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Inducible nitric oxide synthase is involved in corticotropin-releasing hormone-mediated central sympatho-adrenal outflow in rats

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Abstract

Brain nitric oxide (NO), recognized as a neurotransmitter or a neuromodulator, is mainly generated either by neuronal NO synthase (NOS) or by inducible NOS. NO has been shown to activate cyclooxygenase (a prostaglandin-forming enzyme) in addition to guanylate cyclase. Recently, we reported that the intracerebroventricularly (i.c.v.) administered corticotropin-releasing hormone (CRH) increases plasma catecholamines through brain cyclooxygenase-dependent mechanisms in rats [Eur. J. Pharmacol. 419 (2001) 183]. In the present experiments, therefore, we examined whether NO is involved in the CRH-induced increase of plasma catecholamines using urethane-anesthetized rats. I.c.v. administered CRH increased plasma noradrenaline and adrenaline in a dose-dependent manner (0.5, 1.5, and 3.0 nmol/animal). The CRH (1.5 nmol/animal, i.c.v.)-induced increase of plasma catecholamines was reduced by N^{ω}-nitro-L-arginine methyl ester (a non-selective inhibitor of NOS) [111 nmol (30 μg)/animal, i.c.v.], but not by the same dose of N^{ω}-nitro-D-arginine methyl ester (an inactive isomer of N^{ω}-nitro-L-arginine methyl ester). The CRH-induced increase of plasma catecholamines was also reduced either by cycloheximide (an inhibitor of protein synthesis) [107 nmol (30 μg)/animal, i.c.v.] or by *S*-methylisothiourea (an inhibitor of inducible NOS) [71 nmol (20 μg) and 711 nmol (200 μg)/animal, i.c.v.]. These results suggest the involvement of brain inducible NOS in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

Keywords: Noradrenaline; Adrenaline; Plasma; CRH (corticotropin-releasing hormone); Nitric oxide (NO) synthase, inducible, brain

1. Introduction

Previously, we reported that intracerebroventricularly (i.c.v.) administered prostanoids, prostaglandin E_2 and thromboxane A_2, increase plasma noradrenaline and adrenaline, respectively, in rats (Yokotani et al., 1995; Murakami et al., 2002a). In addition, intracerebroventricular pretreatment with indomethacin, an inhibitor of cyclooxygenase, effectively reduced the increase of plasma catecholamines induced by intracerebroventricularly administered corticotropin releasing hormone (CRH) (Yokotani et al., 2001), interleukin-1β (Murakami et al., 1996) and arachidonic acid (Yokotani et al., 2000). These results suggest the involvement of brain cyclooxygenase in the central activation of the sympatho-adrenomedullary outflow in rats. Cyclooxygenase is activated by nitric oxide, which has also been shown to activate guanylate cyclase (Salvemini et al., 1993). Indeed, intracerebroventricularly administered 3-morpholino-sydnonimine, a nitric oxide donor, increases plasma catecholamines in an indomethacin-sensitive manner in rats (Murakami et al., 1998). These results suggest a possibility that brain nitric oxide activates the brain cyclooxygenase-dependent pathways involved in the central activation of the sympatho-adrenomedullary outflow in rats.

Nitric oxide is generated from the amino acid L-arginine by nitric oxide synthase (Palmer et al., 1988; Moncada et al., 1991; Jaffrey and Snyder, 1995). Nitric oxide synthase has been described in three isoforms; neuronal, endothelial and inducible isoforms (Costa et al., 1996). The constitutively expressed neuronal nitric oxide synthase is the most abundant isoform in the brain and it can be also found in subsets of neurons belonging to different anatomical and functional regions. The predominant site of localization of neuronal nitric oxide synthase, apart from the cerebellum, is the hypothalamus, especially the paraventricular nucleus (Vincent and Kimura, 1992). Since the paraventricular nucleus has been recognized as a regulatory center of the sympatho-adrenomedullary system (Swanson and Sawchenko, 1983; Jansen et al., 1995), nitric oxide seems to play a role in

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regulation of the central sympatho-adrenomedullary outflow, as shown for the central regulation of renal sympathetic nerve activity (Zhang et al., 1997). On the other hand, inducible nitric oxide synthase is usually not expressed in the brain except in the inflammatory state (Galea et al., 1992; Romero et al., 1996; Rothwell et al., 1996; Heneka and Feinstein, 2001).

Some selective inhibitors of each nitric oxide synthase isoform have become available to further define the role of each isoform in various biological processes (Moore and Handy, 1997). We reported that the interleukin-1β-induced increase of plasma catecholamine was attenuated by intracerebroventricular pretreatment with either N^{ω}-nitro-L-arginine methyl ester (a non-selective inhibitor of nitric oxide synthase) or *S*-ethylisothiourea (an inhibitor of inducible nitric oxide synthase) (Murakami et al., 1996, 2002b). In the present experiments, therefore, we tried to characterize the mechanisms involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow with regard to the isoforms of nitric oxide synthase in rats.

2. Materials and methods

2.1. Experimental procedures

Male Wistar rats weighing about 350 g were maintained in an air-conditioned room at 22–24 °C on 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. Under urethane anesthesia (1.2 g/kg, i.p.), the femoral vein was cannulated for infusion of saline (1.2 ml/h), and the femoral artery was cannulated for collecting blood samples. After these procedures, the animal was placed in a stereotaxic apparatus, as shown in our previous paper (Yokotani et al., 2001; Okada et al., 2002).

Three hours after the animal had been placed in the stereotaxic apparatus, a stainless-steel cannula (0.35 mm outer diameter) or a double-lumen cannula (0.50 mm outer diameter) was inserted into the right lateral ventricle according to the rat brain atlas of Paxinos and Watson (1986). The stereotaxic coordinates of the tip of the 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). CRH and other reagents were dissolved in sterile saline and slowly injected into the right cerebral ventricle in a volume of 5 μl, using a 10-μl Hamilton syringe. N^{ω}-Nitro-L-arginine methyl ester, N^{ω}-nitro-D-arginine methyl ester, cycloheximide and *S*-methylisothiourea were i.c.v. administered in a volume of 10 μl, 30, 30, 120 and 30 min before administration of CRH, respectively. To verify the correct placement of the cannula tip, 5-μl of Cresyl violet solution was successively injected i.c.v. at the end of experiments. Then, the brain was removed to verify that blue dye had spread throughout the entire ventricular system (Okada et al., 2002).

All experiments were conducted in compliance with the guiding principles for the care and use of laboratory animals approved by the Kochi Medical School. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Measurement of plasma catecholamines

Blood samples (250 μl) were collected through an arterial catheter. Catecholamines in the plasma were extracted by the method of Anton and Sayre (1962) with a slight modification and were assayed electrochemically by high-performance liquid chromatography (Okada et al., 2000). Briefly, after centrifugation, the plasma (100 μl) was transferred to a centrifuge tube containing 30 mg of activated alumina, 2 ml of double deionized water, 1 ng of 3,4-dihydroxybenzylamine as internal standard and 1 ml of 1.5 M Tris buffer (pH 8.6) containing 0.1 M disodium EDTA. The tube was shaken for 10 min and the alumina was washed three times with 4 ml of ice-cold double deionized water. After centrifugation, the supernatant was discarded and samples were evaporated to dryness. Then catecholamines adsorbed onto the alumina were eluted with 300 μl of 4% acetic acid containing 0.1 mM disodium EDTA and 40 μl eluate was injected onto a high-performance liquid chromatography column. The recovery of catecholamines was about 85%. 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. Analytical conditions were as follows: detector, +450 mV potential against a Ag/AgCl reference electrode; column, Eicompack CA-50DS, 2.1 × 150 mm (Eicom); mobile phase, 0.1 M NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0) containing 50 mg/l EDTA dihydrate, 750 mg/l 1-octane sulfate sodium (Nacalai Tesque, Kyoto, Japan) and 15% methanol at a flow rate of 0.22 ml/min. The amount of catecholamines in each sample was calculated using the peak height ratio relative to that of 3,4-dihydroxybenzylamine. This assay could determine 0.5 pg of adrenaline and noradrenaline accurately.

2.3. Treatment of data and statistics

Results were expressed as the means ± S.E.M. of the net changes from the respective basal values. The data were analyzed by repeated-measures analysis of variance (ANOVA), followed by post hoc analysis with the Bonferroni method for comparing a control to all other means (Fig. 1). When only two means were to be compared, an unpaired Student's *t*-test was used (Figs. 2–4). *P* values less than 0.05 were taken to indicate significance.

2.4. Compounds

The following drugs were used: synthetic corticotropin-releasing hormone (rat/human) (Peptide Institute, Osaka,

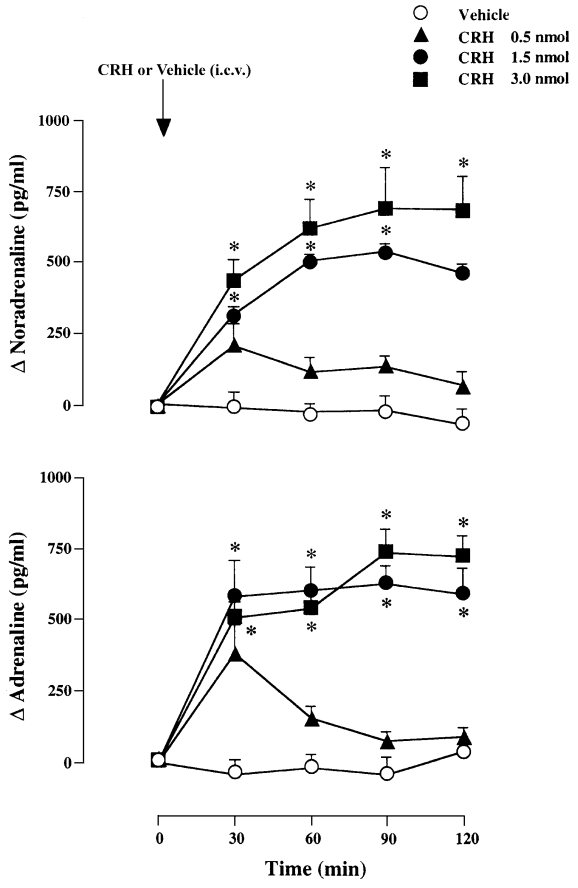

Fig. 1. Effects of corticotropin-releasing hormone (CRH) on plasma levels of noradrenaline and adrenaline. △ Noradrenaline and △ adrenaline, increase of noradrenaline and adrenaline above the basal. Arrow indicates intracerebroventricular (i.c.v.) administration of CRH (0.5, 1.5 and 3.0 nmol/animal) or vehicle (saline 5 μl/animal). Each point represents the mean ± S.E.M. *Significantly different ($P<0.05$) from vehicle-treated control. ○, Vehicle ($n=5$); ▲, 0.5 nmol CRH ($n=4$); ●, 1.5 nmol CRH ($n=7$); ■, 3.0 nmol CRH ($n=5$). The actual values for noradrenaline and adrenaline at 0 min were 332 ± 29 and 150 ± 16 pg/ml ($n=21$), respectively.

Japan); cycloheximide (Biomol Research Laboratories, Plymouth Meeting, PA, USA); *S*-methylisothiourea hemisulfate salt, N^{ω}-nitro-L-arginine methyl ester hydrochloride, N^{ω}-nitro-D-arginine methyl ester hydrochloride, (Sigma-RBI, St. Louis, MO, USA); activated alumina (Wako, Osaka, Japan). All other reagents were of the highest grade available (Nacalai Tesque, Kyoto, Japan).

3. Results

3.1. Effects of corticotropin-releasing hormone (CRH) on plasma levels of catecholamines

Intracerebroventricularly (i.c.v.) administered vehicle (5 μl saline/animal) and blood sampling five times over a 120-min period had no effect on the basal plasma levels of either noradrenaline or adrenaline (Fig. 1).

Administration of corticotropin-releasing hormone (CRH) (0.5, 1.5 and 3.0 nmol/animal, i.c.v.) dose-dependently increased plasma noradrenaline and adrenaline (Fig. 1). The increases of noradrenaline and adrenaline in response to CRH (0.5 nmol/animal, i.c.v.) reached a maximum 30 min after administration of CRH. The increases of plasma catecholamines induced by CRH (1.5 and 3.0 nmol/animal, i.c.v.) reached a maximum 60–90 min after administration of this peptide.

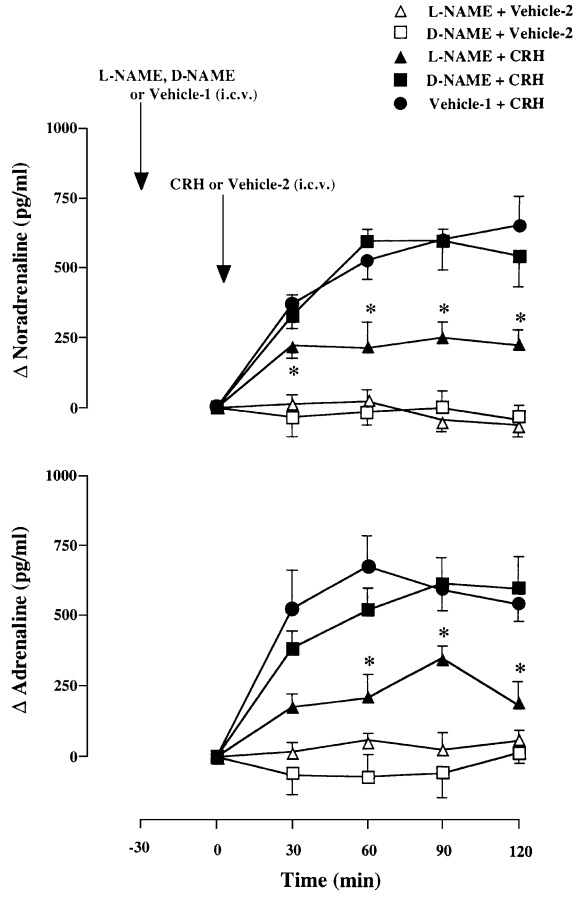

Fig. 2. Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-nitro-D-arginine methyl ester (D-NAME) on the CRH-induced increase of plasma catecholamines. L-NAME [111 nmol (30 μg)/animal, i.c.v.], D-NAME [111 nmol (30 μg)/animal, i.c.v.], or vehicle-1 (10 μl of saline, i.c.v.) was administered 30 min before administration of CRH (1.5 nmol/animal, i.c.v.) or vehicle-2 (5 μl of saline, i.c.v.). △, L-NAME plus vehicle-2 ($n=4$); □, D-NAME plus vehicle-2 ($n=4$); ●, vehicle-1 plus CRH ($n=10$); ▲, L-NAME plus CRH ($n=5$); ■, D-NAME plus CRH ($n=5$). *Significantly different ($P<0.05$) from the group treated with vehicle-1 plus CRH. Other conditions were the same as those in Fig. 1. The actual values for noradrenaline and adrenaline at 0 min were 346 ± 36 and 231 ± 42 pg/ml for vehicle-1-pretreated group ($n=10$), 354 ± 50 and 175 ± 34 pg/ml for L-NAME-pretreated group ($n=9$), 368 ± 37 and 290 ± 43 pg/ml for D-NAME-pretreated group ($n=9$), respectively.

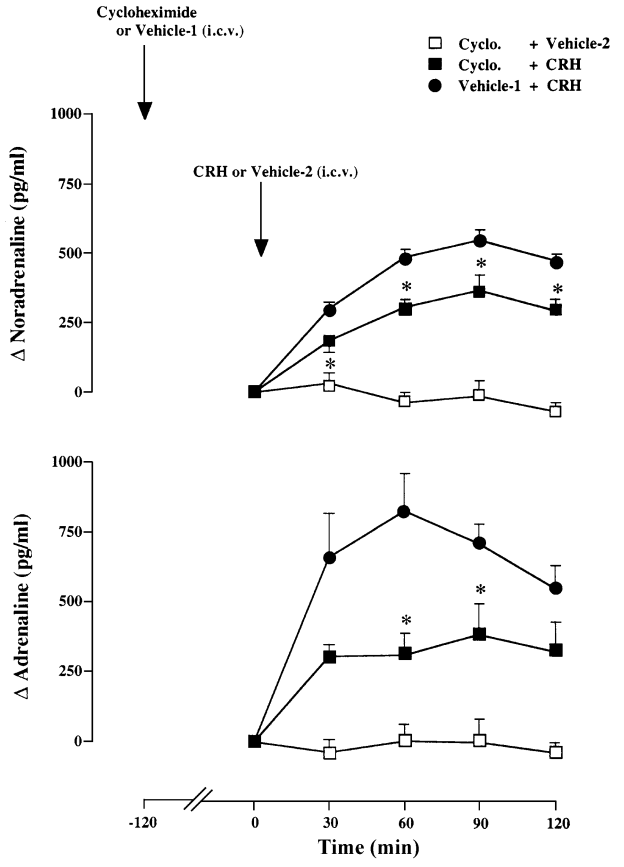

Fig. 3. Effect of cycloheximide, an inhibitor of protein synthesis, on the CRH-induced increase of plasma catecholamines. Cycloheximide [107 nmol (30 μg)/animal, i.c.v.] or vehicle-1 (10 μl of saline, i.c.v.) was administered 120 min before the administration of CRH (1.5 nmol/animal, i.c.v.) or vehicle-2 (5 μl of saline, i.c.v.). □, cycloheximide plus vehicle-2 ($n=4$); ●, vehicle-1 plus CRH ($n=6$); ■, cycloheximide plus CRH ($n=5$). *Significantly different ($P<0.05$) from the group treated with vehicle-1 plus CRH. Other conditions were the same as those in Figs. 1 and 2. The actual values for noradrenaline and adrenaline at 0 min were 219 ± 27 and 176 ± 60 pg/ml for vehicle-1-pretreated group ($n=6$) and 330 ± 36 and 196 ± 48 pg/ml for cycloheximide-pretreated group ($n=9$), respectively.

3.2. Effects of N^{ω}-nitro-L-arginine methyl ester and N^{ω}-nitro-D-arginine methyl ester on the CRH-induced increase of plasma catecholamines

Pretreatment with N^{ω}-nitro-L-arginine methyl ester (an inhibitor of nitric oxide synthase) [111 nmol (30 μg)/animal, i.c.v.] or N^{ω}-nitro-D-arginine methyl ester (an inactive isomer of N^{ω}-nitro-L-arginine methyl ester) [111 nmol (30 μg)/animal, i.c.v.] had no effect on the basal plasma levels of either noradrenaline or adrenaline (Fig. 2).

CRH (1.5 nmol/animal, i.c.v.) increased plasma noradrenaline and adrenaline 30 min after pretreatment with vehicle-1 (10 μl saline/animal, i.c.v.). The CRH (1.5 nmol/animal, i.c.v.)-induced increases of plasma catecholamines were not influenced by N^{ω}-nitro-D-arginine methyl ester [111 nmol (30 μg)/animal, i.c.v.], but were significantly reduced by N^{ω}-nitro-L-arginine methyl ester [111 nmol (30 μg)/animal, i.c.v.] (Fig. 2).

3.3. Effect of cycloheximide, an inhibitor of protein synthesis, on the CRH-induced increase of plasma catecholamines

Pretreatment with cycloheximide [107 nmol (30 μg)/animal, i.c.v.] or vehicle-1 (10 μl saline, i.c.v.) had no effect on the basal plasma levels of either noradrenaline or adrenaline 120 min after administration of this reagent (Fig. 3).

CRH (1.5 nmol/animal, i.c.v.) increased plasma noradrenaline and adrenaline 120 min after pretreatment with vehicle-1 (10 μl saline/animal, i.c.v.). Cycloheximide [107 nmol (30 μg)/animal, i.c.v.] significantly reduced the CRH-induced increase of plasma catecholamines (Fig. 3).

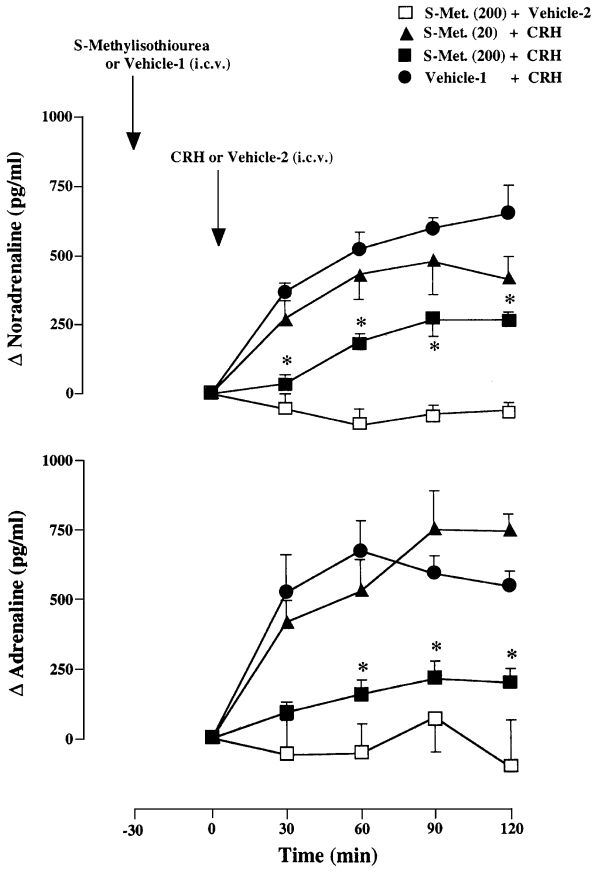

Fig. 4. Effect of *S*-methylisothiourea, an inhibitor of inducible nitric oxide synthase, on the CRH-induced increase of plasma catecholamines. *S*-methylisothiourea [71 nmol (20 μg) and 711 nmol (200 μg) or vehicle-1 (10 μl of saline) was i.c.v. administered 30 min before the administration of CRH (1.5 nmol/animal, i.c.v.). ●, vehicle-1 plus CRH ($n=10$) (cited in Fig. 2); ▲, *S*-methylisothiourea (20 μg/animal, i.c.v.) plus CRH ($n=6$); ■, *S*-methylisothiourea (200 μg/animal, i.c.v.) plus CRH ($n=6$); □, *S*-methylisothiourea (200 μg/animal, i.c.v.) plus vehicle-2 ($n=4$). *Significantly different ($P<0.05$) from the group treated with vehicle-1 plus CRH. Other conditions were the same as those in Figs. 1–3. The actual values for noradrenaline and adrenaline at 0 min were 346 ± 36 and 231 ± 42 pg/ml for vehicle-1-pretreated group ($n=10$), 368 ± 69 and 199 ± 69 pg/ml for *S*-methylisothiourea (20 μg/animal)-pretreated group ($n=6$), and 317 ± 43 and 162 ± 36 pg/ml for *S*-methylisothiourea (200 μg/animal)-pretreated group ($n=10$), respectively.

3.4. Effect of S-methylisothiourea, an inhibitor of inducible nitric oxide synthase, on the CRH-induced increase of plasma catecholamines

Pretreatment with *S*-methylisothiourea [711 nmol (200 μg)/animal, i.c.v.] had no effect on the basal plasma levels of noradrenaline and adrenaline 30 min after administration of this reagent (Fig. 4).

The increase of plasma noradrenaline evoked by CRH (1.5 nmol/animal, i.c.v.) was dose-dependently reduced by *S*-methylisothiourea [71 nmol (20 μg) and 711 nmol (200 μg)/animal, i.c.v.]. The increase of plasma adrenaline was significantly reduced by a higher dose of *S*-methylisothiourea [711 nmol (200 μg)/animal, i.c.v.)] (Fig. 4).

4. Discussion

Centrally administered CRH produced a gradually developing increase of plasma catecholamines. The gradually developing increase of plasma catecholamine was also found for the interleukin-1β-induced increase of plasma noradrenaline in rats (Murakami et al., 1996). The gradual increase of plasma noradrenaline induced by this cytokine seems to be responsible for a prolonged production of nitric oxide by brain inducible nitric oxide synthase in rats (Murakami et al., 2002b). In the present experiments, therefore, we examined the central mechanisms involved in the CRH-induced increase of plasma catecholamines with regard to brain nitric oxide synthase.

The CRH-induced increase of plasma noradrenaline and adrenaline was abolished by N^{ω}-nitro-L-arginine methyl ester (a non-selective inhibitor of nitric oxide synthase), but not by N^{ω}-nitro-D-arginine methyl ester (an inactive isoform of N^{ω}-nitro-L-arginine methyl ester). These results suggest the involvement of brain nitric oxide synthase in the CRH-induced increase of plasma catecholamines. Although N^{ω}-nitro-L-arginine methyl ester appears to inhibit constitutive nitric oxide synthase preferentially over inducible nitric oxide synthase, the degree of selectivity is marginal (Gross et al., 1990). Therefore, a question arises as to which type of nitric oxide synthase isoforms is involved in the CRH-induced increase of plasma catecholamines.

The CRH-induced increase of plasma catecholamines was abolished by cycloheximide, an inhibitor of protein synthesis (Obrig et al., 1971). Cycloheximide inhibits the interleukin-1β-induced mRNA expression of inducible nitric oxide synthase in murine cortical astrocytes (Hewett et al., 1993) and in rat cardiac myocytes (Tsujino et al., 1994). We also reported that central pretreatment with cycloheximide abolishes the interleukin-1β-induced increase of plasma noradrenaline in rats (Murakami et al., 2002b). These results suggest that brain protein synthesis, probably the synthesis of inducible nitric oxide synthase, is involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

S-methylisothiourea has been shown to be a potent and selective inhibitor of inducible nitric oxide synthase. *S*-methylisothiourea potently inhibits inducible nitric oxide synthase activity activated by bacterial endotoxin in J774.2 macrophages with an EC_{50} value 8 times lower than that of N^{ω}-methyl-L-arginine and 200 times lower than that of N^{ω}-nitro-L-arginine methyl ester, while this reagent is equipotent with N^{ω}-methyl-L-arginine to inhibit the constitutive nitric oxide synthase activity of bovine endothelial cells in vitro (Szabó et al., 1994; Southan et al., 1995). In the present experiments, central pretreatment with *S*-methylisothiourea dose-dependently reduced the CRH-induced increase of plasma catecholamines. The results suggest the involvement of brain inducible nitric oxide synthase in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

Inducible nitric oxide synthase has been shown to be expressed in the inflammatory state (Heneka and Feinstein, 2001). Whereas centrally administered CRH upregulates transcription of the neuronal nitric oxide synthase in the paraventricular nucleus of the hypothalamus in rats (Lee and Rivier, 1998), the peptide also potentiates protein expression of inducible nitric oxide synthase in endothelial cells of the human umbilical vein (Cantarella et al., 2001). Since CRH exerts proinflammatory effects, such as proliferation of rat splenocytes (McGillis et al., 1989) and increase of the cytokine production of human monocytes (Karalis et al., 1991), it is possible that brain-inducible nitric oxide synthase is involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

In conclusion, we now demonstrated that brain-inducible nitric oxide synthase is involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

Acknowledgements

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