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Short communication

7-Nitroindazole reduces nitric oxide concentration in rat hippocampus after transient forebrain ischemia

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Abstract

We investigated the effects of 7-nitroindazole, a specific inhibitor of neuronal nitric oxide (NO) synthase, on NO concentration and on blood flow in rat hippocampus after transient forebrain ischemia which was induced by 4-vessel occlusion for 10 min. NO concentration was measured directly by an NO-selective electrode method. Hippocampal blood flow was also estimated by laser Doppler flowmetry. 7-Nitroindazole [0 (vehicle), 12.5, 25, 50 or 100 mg/kg] was administered intraperitoneally 20 min before ischemia. 7-Nitroindazole at any dose used did not affect basal NO levels before ischemia. 7-Nitroindazole (25, 50 and 100 mg/kg) reduced the NO concentration significantly during post-ischemic early reperfusion. Before 10 min of ischemia and during post-ischemic early reperfusion, there were no significant differences in hippocampal basal blood flow and reactive hyperemia between vehicle- and 7-nitroindazole-treated groups. These results demonstrate that the neuronal NO synthase inhibitor, 7-nitroindazole, can effectively inhibit NO synthesis in rat hippocampus during post-ischemic early reperfusion.

Keywords: 7-Nitroindazole; Forebrain ischemia; Nitric oxide (NO); Cerebral blood flow; Hippocampus; (Rat)

1. Introduction

Nitric oxide (NO) is involved in many cerebral physiological processes including circulation and neural transmission. NO also is an important factor in pathophysiological events in the brain. Neuronal NO synthase (nNOS) and inducible NO synthase (iNOS) have a neurotoxic effect, while endothelial NO synthase (eNOS) has a protective effect (Samdani et al., 1997). Excessive production of NO by nNOS is implicated in the neurotoxicity elicited by glutamate acting through *N*-metyl-D-aspartate receptors in vascular stroke (Samdani et al., 1997).

7-Nitroindazole is an nNOS inhibitor, which selectively inhibits nNOS and lacks the capacity to elevate blood pressure in anesthetized animals (Moore et al., 1993). Although in vitro studies have demonstrated that 7-nitroindazole inhibits both eNOS and nNOS (Yoshida et al., 1994), in vivo studies showed no effects of 7-nitroindazole on blood pressure, on endothelium-dependent blood vessel relaxation and on acetylcholine-induced vessel relaxation (Moore et al., 1993; Yoshida et al., 1994).

NO is a free radical, which is one of highly reactive chemical species and its short half-life makes direct measurement difficult in vivo. Malinski et al. (1993) reported the first direct measurement of NO in rat brain during and after transient cerebral focal ischemia, using an NO sensitive porphyrinic microsensor. However, direct measurement of NO in vivo has not been performed in transient ischemic hippocampus. In the present study, therefore, using an NO-selective electrode method and laser Doppler flowmetry, we investigated the effects of 7-nitroindazole on NO concentration and blood flow in rat hippocampus after transient forebrain ischemia.

2. Materials and methods

2.1. Animal preparation

Adult male Wistar rats weighing between 280 and 320 g were allowed free access to food and water in day–night regulated quarters at 25°C. The Guiding Principles for Care and Use of Animals in the Field of Physiological

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Sciences (The Physiological Society of Japan) were strictly followed. On the day before the experiment, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The vertebral arteries were electrocauterized, the common carotid arteries of both sides were dissected free and suture loops were placed around each vessel. On the following day, the rats were anesthetized with urethane (1.2 g/kg, i.p.), fixed in a stereotaxic frame and ventilated with an artificial respirator. Transient forebrain ischemia was induced by hanging a 5-g weight on each loop of the suture thread for 10 min. On the start of reperfusion the weights were taken away. Rectal temperature was maintained at 37°C–38°C with a heating pad.

2.2. Measurement of NO concentration

NO concentration in the hippocampus was determined by an NO monitor (NO-501, InterMedical Nagoya, Japan). An NO-selective electrode of 250-μm tip diameter was inserted into the right dorsal hippocampus (5.5 mm caudal to the bregma, 4 mm lateral to the midline and 4 mm below the cortical surface). A reference electrode was placed on the surface of skull beneath the scalp. The pA-order redox current between the NO-selective electrode and the reference electrode was measured. Before beginning each experiment, the sensitivity of NO electrode was calibrated by NO concentration using *S*-nitroso-*N*-acetyl-D,L-penicillamine as a standard NO generator (Ichimori et al., 1994; Ishida et al., 1996). The current increased linearly as the *S*-nitroso-*N*-acetyl-D,L-penicillamine concentration increased. A current of 100 pA was approximately equivalent to the *S*-nitroso-*N*-acetyl-D,L-penicillamine concentration of 12 μM. Changes in NO concentration were represented as changes in current (pA) (Ioroi et al., 1998). Before measuring NO current, the NO-selective electrode and the reference electrode were soaked in saline for 1 h or more.

2.3. Measurement of hippocampal blood flow

Hippocampal blood flow was measured with laser Doppler flowmetry (TBF-LC 1, Unique Medical Tokyo, Japan). A laser Doppler flowmetry probe (Needle Type ON97-066, tip diameter 500 μm, Unique Medical Tokyo, Japan) was inserted into the left dorsal hippocampus (5.5 mm caudal to the bregma, 4 mm lateral to the midline and 3 mm below the cortical surface). Hippocampal blood flow was expressed as a percentage taking the mean baseline blood flow as 100%.

2.4. Experimental protocol

Eighteen rats, divided into 5 groups, received intraperitoneally peanut oil (vehicle) ($n = 4$), 12.5 ($n = 3$), 25 ($n = 4$), 50 ($n = 4$) or 100 ($n = 3$) mg/kg 7-nitroindazole 20 min before ischemia. After 10 min of ischemia, cerebral reperfusion was achieved by removing the weights hung around the common carotid arteries and continued for 60 min. The experiment was started more than 1 h after the laser Doppler flowmetry probe and the NO-selective electrode had been inserted into the hippocampi.

2.5. Drugs

Peanut oil and 7-nitroindazole were purchased from Sigma (St. Louis, MO, USA). 7-Nitroindazole was suspended in peanut oil by sonication.

2.6. Statistical analysis

All data were expressed as means ± S.E.M. A paired Student's *t*-test was used to compare differences between the groups. P values < 0.05 were considered significant.

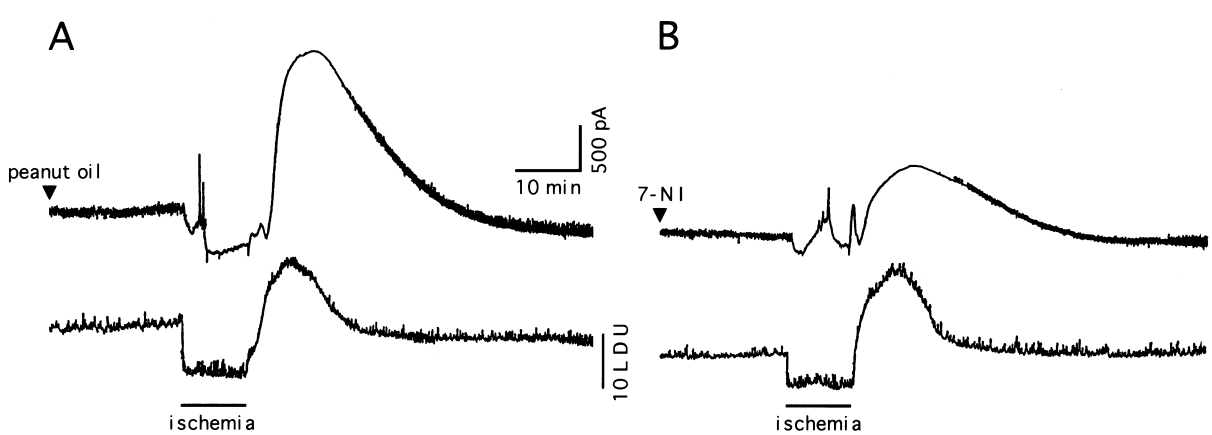

Fig. 1. Representative records of NO concentration (upper trace) and blood flow (lower trace) in the hippocampus before, during and after transient forebrain ischemia in the vehicle (peanut oil) (A) and 25 mg/kg 7-nitroindazole (7-NI) (B) treated rats. LDU, laser Doppler unit. Note that post-ischemic NO concentration was reduced in the 25 mg/kg 7-nitroindazole-treated rats, as compared with that in the vehicle-treated rats.

3. Results

3.1. Effects of 7-nitroindazole on hippocampal basal and post-ischemia-induced NO concentration

Fig. 1 shows representative records of changes in NO concentration and blood flow in rat hippocampus before, during and after transient forebrain ischemia in vehicle- and 25 mg/kg 7-nitroindazole-treated rats. A marked increase in NO concentration (current) was observed during post-ischemic early reperfusion in the vehicle-treated rat. In contrast, application of 25 mg/kg 7-nitroindazole reduced NO concentration.

As NO release during ischemia showed a complex pattern, we analysed only the NO concentration after ischemia, i.e., during early reperfusion for the sake of simplicity. Basal NO levels before ischemia were not affected by 7-nitroindazole (Fig. 2A). Maximum NO concentration during post-ischemic early reperfusion was significantly reduced by 7-nitroindazole in a dose-dependent manner: It was 2520.8 ± 211.6, 1650.0 ± 76.4, 900.0 ± 98.4, 835.5 ± 74.0 and 760.7 ± 105.7 pA in the vehicle-, 12.5, 25, 50 and 100 mg/kg 7-nitroindazole-treated groups, respectively (Fig. 2B). As compared with the vehicle-treated group, 12.5, 25, 50 and 100 mg/kg 7-nitroindazole-treated groups reduced NO concentration significantly by 34.5 ± 3.0 ($P < 0.05$), by 64.3 ± 3.9 ($P < 0.001$), by 66.9 ± 2.9 ($P < 0.001$) and by $69.8 \pm 4.2\%$ ($P < 0.005$), respectively. Peak latencies of maximum NO concentration during post-ischemic early reperfusion were as follows: 11.7 ± 1.9, 12.0 ± 2.0, 8.8 ± 0.9, 7.6 ± 0.8 and 7.2 ± 1.2 min in the vehicle-, 12.5, 25, 50 and 100 mg/kg 7-nitroindazole-treated groups, respectively. There were no significant differences among these groups.

3.2. Effects of 7-nitroindazole on hippocampal basal blood flow and post-ischemia-induced hyperemia

Hippocampal basal blood flow before ischemia was not modified significantly by 7-nitroindazole at any dose used (Fig. 2C). Ischemia reduced hippocampal blood flow to $15.4 \pm 1.6\%$, $14.2 \pm 3.9\%$, $16.2 \pm 1.3\%$, $13.6 \pm 2.8\%$ and $12.3 \pm 1.1\%$ in the vehicle-, 12.5, 25, 50 and 100 mg/kg 7-nitroindazole-treated groups, respectively. No significant differences were found among these groups. Reactive hyperemia during post-ischemic early reperfusion was observed and hippocampal blood flow reached to $219.7 \pm 20.6\%$, $224.1 \pm 6.7\%$, $229.2 \pm 22.9\%$, $254.9 \pm 37.8\%$ and $269.2 \pm 22.7\%$ in the vehicle-, 12.5, 25, 50 and 100 mg/kg 7-nitroindazole-treated groups, respectively (Fig. 2D). No significant differences were found between vehicle- and 7-nitroindazole-treated groups. Sixty min after the onset of reperfusion, hippocampal blood flow was decreased by approximately 20% in all rats (data not shown).

4. Discussion

In the present study, we have demonstrated that the selective inhibitor of nNOS, 7-nitroindazole, reduces the increase in NO concentration in rat hippocampus during early reperfusion after transient forebrain ischemia induced by four vessel occlusion in a dose-dependent manner. The reduction of NO concentration induced by 7-nitroindazole was not accompanied by any significant change in hippocampal blood flow. 7-Nitroindazole affected neither basal NO concentration nor basal blood flow in rat hippocampus.

It is known that 7-nitroindazole inhibits selectively nNOS (Moore et al., 1993; Yoshida et al., 1994) but not eNOS (Faraci and Brian, 1995). Moreover, 7-nitroindazole does not modify the cerebrovascular response to acetylcholine (Yoshida et al., 1994). 7-Nitroindazole (25 mg/kg, i.p.) has a neuroprotective action in 20 min global ischemia in rats (Nanri et al., 1998). Our present in vivo findings that application of 12.5, 25 and 50 mg/kg 7-nitroindazole reduced post-ischemia-induced NO release by 35%, 64% and 67%, respectively, is in accordance with other reports that application of 12.5, 25 and 50 mg/kg 7-nitroindazole reduced NOS activity in hippocampus by

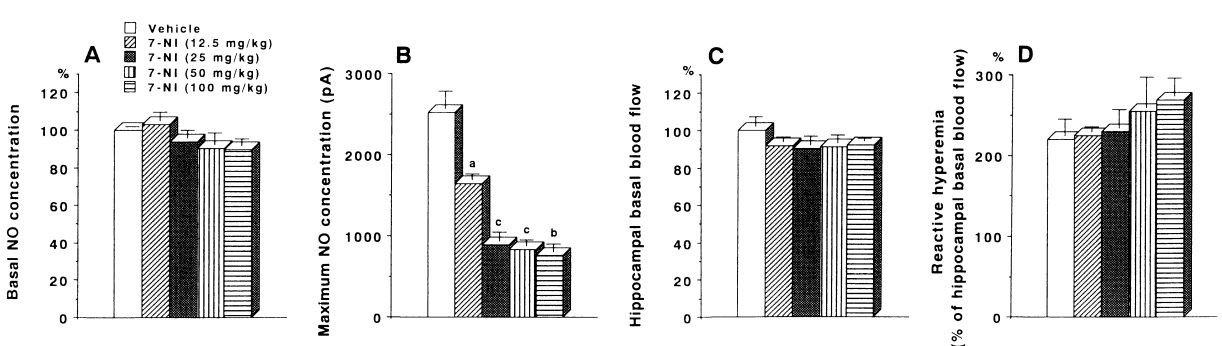

Fig. 2. Effects of 7-nitroindazole (7-NI) on basal (A) and maximum (B) NO concentration during post-ischemic early reperfusion and basal blood flow (C) and reactive hyperemia induced by transient forebrain ischemia (D) in rat hippocampus. In (A) and (B), note that 7-nitroindazole reduces post-ischemia-induced NO concentration in a dose-dependent manner, although 7-nitroindazole does not affect basal NO concentration. (a) $P < 0.05$; (b) $P < 0.005$; (c) $P < 0.001$ vs. vehicle. In (C) and (D), note that 7-nitroindazole does not affect either hippocampal basal blood flow or reactive hyperemia.

37%, 65% and 44%, respectively (Babbedge et al., 1993; Kalisch et al., 1996; Stagliano et al., 1997) and that the ED_{50} value obtained from 7-nitroindazole dose-response curve in the hippocampus was estimated as 25 mg/kg (Kalisch et al., 1996). Moreover, the present result that 7-nitroindazole reduced NO concentration during post-ischemic early reperfusion is also supported by a recent report by Lei et al. (1999). They have observed using microdialysis that 7-nitroindazole (80 mg/kg, i.p.) inhibits the increased NO metabolites in the CA1 field of the gerbil hippocampus after 10 min of forebrain ischemia.

In the present study, the mean latency to the maximum NO concentration during post-ischemic early reperfusion in the 25 mg/kg 7-nitroindazole-treated group was 8.8 min. The timing of NOS inhibitor application affects markedly the outcome of ischemic damage (Shibata et al., 1996). In this respect, we applied 7-nitroindazole 20 min before the onset of ischemia, being consistent with the result that maximum inhibition of nNOS occurred 30 min after application of 7-nitroindazole (Mackenzie et al., 1994). In the present study, we have observed that NO concentration and blood flow were not modified by 7-nitroindazole during pre-ischemic period, indicating that under physiological conditions, 7-nitroindazole may not affect basal activity of eNOS which is found abundantly in hippocampal pyramidal cells (Dinerman et al., 1994). Lei et al. (1999) have recently reported that eNOS may contribute to the basal NO production in the gerbil hippocampus. Post-ischemia-induced NO concentration which was kept unchanged after application of 7-nitroindazole may be due to direct reduction of nitrite to NO under acidic and highly reduced conditions (Zweier et al., 1995).

In the previous investigations, NO concentration was estimated by measuring NOS activity (Babbedge et al., 1993; Kalisch et al., 1996; Stagliano et al., 1997). Kojima et al. (1998) have shown that high expression of NOS does not always imply high NO synthesis. Applying 7-nitroindazole in various doses, we have proved 25 mg/kg to be effective for inhibition of nNOS, which supports other reports of neuroprotective action by application of 25 mg/kg 7-nitroindazole (Nanri et al., 1998).

Our present in vivo finding, that under urethane anesthesia, 7-nitroindazole did not change hippocampal basal blood flow, is in accordance with other reports that 7-nitroindazole did not alter the diameter of cerebral arterioles (Yoshida et al., 1994) and that 7-nitroindazole decreased hippocampal basal blood flow by only 2% (Cholet et al., 1997). As post-ischemic early reperfusion is accompanied by an increase in NO concentration to induce neural damage, reduction of NO concentration induced by 7-nitroindazole may be responsible for the neuroprotection.

In conclusion, the present study has demonstrated that when a suitable dose of 25 mg/kg is applied intraperitoneally, 7-nitroindazole can inhibit excessive NO concentration during post-ischemic early reperfusion in rat hippocampus in vivo. It is reasonable to conclude that 7-nitroindazole should be administered into the experimental animals before or during ischemia to reduce excessive NO concentration, because maximum NO concentration occurs approximately 10 min after the onset of reperfusion. In addition, 7-nitroindazole does not affect either basal NO concentration or basal blood flow under physiological conditions.

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