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Significance of measured elevation of skin temperature induced by calcitonin gene-related peptide in anaesthetized rats

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Abstract

To assess whether peripheral changes related to skin temperature rise were induced by ovarian hormone deficiency, we investigated the effects of anaesthesia on calcitonin gene-related peptide (CGRP)- or luteinizing hormone-releasing hormone (LH-RH)-induced elevation of skin temperature in female rats. CGRP was used as an inducer of peripherally-mediated elevation of skin temperature, whereas LH-RH was used as an inducer of centrally-mediated elevation of skin temperature. Intravenous (i.v.) but not intracerebroventricular injection of CGRP (10 μg kg^{-1}) or intracerebroventricular but not intravenous injection of LH-RH (10 μg/rat) elevated the skin temperature of unanaesthetized rats restrained in a Ballman's cage. The elevation with LH-RH was completely inhibited by urethane anaesthesia, whereas the elevation with CGRP was not. These results suggested that changes in skin temperature measured under anaesthesia reflected a peripherally rather than a centrally mediated mechanism. The CGRP (1.0–30 μg kg^{-1}, i.v.)-induced elevation of skin temperature was potentiated in ovariectomized rats and inhibited by pretreatment with a CGRP receptor antagonist CGRP$_{8-37}$ (1000 μg kg^{-1}, i.v.), suggesting that the potentiation may participate in peripheral factors such as a postsynaptic hypersensitivity to CGRP following ovarian hormone deficiency. Thus, measurement of skin temperature in the anaesthetized rat was a useful procedure to seek the peripheral mechanism of potentiation of skin temperature induced by CGRP, thought to be closely related to menopausal hot flashes.

Introduction

The hot flash is a climacteric disturbance in women. 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). It is thought that the drastic increase in skin temperature is caused by the increase in blood flow to the skin due to peripheral vasodilation (Kronenberg 1994). To date, several hypotheses have been considered to explain the thermoregulatory characteristics of a hot flash (Chen et al 1993; Lomax & Schönbaum 1993; Freedman 2001). Centrally- and peripherally-mediated regulation have been suggested to participate in elevation of skin temperature (Kronenberg & Downey 1987). Luteinizing hormone-releasing hormone (LH-RH) is suggested as an inducer of centrally-mediated elevation of skin temperature because activation of cerebral LH-RH neurons induces a skin temperature rise by secondarily activating autonomic vasodilation through a downward shift in the set point of the hypothalamic thermoregulatory center (Lomax et al 1980; Katovich et al 1989). Calcitonin gene-related peptide (CGRP) is one of the potent vasodilator neuropeptides that is suggested as an inducer of peripherally-mediated elevation of skin temperature because the increase in peripheral blood flow accompanied by vasodilation with CGRP induces a skin temperature rise (Kawasaki et al 1988; Noguchi et al 2002b). In addition, hot flashes are considered to be closely related to ovarian hormone deficiency (Lomax & Schönbaum 1993; Kronenberg 1994). However, the detailed mechanism underlying this symptom has not been clarified.

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Complicated interactions of peripheral and central mechanisms obstruct the clarification of the mechanism.

Until now, to clarify the thermoregulatory mechanism, rats have often been used as the experimental animal (Rand et al 1965; Kobayashi et al 1995; Hosono et al 1997). Since the tail and the plantar face of both hind paws of the rat are 'naked' i.e. without the thermal insulation of fur, and have a comparatively large ratio of surface to volume, these regions are used for measurement of skin temperature (Rand et al 1965; Kobayashi et al 1995; Hosono et al 1997; Noguchi et al 2002a). Most studies in which tail skin temperatures were recorded have required restraint devices to prevent the rats from removing the probes that monitor skin temperature (Kobayashi et al 1995). However, it has been reported that immobilization or restraint is stressful for the animal, and animal-to-animal variation in responses to various pharmacological agents occurs (Wright & Katovich 1996; Merchenthaler et al 1998). Some studies have evaluated changes in skin temperature in anaesthetized rats to solely avoid the influence of stress associated with restraint (Merchenthaler et al 1998). In general, anaesthesia inhibits the activity of the central nervous system (Marshall & Longnecker 1995). This point interests us greatly and so for this investigation, we hypothesized that skin temperature measured under anaesthesia may reflect a selectively peripherally-mediated response. If so, centrally-mediated regulation of skin temperature would be completely blocked by anaesthesia.

In this study we have aimed to clarify the significance of skin temperature measured in anaesthetized rats. For this purpose, LH-RH and CGRP were selected as the inducers to elevate skin temperature. Firstly, we confirmed in unanaesthetized rats that LH-RH or CGRP was a central or peripheral inducer to elevate skin temperature, by injecting both substances into cerebral ventricle and vein. Secondly, to clarify the effect of anaesthesia on the centrally- or peripherally-mediated elevation of skin temperature, we examined whether or not intravenous (i.v.) and intracerebroventricular (i.c.v.) injections of LH-RH or CGRP elevated skin temperature, in anaesthetized rats. Finally, CGRP-induced elevation of skin temperature was compared between ovariectomized and sham-operated rats in an anaesthetized condition.

Materials and Methods

Animals

Ten-week-old female Sprague-Dawley rats (200–250 g; Charles River Laboratories, Yokohama, Japan) were allowed free access to water and standard laboratory food. The animals were housed in stainless steel cages at a temperature of $23 \pm 2\,^{\circ}C$, a relative humidity of $55 \pm 10\%$, with a 12-h light/dark cycle (lights on from 07 00 h to 19 00 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.

Reagents

Rat αCGRP and human $CGRP_{8-37}$, which is a $CGRP_1$ receptor antagonist, were purchased from Peptide Institute Inc. (Osaka, Japan). An LH-RH agonist (Des-Gly^{10}-[im-BZl-D-His^6]LH-RH ethylamide), urethane and chloralose (α-chloralose) were purchased from Sigma Chemical Co. (St Louis, MO). Sodium pentobarbital was purchased from Dinabot Laboratories (North Chicago, IL). Other reagents used for analysis were the highest purity commercially available.

Surgical procedure for implantation of a guide cannula into cerebral ventricle in rats

Rats were anaesthetized with sodium pentobarbital ($50\,mg\,kg^{-1}$, i.p.) and placed on a stereotaxic frame. The guide cannula was implanted into the right lateral ventricle (co-ordinates: posterior 0.80 mm and right lateral 1.20 mm from the bregma, and ventral 3.70 mm from the skull surface) according to a rat brain atlas (Paxinos & Watson 1986). The guide cannula implantation was carried out only to rats that were going to receive LH-RH or CGRP intracerebroventricularly. The rats were used in the experiments seven days after the cannula implantation. The placement of the guide cannula in the brain was verified visually on frontal sections of formalin-fixed brain following injection of 5 μL 5% Evans blue solution into the cannula after completion of the experiment.

Effect of anaesthesia on CGRP or LH-RH-induced elevation of tail skin temperature in rats

Female rats were divided into two groups: unanaesthetized ($n = 34$) and anaesthetized ($n = 30$). In the unanaesthetized group each rat was restrained in a Ballman's cage. In the anaesthetized group each rat was anaesthetized with an intraperitoneal (i.p.) injection of urethane ($0.75\,g\,mL^{-1}\,kg^{-1}$) containing chloralose ($0.06\,g\,mL^{-1}\,kg^{-1}$). A thermistor probe (SXN-54, Technol Seven Co. Ltd, Yokohama, Japan) was taped on the tail of all anaesthetized and unanaesthetized rats. The basal temperatures of all rats were stable 40 min later. During this 40 min, changes in the basal temperature were automatically measured at 5-min intervals. Thereafter, half of the animals in each group were injected with LH-RH ($10.0\,\mu g\,mL^{-1}\,kg^{-1}$) or CGRP ($10.0\,\mu g\,mL^{-1}\,kg^{-1}$) dissolved in saline into a tail vein (i.v.) and the other half were injected with LH-RH (10.0 μg/10.0 μL) or CGRP (10.0 μg/10.0 μL) dissolved in saline into a cerebral ventricle through a guide cannula. Changes in skin temperature following intravenous or intracerebroventricular injection of LH-RH or CGRP were automatically measured at 5-min intervals for 120 min.

CGRP-induced changes in regional skin temperatures in anaesthetized rats

Ten female rats were used. Each animal was anaesthetized with urethane ($0.75\,g\,mL^{-1}\,kg^{-1}$, i.p.) containing

chloralose ($0.06\,g\,mL^{-1}\,kg^{-1}$). Four of five thermistor probes (SXN-54, Technol Seven Co. Ltd) were taped to a hind paw, ear, tail and back skin (fur was removed with a hair clipper), respectively, and the other probe was inserted into the rectum. The basal temperature of all rats was stable 40 min later. During this 40 min, changes in the basal temperature were automatically measured at 5-min intervals. Thereafter, CGRP ($10\,\mu g\,kg^{-1}$) was injected into the tail vein. Changes in skin temperature following injection of CGRP were automatically measured at 5-min intervals for 120 min.

Effect of ovariectomy on CGRP-induced paw skin temperature

Female rats were anaesthetized with sodium pentobarbital ($50\,mg\,kg^{-1}$, i.p.) and bilaterally ovariectomized or sham-operated as controls. We used a surgical technique for ovariectomy that has been established by monitoring decreased concentration of plasma estradiol and decreased weight of uterine tissue (Noguchi et al 2003). These animals were used three weeks after surgery. On the day of the experiment, ovariectomized ($n = 54$) or sham-operated ($n = 54$) rats were anaesthetized with urethane ($0.75\,g\,mL^{-1}\,kg^{-1}$, i.p.) containing chloralose ($0.06\,g\,mL^{-1}\,kg^{-1}$). Two thermistor probes (SXN-54, Technol Seven Co. Ltd) were then taped to the plantar face of both hind paws. The basal temperature of all rats was stable 40 min later. During this 40 min, changes in the basal temperature were automatically measured at 5-min intervals. Thereafter, CGRP (1.0–30 $\mu g\,kg^{-1}$) was injected into the tail vein 10 min after $CGRP_{8-37}$ ($1000\,\mu g\,kg^{-1}$, i.v.) was pre-injected. Changes in skin temperature following injection of CGRP were automatically measured at 5-min intervals for 120 min.

Data analysis

Changes in skin temperature after injection of LH-RH or CGRP were plotted for each animal, and the area under the temperature curve (AUC) was calculated using Pharmacokinetic Analysis and Graphics for Clinical Pharmacology analysis (Medical Research AS Medica, Osaka, Japan).

All values were represented as the mean ± s.e.m. The statistical significance of data was evaluated by a two-way or one-way analysis of variance followed by Dunnett's *t*-test, Scheffe's *F*-test or Student's *t*-test. For all tests the significance level was accepted at $P < 0.05$.

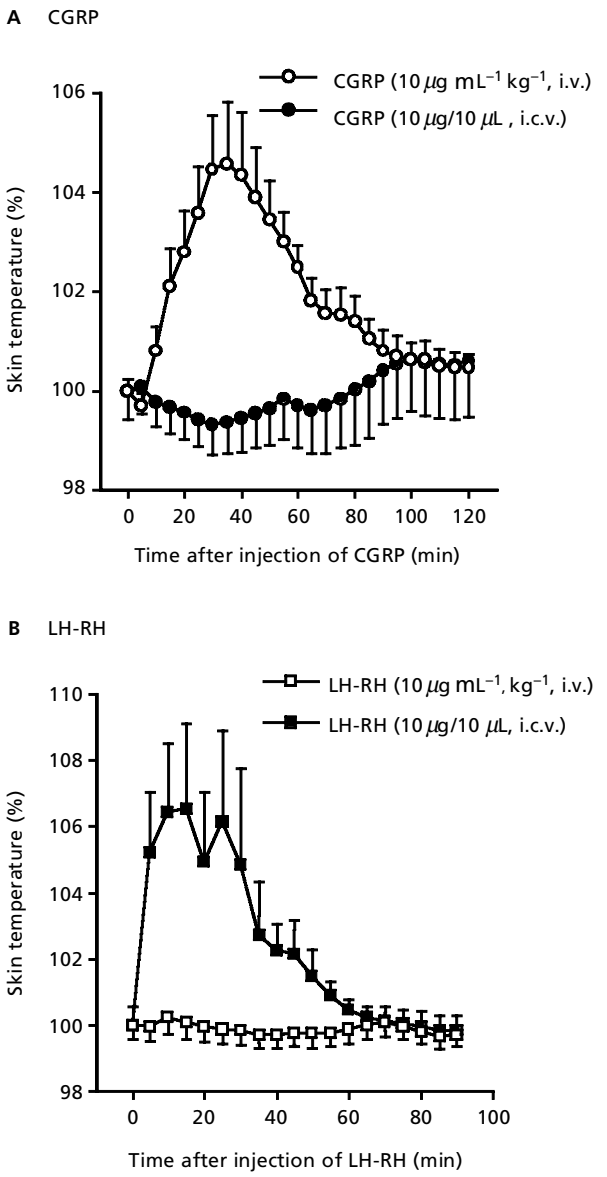

Figure 1 Changes in tail skin temperatures in unanaesthetized rats following intravenous and intracerebroventricular injections of CGRP (A) or LH-RH (B). An awake or conscious rat was restrained in a Ballman's cage. Each value is expressed as the mean ± s.e.m. ($n = 7$–9). Two-way analysis of variance revealed significant effects of different injection routes and times in CGRP-treated or LH-RH-treated rats. The significance between the two groups at each time point was assayed by Student's *t*-test. The significance ($P < 0.05$) was observed during 15–65 min after CGRP injection and during 5–50 min after LH-RH injection.

Results

Effect of anaesthesia on CGRP- or LH-RH-induced elevation of tail skin temperature in rats

Changes in tail skin temperature following intravenous and intracerebroventricular injections of CGRP or LH-RH in unanaesthetized rats are shown in Figure 1. Intravenous but not intracerebroventricular injection of CGRP ($10\,\mu g\,kg^{-1}$) or intracerebroventricular but not intravenous injection of LH-RH ($10\,\mu g$/rat) elevated tail skin temperature. Figure 2 shows the changes in tail skin temperature following intravenous and intracerebroventricular injections of CGRP or LH-RH in anaesthetized rats. Intravenous but not intracerebroventricular injection of CGRP ($10\,\mu g\,kg^{-1}$) elevated tail skin temperature as did the lack of anaesthesia. However, the elevation with

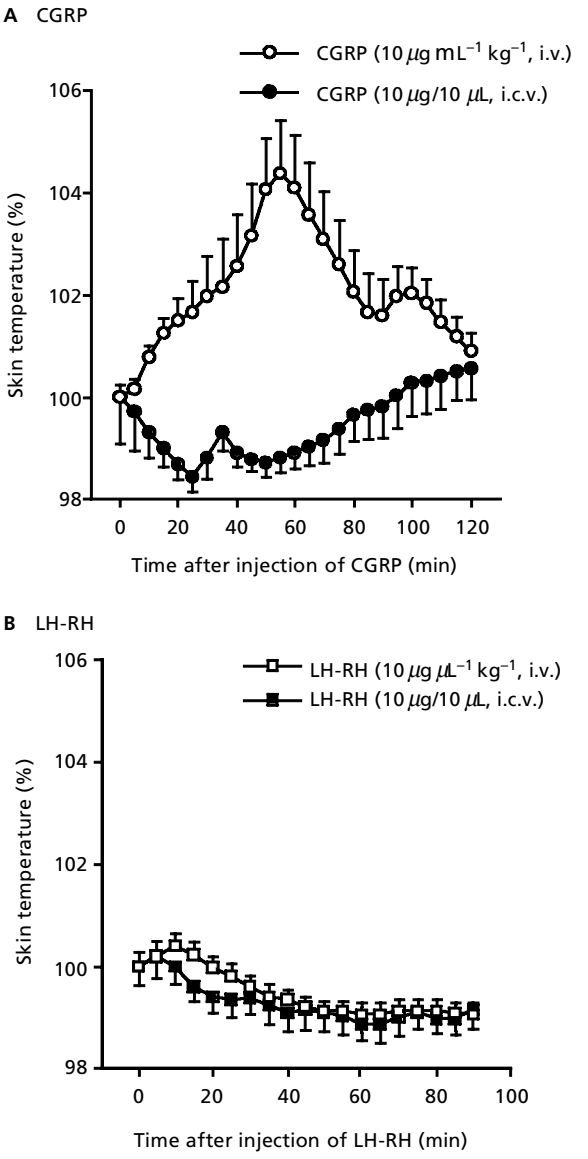

Figure 2 Changes in tail skin temperatures in anaesthetized rats following intravenous and intracerebroventricular injections of CGRP (A) or LH-RH (B). Rats were anaesthetized with intraperitoneal injection of urethane containing chloralose. Each value is expressed as the mean ± s.e.m. (n = 7–8). Factorial two-way analysis of variance revealed significant effects of different injection routes and times in CGRP-treated or LH-RH-treated rats. The significance between the two groups in each time point was assayed by Student's *t*-test. The significance ($P < 0.05$) was observed during 10–80 min after CGRP injection.

intracerebroventricular injection of LH-RH observed in the unanaesthetized condition was completely inhibited by the anaesthesia.

CGRP-induced changes in regional skin temperature in anaesthetized rats

Changes in skin temperature of hind paw, ear, tail and back regions, and of rectal temperature following injection of CGRP (10 μg kg^{-1}, i.v.) were simultaneously measured in anaesthetized rats. The results are shown in Figure 3. Skin temperature of the hind paw (111.09 ± 0.96% increase at 55 min), tail (103.57 ± 0.34% increase at 60 min) and ear (101.92 ± 0.59% increase at 20 min) reached maximal elevation at different times after the injection, and gradually declined. In particular, the ear temperature declined below the basal temperature. When the maximal skin temperature was compared among these regions, the hind temperature was significantly greater ($P < 0.01$) than tail and ear temperatures. The skin temperature on the back gradually decreased after injection of CGRP, as did rectal temperature.

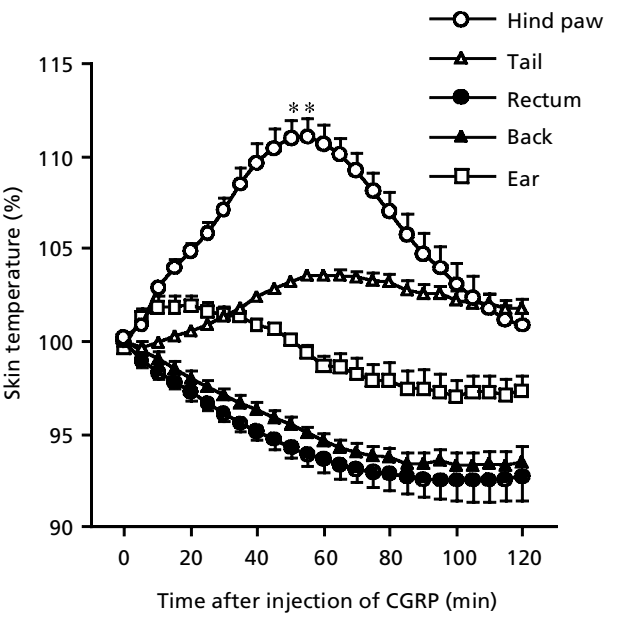

Figure 3 Changes in regional skin temperatures of the hind paw, ear, tail, back and rectal temperature following intravenous injection of CGRP into anaesthetized female rats. Changes in each regional temperature following injection of CGRP (10 μg kg^{-1}, i.v.) were calculated as a percentage of each basal skin temperature. Time-dependent changes in the skin temperatures following injection of CGRP were evaluated by post hoc Dunnett's *t*-test following a one-way analysis of variance. The significance ($P < 0.05$) was observed during 10–110 min in the hind paw, 25–120 min in the tail, 10–30 min in the ear, 10–120 min in the back and 5–120 min in the rectum, compared with the basal level of each region. When the maximal skin temperatures were compared among the hind paw, tail and ear by using post hoc Scheffe's *F*-test following a one-way analysis of variance, the hind paw temperature was significantly greater (**$P < 0.01$) than tail and ear temperatures.

CGRP-induced elevation of skin temperature on the hind paws and antagonism with the CGRP receptor antagonist $CGRP_{8-37}$ in anaesthetized ovariectomized rats

Changes in skin temperature of the hind paws following injection of various doses of CGRP (1.0–30 μg kg^{-1}, i.v.) in sham-operated or ovariectomized rats are shown in

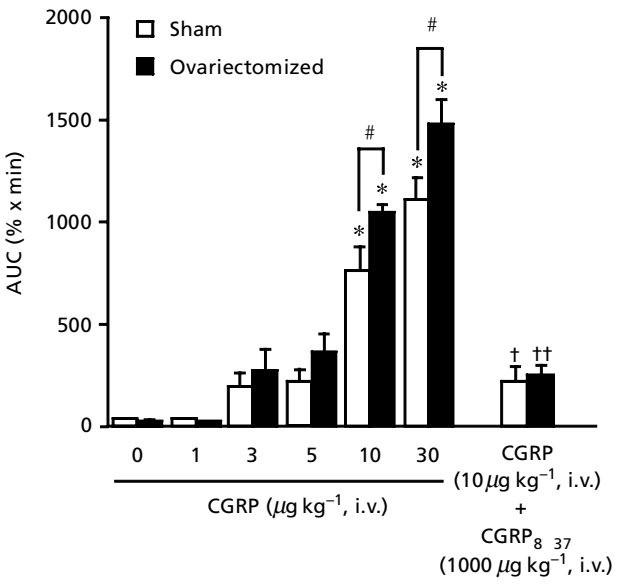

Figure 4 Dose-dependent elevation of skin temperature of hind paws induced by CGRP, and antagonism of the $CGRP_1$ receptor antagonist $CGRP_{8-37}$ in sham-operated and ovariectomized rats under anaesthesia. AUC data was expressed as the mean ± s.e.m. (n = 5–10). Factorial two-way analysis of variance revealed that there were significant effects of group (sham and ovariectomized) and dose. Dose-dependent changes in the skin temperatures following CGRP injection in each group were evaluated by one-way analysis of variance, and then the significance using post hoc Dunnett's *t*-test is indicated as $^{*}P < 0.01$, compared with saline-injected controls in each group. The effect of $CGRP_{8-37}$ ($1000\,\mu g\,kg^{-1}$) on CGRP ($10\,\mu g\,kg^{-1}$)-induced elevation of skin temperature was assessed using Student's *t*-test, and the significance is indicated as $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared with the results induced by $10\,\mu g\,kg^{-1}$ CGRP in each group. The effect of ovariectomy on CGRP-induced elevation of skin temperature in each dose was compared between sham-operated and ovariectomized groups using Student's *t*-test, and the significance is indicated as $^{\#}P < 0.01$.

Figure 4. Elevation of skin temperature was significantly greater in ovariectomized rats than in sham-operated rats, in a dose-dependent manner. The CGRP ($10\,\mu g\,kg^{-1}$, i.v.)-induced elevations of skin temperature in both operated rats were inhibited by pretreatment with $CGRP_{8-37}$ ($1000\,\mu g\,kg^{-1}$, i.v.), which is a $CGRP_1$ receptor antagonist.

Discussion

A rise in skin temperature is considered to be due to the increase in blood flow by vasodilation in the skin and is greatly affected by both central and peripheral regulation (Kronenberg & Downey 1987). This study demonstrated that intracerebroventricular but not intravenous injection of LH-RH resulted in an apparent elevation of tail skin temperature in awake rats. Katovich et al (1989) demonstrated that intravenous injection of LH-RH agonist did not induce the elevation of skin temperature. Taken together, our results suggested that LH-RH was an inducer of centrally-mediated elevation of skin temperature. This is supported by the results of studies by Lomax et al (1980) and Katovich et al (1989); activation of LH-RH neurons was suggested to cause elevation of skin temperature by secondarily activating autonomic vasodilation through a sudden downward shift in the set point of the hypothalamic thermoregulatory centre. Furthermore, the elevation with intracerebroventricular injection of LH-RH observed in the unanaesthetized condition was completely inhibited by the anaesthesia. This result suggested that anaesthesia completely inhibited the centrally-mediated mechanism that raised the skin temperature. On the other hand, CGRP is one of a number of potent vasodilator neuropeptides that has been demonstrated to increase peripheral blood flow and elevate skin temperature (Kawasaki et al 1988; Noguchi et al 2002b). In this study, the peripheral but not the cerebral injection of CGRP elevated skin temperature. In addition, the CGRP-induced elevation of skin temperature was not inhibited by the anaesthesia, unlike that of LH-RH. These results suggested that the elevation of skin temperature induced by intravenous injection of CGRP in anaesthetized rats reflected a selectively peripherally mediated response.

Katovich et al (1986) and Kobayashi et al (1995) suggested that rat-tail skin temperature was a good indicator of skin temperature regulation. However, the results shown in Figure 3 indicated that CGRP-induced elevation of skin temperature was more pronounced in the hind paw than in the tail and ear. In addition, the temperature in the ear showed a biphasic response, and the temperature of the back and rectum declined. These results suggested that different mechanisms may be involved in the different skin regions, although the reasons were unclear in this study. From these results, we selected the hind paw as the region for evaluation of skin temperature-elevation induced by CGRP.

Recently, Chen et al (1993) revealed a positive correlation between plasma levels of CGRP and the frequency of hot flashes in menopausal women and an increase in plasma levels during hot flashes in the same patients, suggesting that CGRP plays an important role in occurrence of hot flashes. In this study, the effect of ovarian hormone deficiency on CGRP-induced elevation of skin temperature was examined in ovariectomized rats three weeks after ovariectomy, because we had already confirmed that estrogen concentration in the serum significantly decreased three weeks after ovariectomy in rats (Noguchi et al 2003). Under an anaesthetized condition, intravenous injection of CGRP caused an elevation of skin temperature in a dose-dependent manner that was significantly greater in the ovariectomized rats than in sham-operated rats. The CGRP-induced elevation of skin temperature was inhibited by $CGRP_{8-37}$. $CGRP_{8-37}$ is the most potent CGRP receptor 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). Therefore, it is

suggested that the elevation of skin temperature induced by exogenous CGRP in ovariectomized rats was due to a response through $CGRP_1$ receptors. This result suggested that estrogen deficiency due to ovariectomy induced peripheral hypersensitivity to exogenous CGRP. More recently, we demonstrated that the endogenous CGRP level in circulation was significantly lower in ovariectomized rats than in sham-operated rats (Noguchi et al 2002a). This agrees with the results of a study by Valentini et al (1996), demonstrating that plasma CGRP levels in postmenopausal women were lower than in fertile women. In addition, we have demonstrated that the decrease of circulatory endogenous CGRP following ovarian hormone deficiency induces up-regulation of CGRP receptors i.e. increases the number of receptors (Noguchi et al 2002a) in peripheral vessels. On the basis of these results, we suggest that ovarian hormone deficiency increased CGRP receptors and consequently amplified the stimulatory effects of CGRP to elevate skin temperature. Thus, the up-regulation of CGRP receptors may be involved in the mechanism underlying menopausal hot flashes.

Estrogen deficiency in the climacteric may affect various factors including peripheral and central mechanisms, and the complexity of their interactions obstructs the clarification of the mechanism. The present method of measuring foot temperature under anaesthesia in ovariectomized rats may serve as a useful procedure to discriminate between the central and peripheral factors underlying hot flash.

Conclusions

We have tried to clarify the significance of measured elevation of skin temperature induced by CGRP in anaesthetized rats. Measurement of the skin temperature on the hind paw in the anaesthetized rat is thought to be a useful procedure to study the peripheral mechanism of potentiation of skin temperature induced by CGRP, which has been suggested to be closely related to menopausal hot flashes.

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