Adrenal adrenaline- and noradrenaline-containing cells and
celiac sympathetic ganglia are differentially controlled by
centrally administered corticotropin-releasing factor and
arginine-vasopressin in rats

Naoko Yamaguchi-Shima^{a,*}, Shoshiro Okada^a, Takahiro Shimizu^a,
Daisuke Usui^{a,b}, Kumiko Nakamura^a, Lianyi Lu^a, Kunihiro Yokotani^a

^a*Department of Pharmacology, Graduate School of Medicine, Kochi
University, Nankoku, Kochi 783-8505, Japan*

^b*Department of Pediatrics, Graduate School of Medicine, Kochi
University, Nankoku, Kochi 783-8505, Japan*

* Correspondence to: Naoko Yamaguchi-Shima, Department of
Pharmacology, Graduate School of Medicine, Kochi University,
Nankoku, Kochi 783-8505, Japan. Tel and Fax: +81-88-880-2328

E-mail address: ynaoko@kochi-u.ac.jp

Abstract

The adrenal glands and sympathetic celiac ganglia are innervated mainly by the greater splanchnic nerves, which contain preganglionic sympathetic nerves originated from thoracic spinal cord. The adrenal medulla has two separate populations of chromaffin cells, adrenaline-containing cells (A-cells) and noradrenaline-containing cells (NA-cells), which have been shown to be differentially innervated by separate groups of the preganglionic sympathetic neurons. The present study was designed to characterize the centrally activating mechanisms of the adrenal A-cells, NA-cells and celiac sympathetic ganglia with expression of cFos (a marker for neural excitation), in regard to the brain prostanoids, in anesthetized rats. Intracerebroventricularly (i.c.v.) administered corticotropin-releasing factor (CRF) induced cFos expression in the adrenal A-cells, but not NA-cells, and celiac ganglia. On the other hand, i.c.v. administered arginine-vasopressin (AVP) resulted in cFos induction in both A-cells and NA-cells in the adrenal medulla, but not in the celiac ganglia. Intracerebroventricular pretreatment with indomethacin (an inhibitor of cyclooxygenase) abolished the CRF- and AVP-induced cFos expression in all regions described above. On the other hand, intracerebroventricular pretreatment with furegrelate (an inhibitor of thromboxane A₂ synthase) abolished the CRF-induced cFos expression in the adrenal A-cells, but not in the celiac ganglia, and also abolished the AVP-induced cFos expression in both A-cells and NA-cells in the adrenal medulla. These results suggest that centrally administered CRF activates adrenal A-

cells and celiac sympathetic ganglia by brain thromboxane A₂-mediated and other prostanoid than thromboxane A₂ (probably prostaglandin E₂)-mediated mechanisms, respectively. On the other hand, centrally administered AVP activates adrenal A-cells and NA-cells by brain thromboxane A₂-mediated mechanisms in rats.

Key words: Brain arachidonic acid cascade; Brain thromboxane A₂; cFos expression; Central sympatho-adrenomedullary outflow

1. Introduction

The relative importance of the adrenal medulla and sympathetic nervous system in various pathophysiological conditions has generally been inferred from measurement of plasma adrenaline and noradrenaline, since plasma adrenaline and noradrenaline have been shown to reflect the activity of adrenal medulla and sympathetic nervous system, respectively. Hypoglycemia causes the elevation of plasma adrenaline (Young et al., 1984; Fujino and Fujii, 1995; Vollmer et al., 1997), while hypotension elevates both catecholamines (noradrenaline > adrenaline) (Brown and Fisher, 1984; Vollmer et al., 2000). Immobilization stress elicits a robust increase in the plasma adrenaline and noradrenaline (Kvetnansky and Mikulaj, 1970; Jezova et al., 1999; Dronjak et al., 2004), while cold stress triggers a robust increase in plasma noradrenaline (Dronjak et al., 2004). Likewise, centrally administered neuropeptides such as corticotropin-releasing factor (CRF), arginine-vasopressin (AVP), bombesin, thyrotropin-releasing hormone, and α -calcitonin gene-related peptide also produce differential changes in the plasma catecholamines (Brown et al., 1979, 1985; Fisher et al., 1983; Brown and Fisher, 1984; Feuerstein et al., 1984; Hasegawa et al., 1993; Okuma et al., 1996; Yokotani et al., 2001; Okada et al., 2002).

In the adrenal medulla of both humans and rats, adrenaline and noradrenaline are localized in separate populations of cells with the number of adrenaline-containing cells (A-cells) about 4-fold greater than that of noradrenaline-containing cells (NA-cells) (Verhofstad et al., 1985; Suzuki and Kachi, 1996).

Despite the constancy of the stored amounts of adrenaline and noradrenaline, the proportion of each catecholamine secreted seems to vary depending on the strength and type of stimulus, suggesting a differential control of A-cells and NA-cells (Vollmer, 1996). Recently, adrenal A- and NA-cells have been shown to be innervated by separate groups of preganglionic sympathetic neurons located in the spinal cord (Edwards et al., 1996; Vollmer et al., 2000). Adrenaline secreted into circulation is almost produced within the adrenal A-cells (Axelrod, 1962; Wurtman, 2002), while plasma noradrenaline seems to reflect the secretion from adrenal NA-cells in addition to the release from sympathetic nerves (Folkow and von Euler, 1954; Vollmer et al., 1997; Yokotani et al., 2001, 2005; Okada et al., 2003a). However, little is known about centrally controlling mechanisms of adrenal A-cells, NA-cells and sympathetic nerves.

Arachidonic acid is metabolized rapidly to oxygenated products, such as prostaglandin E_2 and thromboxane A_2 , by several distinct enzymes including cyclooxygenase, prostaglandin E synthase and thromboxane A synthase (Flower and Blackwell, 1976; Irvine, 1982; Axelrod, 1990). Previously, we reported that centrally administered prostaglandin E_2 evokes noradrenaline release from sympathetic nerves by activation of the brain prostanoid EP_3 receptors (Yokotani et al., 1995, 2005). Recently we also reported that centrally administered CRF evokes the brain thromboxane A_2 -mediated adrenal adrenaline secretion and the other prostanoid (probably prostaglandin E_2)-mediated sympathetic noradrenaline release, while centrally administered AVP, bombesin and histamine evoke the brain thromboxane A_2 -

mediated adrenal secretion of both adrenaline and noradrenaline in rats (Yokotani et al., 2001, 2005; Okada et al., 2003a; Shimizu et al., 2006).

Since the greater splanchnic nerve, which contains sympathetic preganglionic nerves originated from spinal cord, ramifies into the adrenal gland and celiac sympathetic ganglia (Yokotani et al., 1983), these regions are very useful to identify the centrally activated adrenal A-, NA-cells and celiac sympathetic ganglia with nuclear expression of cFos, immediate early gene product (Sagar et al., 1988; Herrera and Robertson, 1996). In the present study, therefore, we examined the effects of centrally administered CRF and AVP on the cFos expression in these regions, in regard to the brain arachidonic acid cascade, to further clarify the presence of differential activating mechanisms for sympathetic nerves and adrenal medulla.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing about 350 g were maintained in an air-conditioned room at 22–24°C under a constant day-night rhythm for more than 2 weeks and given food (laboratory chow, CE-2; Clea Japan, Hamamatsu, Japan) and water ad libitum. All effort was made to minimize animal suffering and the number of animals used. All rats were treated in accordance with the Guiding Principles for the Care and Use of laboratory animals approved by the Graduate School of Medicine, Kochi University.

2.2. Experimental design

Under urethane anesthesia (1.2 g/kg, i.p.), the animal was placed in a stereotaxic apparatus, as shown in our previous paper (Yokotani et al., 2001). The skull was drilled for intracerebroventricular administration of test substances using stainless-steel cannula (0.3 mm outer diameter) or a double lumens cannula (0.5 mm outer diameter). The stereotaxic coordinates of the tip of cannula were as follows (in mm): AP - 0.8, L 1.5, V 4.0 (AP, anterior from the bregma; L, lateral from the midline; V, below the surface of the brain), according to the rat brain atlas (Paxinos and Watson, 1986). Then, 3 h were allowed to elapse before the application of test reagents. The peptides (CRF and AVP) were slowly injected into the right lateral ventricle in a volume of 10 µl using a 10 µl Hamilton syringe. CRF and AVP were dissolved in sterile saline. Water-

soluble indomethacin-Na and furegrelate dissolved in sterile saline were also administered into the right lateral ventricle in a volume of 10 μ l 30 min before the application of CRF or AVP. Correct placement of the cannula was confirmed at the end of each experiment by verifying that a blue dye, injected through the cannula, had spread throughout the entire ventricular system.

After several periods of time from injection of these peptide (CRF and AVP) into the right lateral ventricle (at 0, 10, 60 or 120 min after injection of CRF; at 0, 10 or 60 min after injection of AVP), each rat was perfused through the left cardiac ventricle with 100 ml of 0.1 M phosphate buffered saline (pH 7.4) followed by 300 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), as shown in our previous paper (Shima et al., 2003). Then, celiac ganglia around the celiac artery and adrenal glands were immediately removed, post-fixed over night with the same fixative for the celiac ganglia or with 3% potassium dichromate and 4% paraformaldehyde in 0.1 M phosphate buffer for the adrenal gland with a slight modification of the previously reported methods (Coupland et al., 1964; Piezzi et al., 1983), and then cryoprotected in 20% sucrose in 0.1 M phosphate buffer for 24 h at 4°C. Frozen sections (20 μ m-thickness) were cut on a cryostat, thaw-mounted on silane-coated slides, and then stored at -80°C until used.

2.3. Immunohistochemistry

Sections on the slides were washed in phosphate buffered saline and then pretreated with 5% normal donkey serum (for

adrenal glands) or normal goat serum (for celiac ganglia) in phosphate buffered saline containing 0.3% Triton X-100 for 1 h at room temperature to eliminate non-specific antibody binding. To codetect by double-immunostaining of cFos/phenylethanolamine *N*-methyltransferase (PNMT) in the adrenal medulla or cFos/dopamine- β -hydroxylase (DBH) in the celiac ganglia, sections were incubated in a cocktail of rabbit anti-cFos (1:500) and sheep anti-PNMT (1:200) antibodies for adrenal glands, or rabbit anti-cFos (1:500) and mouse anti-DBH (1:500) antibodies for celiac ganglia for 4 h at room temperature. After incubation, the sections were washed three times for 10 min each in phosphate buffered saline, and then incubated for 2 h at room temperature in a mixture of secondary antisera; fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:200) and rhodamine-conjugated donkey anti-sheep IgG (1:200), or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200) and rhodamine-conjugated goat anti-mouse IgG (1:200). After washing in phosphate buffered saline, nuclei were counterstained with 4',6-diamidino-2'-phenylindole dihydrochloride (1:1000) using sections from adrenal glands for chromaffin cell counting. After these procedures, the sections were coverslipped using mounting medium VECTASHIELD. All antisera were diluted in phosphate buffered saline containing 0.3% Triton X-100 and 10% normal serum produced in the animal species from which the respective secondary antibodies were obtained. Control experiments were performed by omitting primary antibodies as a test of cross-reactivity of secondary antibodies, and resulted in the absence of staining.

2.4. Image analysis and statistics

Photomicrographs were captured using a digital camera (DP70, Olympus, Tokyo, Japan) attached to a fluorescent microscope (AX70, Olympus). The chromaffin reaction method demonstrated chromaffin cells with a yellow cytoplasmic staining. In addition, immunohistochemical method for PNMT divided chromaffin cells into two cell-types, A-cells (PNMT-positive) and NA-cells (PNMT-negative). Therefore, cFos/PNMT double-staining cells indicate activated A-cells, while cFos-staining cells in the PNMT-negative chromaffin cells indicate activated NA-cells. In the celiac ganglia, DBH was used as a marker of noradrenergic neurons, and cFos/DBH double-staining indicate activated noradrenergic neurons. Quantification of the number of immunolabeled cells was carried out within a square of defined size (200 x 200 μ m) placed on each section of adrenal medulla and celiac ganglia. The number of cFos-positive cells, adrenal A-cells, NA-cells and ganglionic noradrenergic neurons were assessed by cell counting in five sections of each area per animal. The percentage of cFos-positive cells in the total A-cells, NA-cells and ganglionic noradrenergic neurons was calculated and expressed as the means $\pm$ S.E.M. Data were analyzed by one-way analysis of variance, followed by post-hoc analysis with the Bonferroni method. When only two means were compared, the data were analyzed by ANOVA followed by unpaired Student's *t*-test. *P* values less than 0.05 were taken to indicate significance.

2.5. Drugs

The following reagents were used: arginine-vasopressin, corticotropin-releasing factor (rat/human) (Peptide Institute, Osaka, Japan); •indomethacin sodium trihydrate (a kind gift from Merck, Rahway, NJ, U.S.A.); furegrelate sodium (Biomol Research Lab., Plymouth Meeting, PA, U.S.A.); anti-cFos (Calbiochem, San Diego, CA, U.S.A.); anti-dopamine- β -hydroxylase, anti-phenylethanolamine *N*-methyltransferase (Chemicon, Temecula, CA, U.S.A.); fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, rhodamine-conjugated donkey anti-sheep IgG, rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.); 4',6-diamidine-2'-phenylindole dihydrochloride (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.); VECTASHIELD (Vector Laboratories, Burlingame, CA, U.S.A.). All other reagents were the highest grade available (Nacalai Tesque, Kyoto, Japan).

3. Results

3.1. CRF- and AVP-induced cFos expression in the adrenal medulla and celiac ganglia

In the adrenal medulla, A-cells (PNMT-staining cells in the total chromaffin cells) were $74.2 \pm 5.9\%$ and NA-cells (PNMT-negative cells in the total chromaffin cells) were $25.8 \pm 5.9\%$ (n=44).

Administration of CRF (1.5 and 3.0 nmol/animal, i.c.v.) effectively increased cFos expression in the adrenal A-cells and noradrenergic neurons in the celiac ganglia, and these responses obtained with CRF (1.5 nmol/animal, i.c.v.) was greater than those obtained with CRF (3.0 nmol/animal, i.c.v.) (Fig. 1A,B,C,D,E,F,K,M). cFos expression in the adrenal A-cells gradually increased and reached a maximum 60 min after administration of CRF (1.5 and 3.0 nmol/animal) (Fig. 1K,M). In noradrenergic neurons in the celiac ganglia, a large dose of CRF (3.0 nmol/animal, i.c.v.) quickly evoked cFos expression than a small dose of CRF (1.5 nmol/animal, i.c.v.): maximal response was obtained at 60 min for CRF (1.5 nmol/animal, i.c.v.) and at 10 min for CRF (3.0 nmol/animal, i.c.v.), respectively (Fig. 1K,M). On the other hand, the peptide (1.5 and 3.0 nmol/animal, i.c.v.) had no effect on the adrenal NA-cells throughout the experimental period (from 0 min to 120 min) (Fig. 1C,E,K,M).

Administration of AVP (0.2 and 0.5 nmol/animal, i.c.v.) effectively increased the levels of cFos expression in the adrenal A-cells and NA-cells, and these responses were almost the same in both doses of AVP. The maximal cFos expressions in

these regions were obtained at 10 min after AVP administration (0.2 and 0.5 nmol/animal, i.c.v.) (Fig. 1G,I,L,N). On the other hand, the peptide (0.2 and 0.5 nmol/animal, i.c.v.) had no effect on noradrenergic neurons in the celiac ganglia throughout the experimental period (from 0 min to 60 min) (Fig. 1H,J,L,N).

In the following experiments, we examined further cFos expression at 60 min after the CRF (1.5 nmol/animal, i.c.v.) administration and 10 min after the AVP (0.2 nmol/animal, i.c.v.) administration, respectively.

3.2. Effects of indomethacin and furegrelate on the CRF-induced cFos expression in the adrenal medulla and celiac ganglia

Sixty min after intracerebroventricular treatments with vehicle-1 (10 μ l saline) plus vehicle-2 (10 μ l saline), indomethacin (an inhibitor of cyclooxygenase) [1.2 μ mol (500 μ g)/animal] plus vehicle-2, or furegrelate [1.8 μ mol (500 μ g)/animal] plus vehicle-2, there were little cFos-positive cells in each cell type in the adrenal medulla (A-cells and NA-cells) and noradrenergic neurons in the celiac ganglia: percentage of cFos-positive A-cells in total adrenal A-cells [4.2 $\pm$ 1.0% for vehicle-1 plus vehicle-2 (n=4), 4.0 $\pm$ 0.6% for indomethacin plus vehicle-2 (n=3), 5.5 $\pm$ 0.3% for furegrelate plus vehicle-2 (n=3)]; percentage of cFos-positive NA-cells in total adrenal NA-cells [3.9 $\pm$ 1.6 for vehicle-1 plus vehicle-2 (n=4), 3.0 $\pm$ 1.0% for indomethacin plus vehicle-2 (n=3), 4.5 $\pm$ 0.1% for furegrelate plus vehicle-2 (n=3)]; percentage of cFos-positive neurons in total noradrenergic neurons in the celiac ganglia

[1.1±1.1% for vehicle-1 plus vehicle-2 (n=4), 2.6±0.4% for indomethacin plus vehicle-2 (n=3), 1.8±0.4% for furegrelate plus vehicle-2 (n=3)] (Fig. 2A,B,I).

CRF (1.5 nmol/animal, i.c.v.) significantly increased cFos expression in the adrenal A-cells and noradrenergic neurons in the celiac ganglia, but had no effect on the adrenal NA-cells: percentage of cFos-positive cells in total cells in each group [A-cells, 32.4±1.1%; NA-cells, 2.1±2.1%; noradrenergic neurons in the celiac ganglia, 48.5±6.4% (n=4)] (Fig. 2C,D,I).

Pretreatment with indomethacin (1.2 µmol/animal, i.c.v.) abolished the CRF (1.5 nmol/animal, i.c.v.)-induced cFos expression in all regions (adrenal A-cells and noradrenergic neurons in the celiac ganglia): percentage of cFos-positive cells in total cells in each group [A-cells, 3.9±1.1%; NA-cells, 3.3±1.7%; noradrenergic neurons in the celiac ganglia, 6.1±4.6 (n=4)] (Fig. 2E,F,I).

Pretreatment with furegrelate (1.8 µmol/animal, i.c.v.) abolished the CRF-induced cFos expression in the adrenal A-cells, but had no effect on the noradrenergic neurons in the celiac ganglia: percentage of cFos-positive cells in total cells in each group [A-cells, 5.1±2.2%; NA-cells, 4.8±3.3%; noradrenergic neurons in the celiac ganglia, 41.4±8.6% (n=4)] (Fig. 2G,H,I).

3.3. Effects of indomethacin and furegrelate on the AVP-induced cFos expression in the adrenal medulla and celiac ganglia

Ten min after intracerebroventricular treatments with vehicle-1 (10 μ l saline) plus vehicle-2 (10 μ l saline), indomethacin (an inhibitor of cyclooxygenase) [1.2 μ mol (500 μ g)/animal] plus vehicle-2, or furegrelate [1.8 μ mol (500 μ g)/animal] plus vehicle-2, there were little cFos-positive cells in each cell type in the adrenal medulla (A-cells and NA-cells) and noradrenergic neurons in the celiac ganglia: percentage of cFos-positive cells in total A-cells [3.5 $\pm$ 0.9% for vehicle-1 plus vehicle-2 (n=4), 5.6 $\pm$ 1.4% for indomethacin plus vehicle-2 (n=3), 4.1 $\pm$ 1.4% for furegrelate plus vehicle-2 (n=3)]; percentage of cFos-positive cells in total NA-cells [5.0 $\pm$ 0.7% for vehicle-1 plus vehicle-2 (n=4), 4.7 $\pm$ 0.9% for indomethacin plus vehicle-2 (n=3), 4.0 $\pm$ 1.2% for furegrelate plus vehicle-2 (n=3)]; percentage of cFos-positive cells in total noradrenergic neurons in the celiac ganglia [2.5 $\pm$ 1.0% for vehicle-1 plus vehicle-2 (n=4), 2.5 $\pm$ 0.6% for indomethacin plus vehicle-2 (n=3), 3.8 $\pm$ 0.8 for furegrelate plus vehicle-2 (n=3)] (Fig. 3A,B,I).

AVP (0.2 nmol/animal, i.c.v.) significantly increased cFos expression in the adrenal A-cells and NA-cells, but had no effect on the noradrenergic neurons in the celiac ganglia: percentage of cFos-positive cells in total cells in each group [A-cells, 37.2 $\pm$ 1.0%; NA-cells, 40.2 $\pm$ 5.8%; noradrenergic neurons in the celiac ganglia, 2.0 $\pm$ 1.0% (n=4)] (Fig. 3C, D and I).

Indomethacin [1.2 μ mol (500 μ g)/animal, i.c.v.] abolished the AVP (0.2 nmol/animal)-induced cFos expression in all regions (the adrenal A-cells and NA-cells): percentage of cFos-positive cells in total cells in each group [A-cells, 5.3 $\pm$ 0.8%; NA-cells,

6.2±3.1%; noradrenergic neurons in the celiac ganglia, 3.2±3.2% (n=4)] (Fig. 3E,F,I).

Furegrelate [1.8 µmol (500 µg)/animal, i.c.v.] also abolished the AVP-induced cFos expression in all regions such as the adrenal A-cells and NA-cells: percentage of cFos-positive cells in total cells in each group [A-cells, 7.9±3.2%; NA-cells, 3.4±2.7%; noradrenergic neurons in the celiac ganglia, 1.1±1.1% (n=4)] (Fig. 3G,H,I).

4. Discussion

In many mammalian species, adrenomedullary chromaffin cells consist of A-cells and NA-cells. The former cells contain the enzyme PNMT, which converts noradrenaline to adrenaline. Previous studies with immunohistochemical staining for PNMT show that A-cells occupied large areas in the adrenal medulla, and NA-cells were scattered among A-cell regions (Edwards et al., 1996; Suzuki and Kachi, 1996; Phillips et al., 2001). In the present experiment, the number of A-cells is about 3-fold greater than that of NA-cells in the rat adrenal medulla. In the celiac ganglia around the celiac artery, there are many noradrenergic neurons containing the enzyme DBH, which converts dopamine to noradrenaline. In the present experiment, we used cFos expression as an indicator of neuronal and cellular activity. Although the DBH and the PNMT promoter regions do not contain an AP-1 site (Ebert et al., 1994; Morita et al., 1995; Sabban and Kvetnansky, 2001), suggesting that direct effects of cFos on the synthesis of these enzymes are unlikely, tyrosine hydroxylase (a rate-limiting enzyme in catecholamine synthesis) contains an AP-1 site in its promotor region (Chambi et al., 1989; Liu et al., 1997). Many previous studies have shown the colocalization of cFos and PNMT immunoreactivities to prove the neural activity in the adrenergic neuron in the brain (Jung et al., 2004; Lee et al., 2000; Ritter et al., 1998; Temel et al., 2002; Yang and Voogt, 2001).

Previously we reported that centrally administered CRF (0.5, 1.5 and 3.0 nmol/animal) and AVP (0.1, 0.2 and 0.5 nmol/animal) dose-dependently elevated plasma levels of noradrenaline and

adrenaline in anesthetized rats (Yokotani et al., 2001; Okada et al., 2002). In the first experiment, we examined the time course of cFos induction in the adrenal A- and NA-cells and noradrenergic neurons in the celiac ganglia in response to centrally administered CRF (1.5 and 3.0 nmol/animal) or AVP (0.2 and 0.5 nmol/animal). CRF effectively induced cFos expression in the adrenal A-cells and noradrenergic neurons in the celiac ganglia, although CRF even at a higher dose had no effect on the adrenal NA-cells throughout the experimental period (120 min). AVP also effectively induced cFos expression in both A- and NA-cells in the adrenal medulla, however AVP even at a higher dose had no effect on noradrenergic neurons in the celiac ganglia throughout the experimental period (60 min). Intravenous administration of these peptides had no effect on cFos expression in any regions. These results suggest that centrally administered CRF activates adrenal A-cells and celiac sympathetic ganglia, while centrally administered AVP activates both the adrenal A-cells and NA-cells in rats. These results are well consistent with our previous paper in which bilateral adrenalectomy abolished the AVP-induced elevations of plasma noradrenaline and adrenaline, while the procedure abolished only the CRF-induced elevation of plasma adrenaline in rats (Okada et al., 2003a). The differences in the time course of cFos expression between CRF- and AVP-treated rats may be, in part, due to the differences in their signal transductions in the brain. We previously reported the involvement of brain inducible nitric oxide synthase in the centrally administered CRF-induced increase of plasma catecholamines (Okada et al., 2003b). Brain nitric oxide has been shown to activate the brain cyclooxygenase,

thereby elevating plasma catecholamines in rats (Murakami et al., 1998).

In the second experiments, we examined the effects of indomethacin, a non-selective inhibitor of cyclooxygenase (Insel, 1996), on the centrally administered CRF- and AVP-induced cFos expression. The central pretreatment with indomethacin abolished the CRF-induced all cFos expression in the adrenal A-cells and noradrenergic neurons in the celiac ganglia and also abolished the AVP-induced all cFos expression in the adrenal A-cells and NA-cells. These results suggest the involvement of the brain arachidonic acid cascade in the CRF- and AVP-induced responses. The present results are well consistent with those of our previous report in which central pretreatment with indomethacin abolished the centrally administered CRF- and AVP-induced elevation of plasma catecholamines in rats (Yokotani et al., 2001; Okada et al., 2003a).

Finally, we examined the effects of furegrelate, a selective inhibitor of thromboxane A₂ synthase (Gorman et al., 1983), on the centrally administered CRF- and AVP-induced cFos expression. Central pretreatment with furegrelate abolished the CRF-induced cFos expression in the adrenal A-cells (but not noradrenergic neurons in the celiac ganglia), and also abolished the AVP-induced all cFos expression in the adrenal A-cells and NA-cells. These results indicate the involvement of brain thromboxane A₂ in the centrally administered CRF-mediated activation of adrenal A-cells and AVP-mediated activation of adrenal A- and NA-cells in rats. The present results are well consistent with our previous paper, in which the central pretreatment with

furegrelate abolished the CRF-induced elevation of adrenaline (but not noradrenaline) and the AVP-induced elevations of both adrenaline and noradrenaline in rats (Yokotani et al., 2001; Okada et al., 2003a).

A question has arisen which type of prostanoids is involved in the centrally administered CRF-induced cFos expression in the noradrenergic neurons in the celiac ganglia. In previous studies, we reported that centrally administered prostaglandin E₂ (but not prostaglandin D₂, prostaglandin F₂ or prostaglandin I₂) elevated plasma levels of noradrenaline alone by activation of the brain prostanoid EP₃ receptors in rats (Yokotani et al., 1995; Murakami et al., 2002) and this elevation was not influenced by bilateral adrenalectomy (Yokotani et al., 2005). These results suggest the involvement of brain prostaglandin E₂ in the centrally administered CRF-induced cFos expression in the noradrenergic neurons in the celiac ganglia.

The hypothalamus, especially the paraventricular nucleus, has been considered to be the control center of the sympatho-adrenomedullary outflow (Swanson and Sawchenko, 1980). Retrograde tracer studies suggest a possible connection between the sympatho-adrenomedullary system and the paraventricular nucleus through the splanchnic nerve and spinal cord (Luiten et al., 1987; Jansen et al., 1995; Ranson et al., 1998). Recently we reported that N-methyl-D-aspartate applied into the rat paraventricular nucleus using microdialysis technique produced thromboxane A₂ release in this region and elevation of plasma catecholamines in rats (Okada et al., 2000). Microinjection of thromboxane A₂ mimetic into the rat paraventricular nucleus

elevated plasma catecholamines (Murakami et al., 2002). Further immunohistochemical studies are required to clarify the brain regions involved in the CRF- or AVP-induced activation of sympatho-adrenomedullary outflow in rats.

In summary, we demonstrated here that centrally administered CRF activates adrenal A-cells and noradrenergic neurons in the celiac ganglia by the brain thromboxane A₂-mediated and the other prostanoid than thromboxane A₂ (probably prostaglandin E₂)-mediated mechanisms, respectively. On the other hand, centrally administered AVP activates adrenal A-cells and NA-cells by brain thromboxane A₂-mediated mechanisms in rats.

Acknowledgements

This work was supported in part by a grant from The Smoking Research Foundation in Japan and The Dean Research Fund of the Kochi University.

References

- Axelrod, J., 1990. Receptor-mediated activation of phospholipase A_2 and arachidonic acid release in signal transduction. *Biochem. Soc. Trans.* 18, 503-507.
- Axelrod, L., 1962. Purification and properties of phenylethanolamine-N-methyl transferase. *J. Biol. Chem.* 237, 1657-1660.
- Brown, M., Tache, Y., Fisher, D., 1979. Central nervous system action of bombesin: mechanism to induce hyperglycemia. *Endocrinology* 105, 660-665.
- Brown, M.R., Fisher, L.A., 1984. Brain peptide regulation of adrenal epinephrine secretion. *Am. J. Physiol.* 247, E41-E46.
- Brown, M.R., Fisher, L.A., Webb, V., Vale, W.W., Rivier, J.E., 1985. Corticotropin-releasing factor: a physiologic regulator of adrenal epinephrine secretion. *Brain Res.* 328, 355-357.
- Chambi, F., Fung, B., Chikaraishi, D., 1989. 5' flanking DNA sequences direct cell-specific expression of rat tyrosine hydroxylase. *J. Neurochem.* 53, 1656-1659.
- Coupland, R.E., Pyper, A.S., Hopwood, D., 1964. A method for differentiating between noradrenaline- and adrenaline-storing cells in the light and electron microscope. *Nature* 201, 1240-1242.

- Dronjak, S., Gavrilovic, L., Filipovic, D., Radojcic, M.B., 2004. Immobilization and cold stress affect sympatho-adrenomedullary system and pituitary-adrenocortical axis of rats exposed to long-term isolation and crowding. *Physiol. Behav.* 81, 409-415.
- Ebert, S.N., Balt, S.L., Hunter, J.P., Gashler, A., Sukhatme, V., Wong, D.L., 1994. Egr-1 activation of rat adrenal phenylethanolamine N-methyltransferase gene. *J. Biol. Chem.* 269, 20885-20898.
- Edwards, S.L., Anderson, C.R., Southwell, B.R., McAllen, R.M. 1996. Distinct preganglionic neurons innervate noradrenaline and adrenaline cells in the cat adrenal medulla. *Neuroscience* 70, 825-832.
- Feuerstein, G., Zerbe, R.L., Faden, A.I., 1984. Central cardiovascular effects of vasotocin, oxytocin and vasopressin in conscious rats. *J. Pharmacol. Exp. Ther.* 228, 348-353.
- Fisher, L.A., Kikkawa, D.O., Rivier, J.E., Amara, S.G., Evans, R.M., Rosenfeld, M.G., Vale, W.W., Brown, M.R., 1983. Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. *Nature* 305, 534-536.
- Flower, R.J., Blackwell, G.J., 1976. The importance of phospholipase A₂ in prostaglandin biosynthesis. *Biochem. Pharmacol.* 25, 285-291.
- Folkow, B., von Euler, U.S., 1954. Selective activation of noradrenaline and adrenaline producing cells in the cat's

- adrenal gland by hypothalamic stimulation. *Cric. Res.* 2, 191-195.
- Fujino, Y., Fujii, T., 1995. Insulin-induced hypoglycemia stimulates both adrenaline and noradrenaline release from adrenal medulla in 21-day-old rats. *Jpn. J. Pharmacol.* 69, 413-420.
- Gorman, R.R., Johnson, R.A., Spilman, C.H., Aiken, J.W., 1983. Inhibition of platelet thromboxane A₂ synthase activity by sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate. *Prostaglandins* 26, 325-342.
- Hasegawa, T., Yokotani, K., Okuma, Y., Manabe, M., Hirakawa, M., Osumi, Y., 1993. Microinjection of α -calcitonin gene-related peptide into the hypothalamus activates sympathetic outflow in rats. *Jpn. J. Pharmacol.* 61, 325-332.
- Herrera, D.G., Robertson, H.A., 1996. Activation of c-Fos in the brain. *Prog. Neurobiol.* 50, 83-107.
- Insel, P.A., 1996. Analgesic-antipyretic and antiinflammatory agents and drugs employed in the treatment of gout. In: Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddonm, R.W., Gilman, A.G. (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, pp. 617-658.
- Irvine, R.F., 1982. How is the level of free arachidonic acid controlled in mammalian cells? *Biochem. J.* 2004, 3-16.

- Jansen, A.S., Nguyen, X.V., Karpitskiy, V., Mettenleiter, T.C., Loewy, A.D., 1995. Central command neurons of the sympathetic nervous system: basis of the fight-or-flight response. *Science* 270, 644-646.
- Jezova, D., Ochedalski, T., Glickman, M., Kiss, A., Aguilera, G., 1999. Central corticotropin-releasing hormone receptors modulate hypothalamic-pituitary-adrenocortical and sympathoadrenal activity during stress. *Neuroscience* 94, 797-802.
- Jung, J., Lee, J., Kim, W., 2004. Enhanced activity of central adrenergic neurons in two-kidney, one clip hypertension in Sprague-Dawley rats. *Neurosci. Lett.* 369, 14-18.
- Kvetnansky, R., Mikulaj, L., 1970. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology* 87, 738-743.
- Lee, E.J., Moore, C.T., Hosny, S., Centers, A., Jennes, L., 2000. Expression of estrogen receptor- α and c-Fos in adrenergic neurons of the female rat during the steroid-induced LH surge. *Brain Res.* 875, 56-65.
- Liu, J., Merlie, J.P., Todd, R.D., 1997. Identification of cell type-specific promotor elements associated with the rat tyrosine hydroxylase gene using transgenic founder analysis. *Mol. Brain Res.* 50, 33-42.
- Luiten, P.G.M., Ter Horst, G.J., Steffens, A.B., 1987. The hypothalamus, intrinsic connections and outflow pathways to

- the endocrine system in relation to the control of feeding and metabolism. *Prog. Neurobiol.* 28, 1-54.
- Morita, K., Ebert, S.N., Wong, D.L., 1995. Role of transcription factor Egr-1 in phorbol ester-induced phenylethanolamine N-methyltransferase gene expression. *J. Biol. Chem.* 270, 11161-11167.
- Murakami, Y., Yokotani, K., Okuma, Y., Osumi, Y., 1998. Thromboxane A₂ is involved in the nitric oxide-induced central activation of adrenomedullary outflow in rats. *Neuroscience* 87, 197-205.
- Murakami, Y., Okada, S., Nishihara, M., Yokotani, K., 2002. Roles of brain prostaglandin E₂ and thromboxane A₂ in the activation of the central sympatho-adrenomedullary outflow in rats. *Eur. J. Pharmacol.* 452, 289-294.
- Okada, S., Murakami, Y., Nishihara, M., Yokotani, K., Osumi, Y., 2000. Perfusion of the hypothalamic paraventricular nucleus with N-methyl-D-aspartate produces thromboxane A₂ and centrally activates adrenomedullary outflow in rats. *Neuroscience* 96, 585-590.
- Okada, S., Murakami, Y., Nakamura, K., Yokotani, K., 2002. Vasopressin V₁ receptor-mediated activation of central sympatho-adrenomedullary outflow in rats. *Eur. J. Pharmacol.* 457, 29-35.
- Okada, S., Murakami, Y., Yokotani, K., 2003a. Role of brain thromboxane A₂ in the release of noradrenaline and adrenaline

- from adrenal medulla in rats. *Eur. J. Pharmacol.* 467, 125-131.
- Okada, S., Yokotani, K., Yokotani, K., 2003b. Inducible nitric oxide synthase is involved in corticotropin-releasing hormone-mediated central sympatho-adrenal outflow in rats. *Eur. J. Pharmacol.* 477, 95-100.
- Okuma, Y., Yokotani, K., Osumi, Y., 1996. Brain prostaglandins mediate the bombesin-induced increase in plasma levels of catecholamines. *Life Sci.* 59, 1217-1225.
- Paxinos, G., Watson, C., 1986. *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Boston.
- Phillips, J.K., Dubey, R., Sesiashvilvi, E., Takeda, M., Christie, D.L., Lipski, J., 2001. Differential expression of the noradrenaline transporter in adrenergic chromaffin cells, ganglion cells and nerve fibres of the rat adrenal medulla. *J. Chem. Neuroanat.* 21, 95-104.
- Piezzi, R.S., Bianchi, R.A., Miranda, J.C., 1983. Chromaffin reactions and fluorometric determination of catecholamines in the neonatal adrenal medulla of the rat. *J. Histochem. Cytochem.* 31, 1351-1355.
- Ranson, R.N., Motawei, K., Pyner, S., Coote, J.H., 1998. The paraventricular nucleus of the hypothalamus sends efferents to the spinal cord of the rat that closely appose sympathetic preganglionic neurones projecting to the stellate ganglion. *Exp. Brain Res.* 120, 164-172.

- Ritter, S., Llewellyn-Smith, I., Dinh, T.T., 1998. Subgroups of hindbrain catecholamine neurons are selectively activated by 2-deoxy-D-glucose induced metabolic challenge. *Brain Res.* 805, 41-54.
- Sabban, E.L., Kvetnansky, R., 2001. Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. *Trends Neurosci.* 24, 91-98.
- Sagar, S.M., Sharp, F.R., Curran, T., 1988. Expression of c-Fos protein in brain: metabolic mapping at the cellular level. *Science* 240, 1328-1331.
- Shima, N., Yamaguchi, Y., Yuri, K., 2003. Distribution of estrogen receptor beta mRNA-containing cells in ovariectomized and estrogen-treated female rat brain. *Ant. Sci. Int.* 78, 85-97.
- Shimizu, T., Okada, S., Yamaguchi, N., Sasaki, T., Lu, L., Yokotani, K., 2006. Centrally administered histamine evokes the adrenal secretion of noradrenaline and adrenaline by brain cyclooxygenase-1- and thromboxane A₂-mediated mechanisms in rats. *Eur. J. Pharmacol.* 541, 152-157.
- Suzuki, T., Kachi, T., 1996. Similarities and differences in supporting and chromaffin cells in the mammalian adrenal medullae: and immunohistochemical study. *Anat. Rec.* 244, 358-365.
- Swanson, L.W., Sawchenko, P.E., 1980. Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* 31, 410-417.

- Temel, S., Lin, W., Lakhlani, S., Jennes, L., 2002. Expression of estrogen receptor- α and cFos in Norepinephrine and epinephrine neurons of young and middle-aged rats during the steroid-induced luteinizing hormone surge. *Endocrinology* 143, 3974-3983.
- Verhofstad, A.A., Coupland, R.E., Parker, T.R., Goldstein, M., 1985. Immunohistochemical and biochemical study on the development of the noradrenaline- and adrenaline-storing cells of the adrenal medulla of the rat. *Cell Tissue Res.* 242, 233-243.
- Vollmer, R.R., 1996. Selective neural regulation of epinephrine and norepinephrine cells in the adrenal medulla-cardiovascular implications. *Clin. Exp. Hypertens.* 18, 731-751.
- Vollmer, R.R., Balcita, J.J., Sved, A.F., Edwards, D.J., 1997. Adrenal epinephrine and norepinephrine release to hypoglycemia measured by microdialysis in conscious rats. *Am. J. Physiol.* 273, R1758-R1763.
- Vollmer, R.R., Balcita-Pedicino, J.J., Debnam, A.J., Edwards, D.J., 2000. Adrenal medullary catecholamine secretion patterns in rats evoked by reflex and direct neural stimulation. *Clin. Exp. Hypertens.* 22, 705-715.
- Wurtman, R.J., 2002. Stress and the adrenocortical control of epinephrine synthesis. *Metabolism* 51, 11-14.

- Yang, S., Voogt, J.L., 2001. Mating-activated brainstem catecholaminergic neurons in the female rat. *Brain Res.* 894, 159-166.
- Yokotani, K., Muramatsu, I., Fujiwara, M., Osumi, Y., 1983. Effects of the sympathoadrenal system on vagally induced gastric acid secretion and mucosal blood flow in rats. *J. Pharmacol. Exp. Ther.* 224, 436-442.
- Yokotani, K., Nishihara, M., Murakami, Y., Hasegawa, T., Okuma, Y., Osumi, Y., 1995. Elevation of plasma noradrenaline levels in urethane-anaesthetized rats by activation of central prostanoid EP₃ receptors. *Br. J. Pharmacol.* 115, 672-676.
- Yokotani, K., Murakami, Y., Okada, S., Hirata, M., 2001. Role of brain arachidonic acid cascade on central CRF₁ receptor-mediated activation of sympatho-adrenomedullary outflow in rats. *Eur. J. Pharmacol.* 419, 183-189.
- Yokotani, K., Okada, S., Nakamura, K., Yamaguchi-Shima, N., Shimizu, T., Arai, J., Wakiguchi, H., Yokotani, K., 2005. Brain prostanoid TP receptor-mediated adrenal noradrenaline secretion and EP₃ receptor-mediated sympathetic noradrenaline release in rats. *Eur. J. Pharmacol.* 512, 29-35.
- Young, J.B., Rosa, R.M., Landsberg, L., 1984. Dissociation of sympathetic nervous system and adrenal medullary responses. *Am. J. Physiol.* 247, E35-E40.

Legends to figures

Fig. 1. CRF- and AVP-induced cFos expression in the adrenal medulla and celiac ganglia. CRF (1.5 and 3.0 nmol/animal, i.c.v.) or AVP (0.2 and 0.5 nmol/animal, i.c.v.) was administered in anesthetized rats. cFos expression in the adrenal medulla (A,C,E,G,I) and celiac ganglia (B,D,F,H,J) was examined at 0 min (A,B) and 60 min (C,D) after CRF (1.5 nmol/animal) administration and at 120 min (E,F) after CRF (3.0 nmol/animal) administration, or 0 min (A,B) and 10 min (G,H) after AVP (0.2 nmol/animal) administration and at 60 min (I,J) after AVP (0.5 nmol/animal) administration. In the adrenal medulla, cFos is shown in the left panel, PNMT is shown in the middle panel and both in the right panel. In the celiac ganglia, cFos is shown in the left panel, DBH is shown in the middle panel and both in the right panel. Scale bar = 100 μ m. (K-N) Quantitative analysis of the percentage of cFos-positive cells in all cells in each group (adrenal A-cells and NA-cells and noradrenergic neurons in the celiac ganglia) after intracerebroventricular administration of CRF (1.5 nmol/animal, K; 3.0 nmol/animal, M) or AVP (0.2 nmol/animal, L; 0.5 nmol/animal, N). ■ , adrenal A cells; ● , adrenal NA cells; ▲ , celiac ganglia. Results represent the means $\pm$ S.E.M. (n=4 in each group). #Significantly different (P<0.05) from 0 min before intracerebroventricular administration of CRF or AVP with the Bonferroni method.

Fig. 2. Effects of indomethacin and furegrelate on the CRF-induced cFos expression in the adrenal medulla and celiac

ganglia. Indomethacin (IND) [1.2 μ mol (500 μ g)/animal], furegrelate [1.8 μ mol (500 μ g)/animal] (FUR) or vehicle-1 (10 μ l saline) was administered 30 min before application of CRF (1.5 nmol/animal, i.c.v.) or vehicle-2 (10 μ l saline, i.c.v.). cFos expression in the adrenal medulla (A,C,E,G) and celiac ganglia (B,D,F,H) was examined 60 after CRF (or vehicle-2) administration. (A,B) vehicle-1 plus vehicle-2; (C,D) vehicle-1 plus CRF; (E,F) indomethacin (IND) plus CRF; (G,H) furegrelate (FUR) plus CRF. (I) Quantitative analysis of the percentage of cFos-positive cells in all cells in each group (adrenal A-cells and NA-cells and noradrenergic neurons in the celiac ganglia) after intracerebroventricular administration of CRF (n=4 in each group). Other conditions were the same as those in Fig. 1. *Significantly different ($P<0.05$) from the group treated with vehicle-1 plus vehicle-2 with the Student's *t*-test; #significantly different ($P<0.05$) from the group treated with vehicle-1 plus CRF with the Bonferroni method.

Fig. 3. Effects of indomethacin and furegrelate on the AVP-induced cFos expression in the adrenal medulla and celiac ganglia. Indomethacin (IND) [1.2 μ mol (500 μ g)/animal], furegrelate [1.8 μ mol (500 μ g)/animal] (FUR) or vehicle-1 (10 μ l saline) was i.c.v. administered 30 min before the application of AVP (0.2 nmol/animal) or vehicle-2 (10 μ l saline). cFos expression in the adrenal medulla (A,C,E,G) and celiac ganglia (B,D,F,H) was examined 10 min after AVP (or vehicle-2) administration. (A,B) vehicle-1 plus vehicle-2; (C,D) vehicle-1 plus AVP; (E,F) indomethacin (IND) plus AVP; (G,H) furegrelate

(FUR) plus AVP. (I) Quantitative analysis of the percentage of cFos-positive cells in all cells in each group (adrenal A-cells and NA-cells and noradrenergic neurons in the celiac ganglia) after intracerebroventricular administration of AVP (n=4 in each group). Other conditions were the same as those in Figs. 1 and 2. *Significantly different ($P<0.05$) from the group treated with vehicle-1 plus vehicle-2 with the Student's *t*-test; #significantly different ($P<0.05$) from the group treated with vehicle-1 plus CRF with the Bonferroni method.

Figure
[Click here to download high resolution image](#)

Figure
[Click here to download high resolution image](#)

Figure
[Click here to download high resolution image](#)