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CYCLOOXYGENASE AND NITRIC OXIDE SYNTHASE IN THE PRESYMPATHETIC NEURONS IN THE PARAVENTRICULAR HYPOTHALAMIC NUCLEUS ARE INVOLVED IN RESTRAINT STRESS-INDUCED SYMPATHETIC ACTIVATION IN RATS

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Abstract—Stress is one of the important factors to activate the sympathetic nervous system. We recently reported that central administration of corticotropin-releasing factor (CRF), known as a stress-related neuropeptide, increases the expression of both cyclooxygenase (COX) and nitric oxide synthase (NOS) in presympathetic neurons in the paraventricular hypothalamic nucleus (PVN). In the present study, therefore, we investigated whether brain COX and NOS can also mediate restraint stress (RS)-induced sympathetic activation by assessing the plasma catecholamine levels and neuronal activation of presympathetic neurons in the PVN. In addition, we examined effects of RS on the expression of both COX and NOS isozymes in the presympathetic PVN neurons. Intraperitoneal administration of an inhibitor for COX-1, COX-2 or inducible NOS (iNOS), but not for neuronal NOS (nNOS), reduced RS-induced elevation of plasma catecholamine levels and Fos expression in the presympathetic PVN neurons. Moreover, RS increased the expression of COX-1, COX-2 and iNOS in the presympathetic PVN neurons, whereas nNOS expression did not change. These results suggest that COX-1, COX-2 and iNOS in the presympathetic PVN neurons mediate acute RS-induced sympathetic activation.

Key words: stress, COX, NOS, sympathetic nervous system, retrograde tracing.

Stress is one of the important factors to activate the sympathetic nervous system as well as the hypothalamic-pituitary-adrenocortical axis (Pacak et al., 1995; Sabban and Kvetnansky, 2001). Exposure to stress leads to various sympathetic responses such as increase of plasma catecholamine level, catecholamine-related genes expression (tyrosine hydroxylase, dopamine β-hydroxylase and phenylethanolamine N-methyltransferase) in the sympathetic ganglia (Sabban and Kvetnansky, 2001; Pajovic et al., 2006), and cardiovascular responses such as heart rate and blood pressure (Bhatnagar et al., 1998; Morimoto et al., 2004; Uji et al., 2007). In the brain, a separate population of neurons in several central autonomic structures including the paraventricular hypothalamic nucleus (PVN), raphe nuclei and rostral ventrolateral medulla regulates the sympathoexcitatory responses. The presympathetic neurons localized in these brain regions innervate the target organs via the sympathetic preganglionic neurons located in the intermediolateral cell column (IML) of the spinal cord (Appel and Elde, 1988; Fritschy and Grzanna, 1990; Pyner and Coote, 1999, 2000; Lee et al., 2006b). It has been reported that restraint stress (RS) induces Fos expression, a marker for neuronal activation, in these regions associated with central sympathetic regulation (Ueyama et al., 2006), suggesting that the activation of presympathetic neurons is involved in the stress-induced sympathetic activation.

Corticotropin-releasing factor (CRF) is well known as a stress-related neuropeptide. Indeed, stress exposure increases endogenous levels of CRF mRNA and protein in various brain regions including the PVN (Kalin et al., 1994; Li et al., 1996). Moreover, acute RS induces c-fos mRNA expression, which is suppressed by α-helical CRF (9–41), a non-selective CRF receptor antagonist, in the PVN (Imaki et al., 1995). In addition to the role in initiation/modulation of stress responses, it has been reported that exogenous CRF elicits central activation of the sympathetic nervous system, which leads to increase in heart rate, blood pressure, plasma catecholamine levels and neuronal/neural activation in the sympathetic nerve and the adrenal medulla (Brown et al., 1982; Fisher et al., 1982; Brown and Fisher, 1984; Egawa et al., 1990; Okada et al., 2003a; Yamaguchi-Shima et al., 2007; Usui et al., 2009). Taken together, the previous findings indicate that stress-induced CRF contributes to the sympathoexcitatory responses during and/or after stress exposure.

Recently we have reported that cyclooxygenase (COX) and nitric oxide synthase (NOS) are involved in regulation of brain CRF-induced sympathetic activation. COX is the rate-limiting enzyme in the synthesis of prostanoids, and has two isozymes; COX-1, a constitutive type, and COX-2, an inducible type (Fu et al., 1990; Xie et al., 1991). Nitric oxide (NO) synthesized by NOS acts as a gaseous neurotransmitter in the brain (Bredt and Snyder, 1992). In the brain, neuronal NOS (nNOS), a constitutive isozyme, is the major isozyme of NOS, whereas iNOS is undetectable under physiological condition (Nathan and Xie, 1994). We showed

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Abbreviations: COX, cyclooxygenase; CRF, corticotropin-releasing factor; FG, fluoro-gold; IML, intermediolateral cell column; iNOS, inducible NOS; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; PVN, paraventricular hypothalamic nucleus; RS, restraint stress.

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that intracerebroventricularly administered CRF-induced elevation of plasma catecholamine level is attenuated by central pretreatment with indomethacin, a non-selective COX inhibitor, or *S*-methylisothiourea, an inducible NOS (iNOS) inhibitor, in rats (Okada et al., 2003a,b). Furthermore, centrally administered CRF induces neuronal activation of presympathetic PVN neurons expressing COX isozymes (COX-1 and COX-2) and presympathetic PVN neurons expressing NOS isozymes (iNOS and nNOS) (Yamaguchi and Okada, 2009; Yamaguchi et al., 2009). These findings raise the possibility that COX and NOS in the PVN are involved in the sympathetic activation caused by CRF-mediated stress responses.

The PVN is one of the important regions related to both central organization of the stress-triggered processes and the sympathetic regulation (Swanson and Sawchenko, 1980; Valentino and Van Bockstaele, 2008). However, there have been no detailed studies on roles of these two enzymes in the presympathetic PVN neurons in response to RS. In the present study, therefore, to elucidate the involvement of COX and NOS isozymes in RS-induced sympathetic activation, we investigated the effect of inhibitors of COX and NOS isozymes on plasma catecholamine level and neuronal activation of presympathetic PVN neurons. In addition, we examined RS-induced changes in the expression of each isozyme in presympathetic PVN neurons in rats.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats weighing approximately 250 g were used. Rats were purchased from SLC (Shizuoka, Japan) and were maintained in an air-conditioned room at 22–24 °C under a constant day-night rhythm for more than 2 weeks with food and water provided *ad libitum*. All rats were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Kochi University. All efforts were made to minimize animal suffering and the number of animals needed to obtain reliable results.

Retrograde tracer injection

For labeling both presympathetic PVN neurons innervating preganglionic neurons and those innervating the adrenal medulla, the retrograde tracer was microinjected into the spinal cord at the T8-T9 levels. Rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.; Kyoritsu Seiyaku Corporation, Tokyo, Japan) and were microinjected with Fluoro-Gold (FG) (Fluorochrome, Denver, CO, USA), a monosynaptic retrograde tracer, into the IML of the thoracic spinal cord by the methods reported previously (Yamaguchi and Okada, 2009; Yamaguchi et al., 2009). Briefly, the spinal cord at the T8-T9 levels was exposed by dorsal laminectomy using an aseptic surgical procedure. FG (4% in sterile saline) was microinjected into the right side of the spinal cord (0.5 mm lateral from the midline and 1.0 mm below the surface of the spinal cord) in a volume of 50 nl each at the T8 and T9 levels using a glass micropipette (tip outer diameter, 50 μm) connected to a 0.5-μl Hamilton microsyringe at a rate of 10 nl/min. The cannula was left in each injection site for 5 min following injection to avoid being spread. Afterwards, the muscle overlying the spinal cord was sutured, and the wound was closed; then the rats were returned to their home cages. The rats were inspected daily for motor activity, signs of infection and food and water intake to assess their health status.

Reagents

SC-560 (a selective COX-1 inhibitor; Cayman Chemical, Ann Arbor, MI, USA), NS-398 (a selective COX-2 inhibitor; Cayman Chemical, Ann Arbor, MI, USA) and 7-nitroindazole (a selective nNOS inhibitor; Cayman Chemical, Ann Arbor, MI, USA) were dissolved in N,N-dimethylformamide, and *S*-methylisothiourea (a selective iNOS inhibitor; Sigma, Saint Louis, MO, USA) was dissolved in sterile saline. Thirty minutes before RS, these reagents or vehicle were i.p. administered in a volume of 0.1 ml/kg body weight.

Restraint stress

Fourteen days after tracer injection, rats were exposed to RS between 9:00 and 12:00. We have examined the effect of RS for one session of 30 min 1 h 3 h or 6 h on Fos expression in the presympathetic PVN neurons. Since the level of stress-induced Fos peaked at 1 h after RS (data not shown), we performed stress paradigm for 1 h in the present study. Rats were restrained for 1 h by binding gently but securely all four limbs to a board using strings and placing the head through a stainless steel loop covered by absorbent cotton to prevent excessive head movements (Ma and Morilak, 2005; Dhir et al., 2006; Lee et al., 2006a; Matsuwaki et al., 2006; Ueyama et al., 2006). Restrained rats were sacrificed immediately afterwards, while control groups were sacrificed after removal from their home cages without any handling. All rats were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.; Kyoritsu Seiyaku Corporation, Tokyo, Japan). Blood samples (300 μl) for plasma catecholamine determinations were collected by cardiac puncture, and then the rats were 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). Then, brains were removed and were post-fixed overnight in the same fixative. After cryoprotection in 20% sucrose in 0.1 M phosphate buffer at 4 °C, frozen sections (20 μm thickness) were cut on a cryostat, thaw-mounted on silane-coated slides, and then stored at −80 °C until use.

Measurement of plasma catecholamines

Blood samples were preserved on ice during the experimental periods. Plasma catecholamines were extracted by the method of Anton and Sayre (1962) with a slight modification and were assayed electrochemically using high performance liquid chromatography (Okada et al., 2003a; Yamaguchi and Okada, 2009). Briefly, the plasma (100 μl) was transferred to a tube containing 30 mg of activated alumina, 2 ml of double deionized water, 1 ng of 3,4-dihydroxybenzylamine as an internal standard and 1 ml of 1.5 M Tris buffer (pH 8.6) containing 0.1 M disodium EDTA, and was shaken for 10 min. After three washes with double deionized water, catecholamines adsorbed onto the alumina were eluted with 300 μl of 4% acetic acid containing 0.1 mM disodium EDTA. A pump (EP-300: Eicom, Kyoto, Japan), a sample injector (Model-231XL; Gilson, Villiers-le-Bel, France) and an electrochemical detector (ECD-300: Eicom) equipped with a graphite electrode were used with high performance liquid chromatography. The amount of catecholamines in each sample was calculated using the peak height ratio relative to that of 3,4-dihydroxybenzylamine. This assay can accurately determine levels of 0.5 pg of noradrenaline and adrenaline.

Immunohistochemistry

Sections on the slides were washed in phosphate buffered saline and then pretreated with 5% normal serum produced in the animal