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

M Noguchi, Y Ikarashi, M Yuzurihara, K Mizoguchi, K Kurauchi, J-T Chen¹ and A Ishige

Kampo and Pharmacognosy Laboratories, Tsumura & Co., 3586 Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki 300-1192, Japan

¹J T Chen Clinic, Sunbright Twin 3F, 2-46-1 Honcho, Nakano-ku, Tokyo 164-0012, Japan

(Requests for offprints should be addressed to M Noguchi; Email: noguchi_masamichi@mail.tsumura.co.jp)

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 α CGRP (10 μ g/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 μ g/kg i.v.), a CGRP receptor antagonist, in a dose-dependent manner. In addition, ovariectomy not only potentiated vasorelaxation due to α CGRP 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 α CGRP-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 $23 \pm 2^\circ\text{C}$, relative humidity of $55 \pm 10\%$ and a 12 h light:12 h darkness cycle, with lights on from 0700 to 1900 h daily.

The rats were anesthetized with 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 α CGRP and human CGRP₈₋₃₇ were purchased from Peptide Institute Inc. (Osaka, Japan). 125 I-CGRP (specific activity 2000 Ci/mmol) was purchased from Peninsula Laboratories Inc. (San Carlos, CA, USA). Prostaglandin (PG) F_{2 α} , 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 ($n=33$) or sham-operated ($n=34$) 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 CGRP₈₋₃₇ (100, 500 or 1000 μ g/kg) dissolved in saline was injected i.v. into the tail vein after basal temperature was stable, and α CGRP (10 μ g/kg i.v.) dissolved in saline was injected 10 min later.

The area under the temperature curve (AUC) following the injection of α CGRP was calculated using PAG-CP (Pharmacokinetic Analysis and Graphics for Clinical Pharmacology) analysis (Medical Research AS Medica, Osaka, Japan).

Measurement of vasorelaxation response in isolated rat mesenteric vascular beds

Changes in the resistance of the peripheral vascular bed were evaluated according to the method described by Kawasaki *et al.* (1988). In brief, a section of mesenteric vascular bed that included small resistance arteries was isolated from OVX ($n=11$) or sham-operated ($n=9$) rats anesthetized with pentobarbital (50 mg/kg i.p.). The isolated vascular bed was 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% CO₂ in O₂. The modified Krebs solution was composed of (in mM): NaCl 120.0, KCl 5.0, MgSO₄ 1.2, NaHCO₃ 25.0, CaCl₂ 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 μ M PGF_{2 α} was continuously perfused to elevate the pressure by vasoconstriction of the vascular bed. After the PGF_{2 α} -induced vasocontraction reached a plateau, various concentrations of α CGRP (10⁻¹¹–10⁻⁹ mol/10.0 μ l) 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_{2 α} -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 ($n=20$) or sham-operated ($n=20$) 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 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.

CGRP receptor binding assay was performed by a modification of the method of Wimalawansa *et al.* (1987). In brief, the membrane suspension (20–25 μ g protein) was incubated with various concentrations of 125 I-CGRP in 100 μ l 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 polyethylenamine 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. The maximal number of binding sites ($B_{\max}$) and the dissociation constant (K_d) were calculated by Scatchard analysis.

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).

Statistical analysis

All values were represented as means $\pm$ 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 $P<0.05$.

Results

α CGRP-induced elevation of skin temperature and antagonism with the CGRP receptor antagonist human CGRP₈₋₃₇ in OVX rats

Changes in skin temperature following injection of α 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 α 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 CGRP₈₋₃₈ (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 CGRP₈₋₃₇ (100–1000 µg/kg i.p.) in a dose-dependent manner.

Vasorelaxation effect of α CGRP on PGF_{2 α} -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 α} (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 α} -induced contraction between vascular beds obtained from OVX (40.27 $\pm$ 0.71 mmHg) and sham-operated rats (41.57 $\pm$ 1.42 mmHg). As shown in Fig. 2, α CGRP (10⁻¹¹–10⁻⁹ mol) relaxed the PGF_{2 α} -induced contraction in the vascular bed isolated 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 ($F(1,108)=29.812$, $P<0.001$).

Changes in ¹²⁵I-CGRP binding in isolated mesenteric arteries of OVX rats

The effect of ovariectomy on ¹²⁵I-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 ¹²⁵I-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.

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 $\pm$ 0.53 pmol/l) was significantly lower ($P<0.01$) than that in sham-operated rats (13.46 $\pm$ 1.56 pmol/l).

Discussion

We first of all demonstrated in the present study that i.v. injection of α 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 α CGRP-induced elevation of skin temperature was inhibited by human CGRP₈₋₃₇ in both OVX and sham-operated rats. Human

CGRP₈₋₃₇ is the most potent CGRP antagonist available to date and appears to differentiate between the two CGRP receptors called CGRP₁ and CGRP₂. CGRP₁

receptors are characterized by their sensitivity to the antagonistic actions of CGRP₈₋₃₇, whereas CGRP₂ receptors are resistant to CGRP₈₋₃₇ (Dennis *et al.* 1990, Mimeault *et al.* 1991, Holzer 1992). It has been demonstrated that CGRP₁ receptor antagonist human CGRP₈₋₃₇ exhibits higher affinity for the CGRP receptors in rat left atrium, when human α CGRP and rat α CGRP are used as an agonist; human CGRP₈₋₃₇ blocks human α CGRP- and rat α CGRP-induced responses with pA₂ values of 7.17 and 7.35 respectively (Juaneda *et al.* 2000, Wu *et al.* 2000). In addition, human CGRP₈₋₃₇ inhibits rat α CGRP-induced vasorelaxation in isolated rat mesenteric vascular beds (Han *et al.* 1990). These suggest that human CGRP₈₋₃₇ is an effective antagonist against rat and human CGRP₁ receptors. Therefore, it is suggested that the α CGRP-induced elevation of skin temperature in the present study may be due to the response through CGRP₁ 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 CGRP₁ subtype. The ¹²⁵I-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 CGRP₁ receptor and CGRP-mediated responses, in place of skin arteries. To clarify the mechanism underlying the potentiation of α CGRP (CGRP₁

Figure 1 Effects of the CGRP antagonist human CGRP₈₋₃₇ on the skin temperature response to α CGRP in sham-operated (A) and OVX rats (B). AUC data following injection of α CGRP (10 µg/kg i.v.) in each treatment are shown in (C). α CGRP was administered 10 min after pretreatment with saline (○) or human CGRP₈₋₃₇ (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 $\pm$ 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 CGRP₈₋₃₇ 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 α CGRP-induced elevation of skin temperature was compared between sham-operated control and OVX control using Student's *t*-test; † $P<0.05$, †† $P<0.01$.

Figure 2 Vasodilator response of α CGRP on $\text{PGF}_{2\alpha}$ -induced contraction of mesenteric vascular beds isolated from sham-operated and OVX rats. Data calculated as percent relaxation of the maximum pressure obtained by $\text{PGF}_{2\alpha}$ -induced contraction are expressed as means $\pm$ S.E.M. Factorial two-way ANOVA analysis revealed that there were significant effects of group ($F(1,108)=29.812$, $P<0.001$), α CGRP dose ($F(5,108)=210.201$, $P<0.001$) and interaction of group $\times$ dose ($F(5,108)=3.836$, $P<0.01$). In this Figure, significance with post hoc analyses (Dunnett's *t*-test) following the ANOVA analysis are indicated; ** $P<0.01$ compared with corresponding values in sham-operated rats.

Figure 3 Scatchard plot of CGRP receptor binding assay in mesenteric arteries isolated from sham-operated and OVX rats. Each value is the mean of duplicate determinations using the pooled plasma membrane isolated from sham-operated ($n=20$) or OVX ($n=20$) rats.

activator)-induced elevation of skin temperature in OVX rats, therefore, we investigated the changes in α CGRP-induced relaxation and the number of CGRP receptors using mesenteric arteries isolated from OVX rats. We demonstrated that infusion of α CGRP induced vasodilation (or vasorelaxation) of isolated arteries contracted by $\text{PGF}_{2\alpha}$. 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 α CGRP 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 α CGRP-induced vasorelaxation was significantly greater in OVX rats than in sham-operated rats, suggesting that ovarian hormone deficiency following OVX 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 perivascular 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 circulatory 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, α CGRP-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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