et al. 2000a). Plasma CGRP levels in rats and humans have been reported to increase with pregnancy and decrease postpartum (Stevenson et al. 1986, Saggese et al. 1990, Gangula et al. 2000b). These findings suggest that ovarian hormones may be responsible for increased CGRP levels in the circulation. Therefore, it is considered that the decrease in plasma CGRP level in OVX rats is due to ovarian hormone deficiency following ovariectomy. Taken together, the present study suggests that the decrease of circulating endogenous CGRP following ovarian hormone deficiency may induce up-regulation of CGRP receptors, i.e. the increase in number of the receptors.

Other vasoactive peptides such as substance P and vasoactive intestinal peptide have been shown to induce vasodilation in mesenteric arteries, as well as CGRP (Kawasaki et al. 1988, Ishida-Yamamoto & Tohyama 1989). The CGRP family, such as adrenomedullin and amylin that have structural similarity to CGRP, is also widely distributed in various peripheral tissues as well as in the central nervous system, and induces a broad variety of biogenic effects including the most potent vasodilatory action (Juaneda et al. 2000). However, effects of these vasoactive neuropeptides on skin temperature have been little investigated. Therefore, we do not rule out the possibility that these neuropeptides induce potentiation of
the increase in skin temperature in OVX. They should be considered in future studies.

In conclusion, aCGRP-induced elevation of skin temperature was potentiated in OVX rats. This result was accompanied by parallel increases in blood flow caused by vasodilation and up-regulation of CGRP receptors following the lower concentration of plasma CGRP in OVX rats. On the basis of these results, we speculate that ovarian hormone deficiency increases CGRP receptors and consequently amplifies the stimulatory effects of CGRP to elevate skin temperature. A majority of hot flashes are reported by women around menopause, and are a consequence of decreasing ovarian hormone concentrations (Sturdee & Reece 1979, Rebar & Spitzer 1987, Kronenberg 1994, Freedman 2001). Valentini et al. (1996) reported that plasma CGRP levels in postmenopausal women are lower than in fertile women. Thus, the up-regulation of CGRP receptors may be involved in the mechanism underlying menopausal hot flashes.

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