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European Journal of Pharmacology 455 (2002) 73–78

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Brain inducible nitric oxide synthase is involved in interleukin-1β-induced activation of the central sympathetic outflow in rats

Yoshinori Murakami[1], Shoshiro Okada[1], Kunihiko Yokotani*

Department of Pharmacology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan

Received 30 May 2002; received in revised form 1 October 2002; accepted 8 October 2002

Abstract

Nitric oxide (NO) has been recognized as a neurotransmitter or a neuromodulator in the central nervous system. Brain NO is mainly generated either by neuronal NO synthase (NOS) or by inducible NOS. Previously we reported that central NO is involved in the elevation of plasma noradrenaline levels induced by intracerebroventricularly (i.c.v.) administered interleukin-1β in rats [Eur. J. Phamacol. 317 (1996) 61]. In the present study, therefore, we tried to characterize which type of NOS isoforms is involved in the cytokine-induced responses using selective inhibitors of each NOS isoform in urethane-anesthetized rats. I.c.v. administered interleukin-1β (100 ng/animal) elevated plasma levels of noradrenaline but not adrenaline. The cytokine-induced elevation of plasma noradrenaline levels was attenuated by cycloheximide, an inhibitor of protein synthesis, in a dose-dependent manner (10 and 20 μg/animal, i.c.v.). *S*-ethylisothiourea (0.1 and 0.5 μg/animal, i.c.v.), an inhibitor of inducible NOS, dose-dependently reduced the cytokine-induced elevation of plasma noradrenaline levels, while 7-nitroindazole (5 and 10 μg/animal, i.c.v.), an inhibitor of neuronal NOS, had no effect. These results suggest the involvement of brain inducible NOS in the interleukin-1β-induced activation of the central sympathetic outflow in rats.

Keywords: Noradrenaline; Plasma; Interleukin-1β; Nitric oxide (NO) synthase, inducible; Brain

1. Introduction

Nitric oxide (NO) has been recognized as a neurotransmitter or a neuromodulator in the central nervous system (Moncada et al., 1991). NO is generated from the amino acid L-arginine by NO synthase (NOS) (Palmer et al., 1988). NOS has been described in three isoforms: neuronal NOS, endothelial NOS and inducible NOS (Costa et al., 1996). The constitutively expressed neuronal NOS 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 most predominant site of localization of neuronal NOS, apart from the cerebellum, is the hypothalamus, especially the paraventricular nucleus (Vincent and Kimura, 1992). The paraventricular nucleus has been recognized as a regulatory center of the sympathetic nervous system, suggesting a role for NO in mediating central sympathetic outflow in addition to the hypothalamic–pituitary–adrenal axis (Swanson and Sawchenko, 1983; Jansen et al., 1995; Zhang et al., 1997). Inducible NOS is formed in response to various cytokines and endotoxin in various cells including neurons and glial cells in the brain (Galea et al., 1992; Romero et al., 1996; Rothwell et al., 1996).

Previously we reported that intracerebroventricularly (i.c.v.) administered interleukin-1β selectively elevated plasma levels of noradrenaline, and this elevation was abolished by L-N^G-nitroarginine methyl ester, a nitric oxide synthase inhibitor, in urethane-anesthetized rats (Murakami et al., 1996). These results clearly indicate an excitatory role of brain NOS in regulation of the central sympathetic outflow. Although L-N^G-nitroarginine methyl ester appears to inhibit constitutive NOS preferentially over inducible NOS, the degree of selectivity is marginal (Gross et al., 1990). Recently some selective inhibitors of each NOS isoform are available to further define a role of each NOS isoform in various biological processes (Moore and Handy, 1997). In the present experiments, therefore, we tried to characterize which type of NOS isoforms is involved in the

* Corresponding author. Tel./fax: +81-88-880-2328.
E-mail address: yokotani@dtn.am400gw.kochi-ms.ac.jp (K. Yokotani).
[1] Both authors contributed equally to this work.

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PII: S0014-2999(02)02580-3

interleukin-1β-induced activation of the central sympathetic outflow using several kinds of selective inhibitors of NOS isoforms.

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 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. 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 (Murakami et al., 1998; Yokotani et al., 2001).

Three hours after the animal had been placed in the stereotaxic apparatus, a stainless-steel cannula (0.35 mm outer diameter) or a double lumens 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, H 4.0 (AP, anterior from the bregma; L, lateral from the midline; H, below the surface of the brain). Interleukin-1β and other reagents were dissolved in sterile saline and slowly injected into the right cerebral ventricle in a volume of 10 μl, using 50-μl Hamilton syringe. *S*-ethylisothiourea, 7-nitroindazole and cycloheximide were i.c.v. administered 15, 30 and 90 min before administration of interleukin-1β, respectively.

To verify the correct placement of the tip of cannula, 5 μl of cresyl violet solution was successively injected i.c.v. at the end of experiments. The brain was then removed and fixed in the 10% formalin and sections sliced at 50 μm were prepared for microscopic study of the location of the injector tip as shown in our previous paper (Murakami et al., 1998).

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. 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 ×

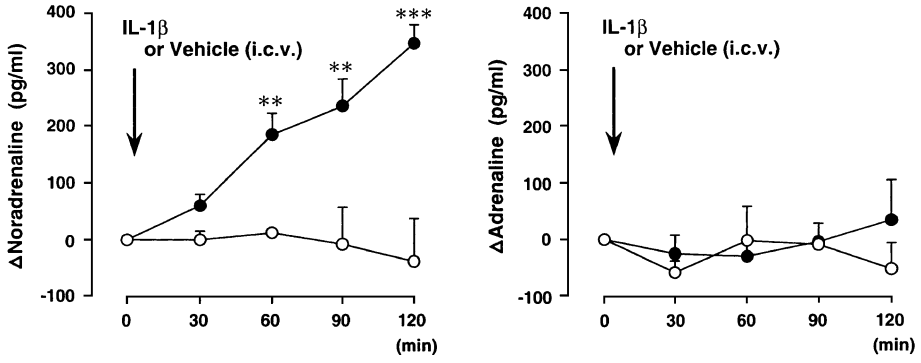

Fig. 1. Effect of interleukin-1β on plasma levels of noradrenaline and adrenaline. Interleukin-1β (IL-1β) or vehicle was intracerebroventricularly (i.c.v.) administered. ○, Vehicle (saline 10 μl/animal); ●, interleukin-1β (100 ng/animal). ΔNoradrenaline and ΔAdrenaline: Net changes in plasma levels of noradrenaline and adrenaline above the respective basal levels. Each point represents the mean ± S.E.M. Asterisks indicate significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$) from the respective vehicle-treated control. The actual values for noradrenaline and adrenaline at 0 min were 447 ± 40 and 351 ± 55 pg/ml for vehicle-treated group ($n=6$), and 437 ± 56 and 347 ± 92 pg/ml for interleukin-1β-treated group ($n=7$), respectively.

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 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 mean ± S.E.M. of the net changes above the respective basal values, because of individual variations. The area under the curve (AUC) is used to compare the effects of cycloheximide, *S*-ethylisothiourea and 7-nitroindazole on the interleukin-1β-induced elevation of plasma noradrenaline (Figs. 2–4). 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 (Figs. 2–4). When only two means were compared, an unpaired Student's *t*-test was used (Fig. 1). *P* values less than 0.05 were taken to indicate significance.

2.4. Compounds

The following drugs were used: human recombinant interleukin-1β (Endogen Chemical, Woburn, MA, USA); cycloheximide and *S*-ethylisothiourea (Biomol Research Laboratories, Plymouth Meeting, PA, USA); 7-nitroindazole monosodium salt (Tocris Cookson, Bristol, UK); alumina activated (Wako, Osaka, Japan). All other reagents were of the highest grade available (Nacalai Tesque, Kyoto, Japan).

3. Results

3.1. Effect of interleukin-1β on plasma levels of catecholamines

I.c.v. administered vehicle (10 μl saline/animal) and blood sampling for five times over a 120-min period did not affect the basal plasma levels of either noradrenaline or adrenaline (Fig. 1A and B).

Since we previously reported that interleukin-1β (10, 50, 100 and 200 ng/animal, i.c.v.) dose-dependently activated the central sympathetic outflow in rats (Yokotani et al., 1995b; Murakami et al., 1996), one dose of interleukin-1β (100 ng/animal, i.c.v.) was used in the present experiments. Administration of this cytokine (100 ng/animal, i.c.v.) induced a gradually developing elevation of plasma levels of noradrenaline, while the levels of adrenaline were not influenced at all (Fig. 1A and B). On the other hand, intravenous administration of interleukin-1β (100 ng/animal, i.v.) did not affect the plasma levels of catecholamines (data not shown).

3.2. Effect of cycloheximide, an inhibitor of protein synthesis, on the interleukin-1β-induced elevation of plasma noradrenaline levels

Pretreatment with cycloheximide alone (10 and 20 μg/animal, i.c.v.) slightly elevated the basal plasma levels of both noradrenaline and adrenaline; however, these elevations were gradually declined to their pre-administered level

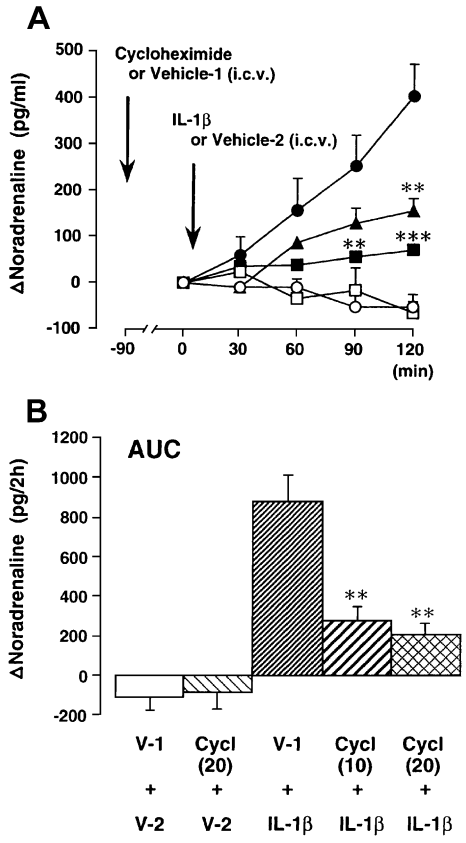

Fig. 2. Effect of cycloheximide, an inhibitor of protein synthesis, on the interleukin-1β-induced elevation of plasma noradrenaline levels. Cycloheximide (Cycl) (10 and 20 μg/animal, i.c.v.) or vehicle-1 (V-1) (10 μl of saline, i.c.v.) was administered 90 min before the administration of interleukin-1β (100 ng/animal, i.c.v.) or vehicle-2 (V-2) (10 μl of saline, i.c.v.). (A) ○, V-1 plus V-2; □, Cycl (20 μg/animal) plus V-2; ●, V-1 plus interleukin-1β; ▲, Cycl (10 μg/animal) plus interleukin-1β; ■, Cycl (20 μg/animal) plus interleukin-1β. (B) The area under the curve (AUC) of the interleukin-1β-induced elevation of plasma noradrenaline levels above the basal in the presence or absence of Cycl is expressed as pg/2 h. Asterisks indicate significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$) from those treated with V-1 plus interleukin-1β. Other conditions were the same as those for Fig. 1. The actual values for noradrenaline at 0 min in A were 402 ± 94 pg/ml for the V-1- plus V-2-treated group ($n=6$); 475 ± 59 pg/ml for the Cycl (20 μg/animal)- plus V-2-treated group ($n=6$); 431 ± 94 pg/ml for the V-1- plus interleukin-1β-treated group ($n=6$); 391 ± 41 pg/ml for the Cycl (10 μg/animal)- plus interleukin-1β-treated group ($n=5$); and 450 ± 50 pg/ml for the Cycl (20 μg/animal)- plus interleukin-1β-treated group ($n=7$), respectively.

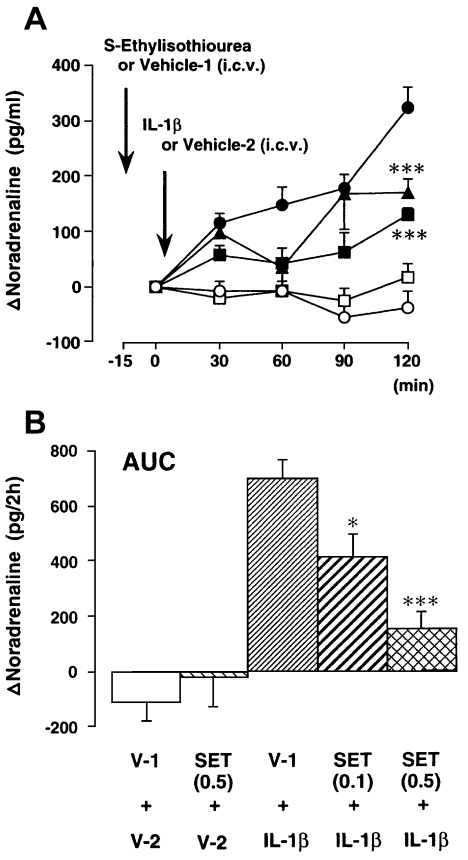

Fig. 3. Effect of *S*-ethylisothiourea, an inhibitor of inducible nitric oxide synthase, on the interleukin-1β-induced elevation of plasma noradrenaline levels. *S*-ethylisothiourea (SET) (0.1 and 0.5 μg/animal) or vehicle-1 (V-1) (10 μl of saline) was i.c.v. administered 15 min before the administration of interleukin-1β (100 ng/animal, i.c.v.) or vehicle-2 (V-2) (10 μl of saline, i.c.v.). (A) ○, V-1 plus V-2; □, SET (0.5 μg/animal) plus V-2; ●, V-1 plus interleukin-1β; ▲, SET (0.1 μg/animal) plus interleukin-1β; ■, SET (0.5 μg/animal) plus interleukin-1β. (B) The area under the curve (AUC) of the interleukin-1β-induced elevation of plasma noradrenaline levels above the basal in the presence or absence of SET is expressed as pg/2 h. Asterisks indicate significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$) from V-1 plus interleukin-1β. Other conditions were the same as for Figs. 1 and 2. The actual values for noradrenaline at 0 min in A were 402 ± 94 pg/ml for the V-1- plus V-2-treated group ($n=5$); 379 ± 27 pg/ml for the SET (0.5 μg/animal)- plus V-2-treated group ($n=6$); 485 ± 52.5 pg/ml for the V-1- plus interleukin-1β-treated group ($n=6$); 391 ± 41 pg/ml for the SET (0.1 μg/animal)- plus interleukin-1β-treated group ($n=5$); and 348 ± 32 pg/ml for the SET (0.5 μg/animal)- plus interleukin-1β-treated group ($n=6$), respectively.

within 60 min. Therefore, this reagent was injected 90 min before administration of interleukin-1β (100 ng/animal, i.c.v.) or vehicle-2 (10 μl saline, i.c.v.).

Pretreatment with cycloheximide (10 and 20 μg/animal, i.c.v.) reduced the interleukin-1β-induced elevation of plasma noradrenaline levels in a dose-dependent manner (Fig. 2A). When using the area under the curve to observe the effect of cycloheximide, this reagent significantly and dose-dependently reduced the interleukin-1β-induced elevation of plasma noradrenaline levels (Fig. 2B).

3.3. Effect of S-ethylisothiourea, an inhibitor of inducible nitric oxide synthase, on the interleukin-1β-induced elevation of plasma noradrenaline levels

Since pretreatment with *S*-ethylisothiourea (0.1 and 0.5 μg/animal, i.v.) or vehicle (10 μl saline) had no affect on the basal plasma levels of noradrenaline, this reagent was injected 15 min before administration of interleukin-1β (100 ng/animal, i.c.v.) or vehicle-2 (10 μl saline, i.c.v.).

S-ethylisothiourea (0.1 and 0.5 μg/animal, i.c.v.) effectively reduced the interleukin-1β-induced elevation of plasma noradrenaline levels in a dose-dependent manner (Fig. 3A). When using the area under the curve to observe

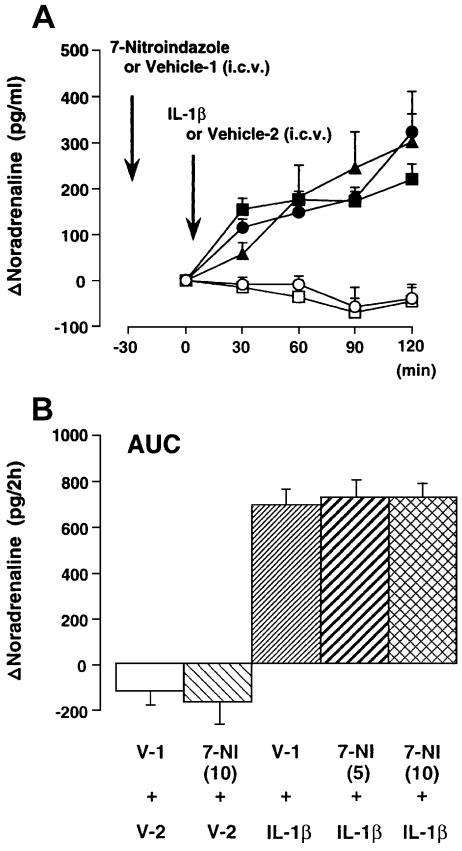

Fig. 4. Effect of 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase, on the interleukin-1β-induced elevation of plasma noradrenaline levels. 7-Nitroindazole (7-NI) (5 and 10 μg/animal) or vehicle-1 (V-1) (10 μl of saline) was i.c.v. administered 30 min before the administration of interleukin-1β (100 ng/animal, i.c.v.) or vehicle-2 (V-2) (10 μl of saline, i.c.v.). (A) ○, V-1 plus V-2 (cited from Fig. 3); □, 7-NI (5 μg/animal) plus V-2; ●, V-1 plus interleukin-1β (100 ng/animal) (cited from Fig. 3); ▲, 7-NI (10 μg/animal) plus interleukin-1β; ■, 7-NI (20 μg/animal) plus interleukin-1β. (B) The area under the curve (AUC) of the interleukin-1β-induced elevation of plasma noradrenaline levels above the basal in the presence or absence of 7-NI is expressed as pg/2 h. Other conditions were the same as those for Figs. 1–3. The actual values for noradrenaline at 0 min in A were 368 ± 21 pg/ml for the 7-NI (10 μg/animal)- plus V-2-treated group ($n=5$); 361 ± 29 pg/ml for the 7-NI (5 μg/animal)- plus interleukin-1β-treated group ($n=5$); and 499 ± 55 pg/ml for the 7-NI (10 μg/animal)- plus interleukin-1β-treated group ($n=5$), respectively.

the effect of *S*-ethylisothiourea, this reagent effectively and dose-dependently attenuated the interleukin-1β-induced elevation of plasma noradrenaline levels (Fig. 3B).

3.4. Effect of 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase, on the interleukin-1β-induced elevation of plasma noradrenaline levels

Since pretreatment with 7-nitroindazole (5 and 10 μg/animal, i.c.v.) or vehicle (10 μl saline, i.c.v.) had no effect on the basal plasma levels of noradrenaline, this reagent was injected 30 min before administration of interleukin-1β (100 ng/animal, i.c.v.) or vehicle-2 (10 μl saline, i.c.v.).

7-Nitroindazole (5 and 10 μg/animal, i.c.v.) had no effect on the interleukin-1β-induced elevation of plasma noradrenaline levels (Fig. 4A). When using the area under the curve to observe the effect of 7-nitroindazole, this reagent also had no effect on the cytokine-induced elevation of plasma noradrenaline levels (Fig. 4B).

4. Discussion

In the present experiments, we used anesthetized rats to clarify the mechanisms of central activation of the sympatho-adrenomedullary outflow (probably located on the hypothalamus), since several stressors (such as anxiety, fear and pain)-induced input from the descending limbic system and ascending brainstem cell groups to the hypothalamus probably modulate the activation of the hypothalamus in awake rats as shown in the hypothalamic–pituitary–adrenal axis (Herman et al., 2002).

I.c.v. administered interleukin-1β produced gradually developing elevation of plasma noradrenaline levels, as shown in our previous paper (Murakami et al., 1996). The cytokine-induced elevation of plasma noradrenaline levels was attenuated by i.c.v. administered cycloheximide, an inhibitor of protein synthesis (Obrig et al., 1971). This reagent has been shown to inhibit the interleukin-1β-induced inducible NOS mRNA expression and/or NO production in rat cardiac myocytes (Tsujino et al., 1994), in murine cortical astrocytes (Hewett et al., 1993) and in mouse pituitary tumour cell line AtT20/D16 (Ohta et al., 1993). From these evidence, it is suggested that the synthesis of brain inducible NOS is required for the interleukin-1β-induced activation of the central sympathetic outflow. A gradual elevation of plasma noradrenaline levels induced by interleukin-1β seems to be responsible for a prolonged production of nitric oxide by inducible NOS (Jaffrey and Snyder, 1995).

S-ethylisothiourea is a potent and selective inhibitor of inducible NOS with a K_i value of 14.7 nM for partially purified inducible NOS obtained from lipopolysaccharide- and interferon-γ-treated RAW 264.7 macrophages and with about 20-fold more selectivity for murine inducible NOS than rat neuronal NOS (Nakane et al., 1995). In the next experiment, we examined the effect of *S*-ethylisothiourea on the interleukin-1β-induced elevation of plasma noradrenaline levels. I.c.v. administered *S*-ethylisothiourea (0.1 and 0.5 μg/animal, i.c.v.) effectively and dose-dependently reduced the interleukin-1β-induced elevation of plasma noradrenaline levels. These results further support the hypothesis that the brain inducible NOS is involved in the interleukin-1β-induced activation of the central sympathetic outflow in rats.

7-Nitroindazole is a potent and selective inhibitor of neuronal NOS with a K_i value of 0.09 μM in purified porcine brain (Mayer et al., 1994) and with a K_i value of 1.6 μM in bovine brain (Wolff and Gribin, 1994). 7-Nitroindazole has been shown to reduce the severity of pilocarpine-induced seizures in mice (Van Leeuwen et al., 1995) and to decrease NO production in the rat hippocampus (Bush and Pollack, 2001). In the last experiment, we examined the effect of 7-nitroindazole on the interleukin-1β-induced elevation of plasma noradrenaline levels. 7-Nitroindazole (5 and 10 μg/animal, i.c.v.) had no effect on the cytokine-induced elevation of plasma noradrenaline levels. From these results, it is suggested that the brain neuronal NOS is not involved in the interleukin-1β-induced activation of the central sympathetic outflow in rats.

A question has arisen as to how NO produced by interleukin-1β activates the central sympathetic outflow. NO has been shown to activate cyclooxygenase in addition to guanylate cyclase (Salvemini et al., 1993). We have already reported that i.c.v. administered prostaglandin E_2 selectively activates central sympathetic outflow by prostanoid EP_3 receptor-mediated mechanisms (Yokotani et al., 1988, 1995a, 1996). I.c.v. administered nitric oxide donors, 3-morpholino-sydnonimine and sodium nitroprusside, activated central sympathetic outflow and these responses were abolished by indomethacin, an inhibitor of cyclooxygenase (Yokotani et al., 1997; Murakami et al., 1998). Furthermore, the activation of central sympathetic outflow induced by i.c.v. administered interleukin-1β was also attenuated by i.c.v. administered indomethacin (Yokotani et al., 1995b; Murakami et al., 1996). Inducible NOS has been shown to be formed in the hypothalamic paraventricular nucleus in response to various cytokines and endotoxin (Harada et al., 1999; Wakita et al., 2001). These evidence suggests that interleukin-1β-induced activation of the central sympathetic outflow is mediated by inducible NOS- and cyclooxygenase-mediated mechanisms in rat brain. These mechanisms have already shown in rat islets in which interleukin-1β induces coexpression of both inducible NOS and cyclooxygenase and accumulates prostaglandin E_2 by NO-mediated mechanisms (Corbett et al., 1993).

In conclusion, we demonstrated here that the brain inducible NOS is involved in the interleukin-1β-induced activation of the central sympathetic outflow in rats.

Acknowledgements

This work was supported in part by a grant from The President Research Fund of Kochi Medical School.

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