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Short communication

Increase in nitric oxide in the hypoxic–ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine

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Abstract

We measured the changes in nitric oxide (NO) metabolites in the brains of neonatal rats with hypoxic–ischemic damage. There were two peaks of NO metabolites in the lesioned side of the cortex without treatment: one during hypoxia and the other during the re-oxygenation period. Prehypoxic treatment with 7-nitroindazole, a selective neuronal NO synthase inhibitor, suppressed both peaks of NO metabolites, whereas prehypoxic treatment with aminoguanidine, a selective inducible NO synthase inhibitor, partially suppressed only the peak in the re-oxygenation period. These data suggest different roles of neuronal and inducible NO synthases in the pathogenesis of hypoxic–ischemic encephalopathy. © 1998 Elsevier Science B.V.

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1. Introduction

There is increasing evidence that nitric oxide (NO) plays an important role in neuronal damage in neonatal hypoxic–ischemic encephalopathy (Trifiletti, 1992; Hamada et al., 1994; Higuchi et al., 1996) as well as in cerebral ischemia in adults. There are three types of NO synthase. Neuronal and inducible NO synthases have a neurotoxic effect and endothelial NO synthase has a protective effect (Yoshida et al., 1994; Huang et al., 1994; Zhang et al., 1996; Huang et al., 1996).

The kinetics of NO itself or its metabolites during and after an ischemic insult have been reported only in adult animals (Kader et al., 1993; Malinski et al., 1993; Salter et al., 1996; Shibata et al., 1996). Here we report the temporal profile of NO metabolites in brains of neonatal rats with hypoxic–ischemic brain damage, as a model of hypoxic–ischemic encephalopathy, and the effect of selective inhibitors against inducible and neuronal NO synthases.

2. Materials and methods

We used the method of Rice and Vannuci (Vannuci, 1990; Rice et al., 1991) to induce hypoxic–ischemic brain damage in neonatal rats. All procedures were in accordance with the Guidelines for Animal Experiments of Kyoto University. Under ether anesthesia, the left common carotid artery of a 7-day-old Wistar rat was ligated. After recovering from the anesthesia, the pup was exposed to an 8% oxygen and 92% nitrogen environment for 2.5 h.

As test agents, 7-nitroindazole was dissolved in dimethylformamide at a concentration of 100 mg/ml and then diluted to 10 mg/ml with peanut oil. Aminoguanidine was dissolved in phosphate-buffered saline to 20 mg/ml. Rat pups were randomly assigned to one of three groups: the no-treatment, 7-nitroindazole, or aminoguanidine group. The 7-nitroindazole and aminoguanidine groups were given 50 mg/kg of 7-nitroindazole or 100 mg/kg of aminoguanidine, respectively, intraperitoneally 1 h before hypoxia.

We measured NO metabolites at 0 h, 1 h (during hypoxia), 2.5 h (end of hypoxia), and 6 h after the start of hypoxia. At each time point, the rat was decapitated and each side of the cerebral cortex was removed on to an ice-cooled glass-plate. After addition of 1.5 ml of buffer

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(0.1 M, pH 7.5, potassium phosphate, 20 mM of EDTA), the tissue was homogenized. The supernatant was obtained through centrifugation (14,000 rpm × 10 min, then 20 min) and was filtered to remove hemoglobin with Centricon 10 (Amicon, MA, USA), 5 G × 1 h (Misko et al., 1993). NO metabolites in the supernatant were measured by the chemiluminescence method, using an NO analyzer (FES-450, ScholaTech, Osaka, Japan). Samples were injected into a sealed vial containing saturated ascorbic acid solution. All NO metabolites, most of which are nitrite and nitrate (Gross, 1995), are reduced to NO. NO was carried on a constant stream of argon gas to a photomultiplier tube and photon counter. The values of NO metabolites are expressed as picomoles per milligram soluble protein. Protein was assayed by using the method of Bradford (1976).

The levels of NO metabolites in the cortices of ligated and non-ligated sides were compared by two-tailed, paired, Student's *t*-test. The comparisons of NO metabolites in the ligated side of the cortex among the three groups were done by using non-paired Student's *t*-test with Bonferroni's correction, using $P < 0.05$ as the level of significance.

3. Results

The results are shown in Fig. 1. In the no-treatment group, there were two significant peaks in NO metabolites in the ligated side compared to the non-ligated side. One peak occurred during hypoxia, $t = 1$ h (the hypoxic phase), and the other occurred after hypoxia, $t = 6$ h (the re-oxygenation phase). In the 7-nitroindazole group, there was no increase in NO metabolites in the ligated side during either phase. In the aminoguanidine group, the level of NO metabolites in the ligated side was significantly increased during both phases compared with the non-ligated side. In the re-oxygenation phase, the level of NO metabolites in the ligated side in the aminoguanidine group was significantly lower than that in the no-treatment group, but significantly higher than that in the 7-nitroindazole group. There was no significant temporal change in NO metabolites in the non-ligated side in any group. The levels of NO metabolites in the rats treated with the vehicle (phosphate-buffered saline or 10% dimethylformamide in peanut oil) did not differ from those in the no-treatment group (data not shown).

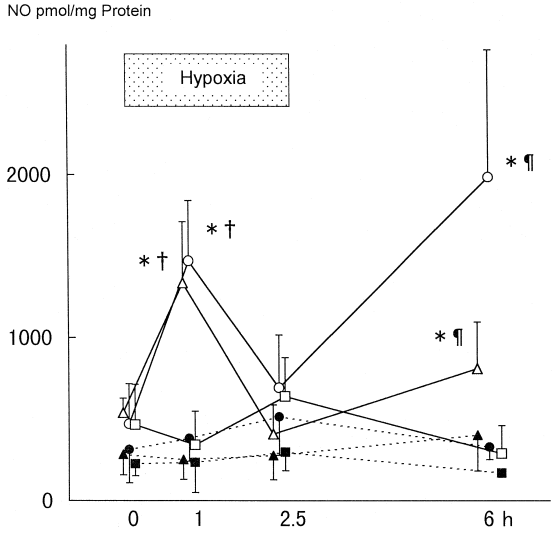

Fig. 1. NO metabolites in the ligated side (open marks and bold lines) and in the non-ligated side (closed marks and dashed lines), mean ± S.D. The no-treatment group (○, ●), the 7-nitroindazole group (□, ■), and the aminoguanidine group (△, ▲). At 1 h and 6 h, NO metabolites increased in the ligated side compared to those in the non-ligated side in the no-treatment and the aminoguanidine group ($^{*}P < 0.05$). At 1 h, NO metabolites in the ligated side in the no-treatment and the aminoguanidine groups were increased compared to those in the 7-nitroindazole group ($^{\dagger}P < 0.05$). At 6 h, NO metabolites in the ligated side increased in the no-treatment group and the aminoguanidine group as compared to the 7-nitroindazole group ($^{\P}P < 0.05$).

4. Discussion

This is the first report showing the temporal profile of NO metabolites in a neonatal rat model of hypoxic–ischemic encephalopathy. NO may be involved in the neuronal injury because an NO synthase inhibitor, L-nitroarginine, has a neuroprotective effect in this neonatal model (Trifiletti, 1992; Hamada et al., 1994). The results of the present study showed that NO metabolites were increased only in the ligated side of the cortex, where the brain injury occurs (Vannuci, 1990; Rice et al., 1991). This provides further evidence of there being a link between excessive NO and neonatal brain injury.

The increase in NO metabolites showed two peaks in the neonatal rats, during hypoxia and re-oxygenation. Similar temporal changes have been reported in adult models of cerebral ischemia and reperfusion. In a middle cerebral artery occlusion model in adult rats, NO, measured by a microsensor placed in the cortex, increased during occlusion and after reperfusion (Malinski et al., 1993). An enhancement of nitrite levels in the striatum following reperfusion was also observed in a rat model of global cerebral ischemia (Shibata et al., 1996). In addition, the increases in NO and its metabolites were prevented by *N*-nitro-L-arginine methyl ester, a non-selective NO synthase inhibitor.

In this study, 7-nitroindazole and aminoguanidine were used as selective inhibitors of NO synthase. 7-Nitroindazole is a specific inhibitor of neuronal NO synthase (Babbedge et al., 1993) and has neuroprotective effects without causing any blood pressure change (Yoshida et al., 1994). Aminoguanidine attenuated inducible NO synthase activity in the infarcted area and reduced infarct volume without affecting the resting cerebral blood flow (Iadecola et al., 1995). The possible neuroprotective effect of these agents has not been shown in a neonatal hypoxic–ischemic encephalopathy model. We are now exploring the neuropro-

tective efficacy of selective NO synthase inhibitors. For the experiments, the temporal profile of NO during and after the ischemic insult shown in this study should be taken into account as well as the short half-lives of the selective inhibitors (Salter et al., 1996; Corbett and MacDaniel, 1996).

7-Nitroindazole and aminoguanidine suppressed the increase in NO metabolites, but in different ways. The increase in NO metabolites was suppressed by 7-nitroindazole during both the hypoxic and the re-oxygenation phases, while aminoguanidine only partially suppressed the peak in the re-oxygenation phase alone.

This suppression by aminoguanidine implies that inducible NO synthase is involved in the increase in NO metabolites during the re-oxygenation phase but not in the hypoxic phase. This selective suppression exerted by aminoguanidine is consistent with the reported temporal changes in neuronal NO synthase and inducible NO synthase. Neuronal NO synthase is upregulated or has increased enzymatic activity in the early phase of cerebral ischemia in both adult (Kader et al., 1993; Zhang et al., 1994) and neonatal (Higuchi et al., 1996) rats. The induction of inducible NO synthase occurs in the late phase of ischemic brain injury in adult (Zhang et al., 1996) and neonatal (Muramatsu et al., 1996) rats. Inducible NO synthase seems to be activated by inflammatory cytokines released from damaged brain tissue (Iadecola, 1997).

Inhibition of neuronal NO synthase abolished the peak of NO metabolites not only during hypoxia but also during re-oxygenation. A direct suppression of inducible NO synthase activity in the later phase by 7-nitroindazole is unlikely, judging by its short half-life and lack of potency against inducible NO synthase (Salter et al., 1996; Southan and Szabo, 1996). We consider that there is a cascade of neuronal injury induced by NO, which is generated initially by neuronal NO synthase during hypoxia and subsequently by inducible NO. In our study, 7-nitroindazole must have suppressed the initial increase in NO by its direct inhibition of neuronal NO synthase. Then, it may have indirectly suppressed the surge in NO during re-oxygenation by inhibiting the induction of inducible NO synthase.

The elucidation of the different roles of NO synthase isoforms may lead to the development of therapeutic strategies for brain protection.

References

Babbedge, R.C., Bland-Wald, P.A., Hart, S.L., Moore, P.K., 1993. Inhibition of rat cerebellar nitric oxide synthase by 7-nitroindazole and related substituted indazoles. Br. J. Pharmacol. 110, 225–228.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram qualities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 246–254.

Corbett, J.A., MacDaniel, M.L., 1996. Selective inhibition of inducible nitric oxide synthase by aminoguanidine. Methods Enzymol. 268, 398–408.

Gross, S.S., 1995. Nitric oxide: Pathophysiological mechanisms. Annu. Rev. Physiol. 57, 737–769.

Hamada, Y., Hayakawa, T., Hattori, H., Mikawa, H., 1994. Inhibitor of nitric oxide synthesis reduces hypoxic–ischemic brain damage in the neonatal rat. Pediatr. Res. 35, 10–14.

Higuchi, Y., Hattori, H., Hattori, R., Furusho, K., 1996. Increased neurons containing neuronal nitric oxide synthase in the brain of a hypoxic–ischemic neonatal rat model. Brain Dev. 18, 369–375.

Huang, Z., Huang, P.L., Panahian, H., Dalkara, T., Fishman, M.C., Moskowitz, M.A., 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265, 1883–1885.

Huang, Z., Huang, P.L., Ma, J., Meng, W., Ayata, C., Fishman, M.C., Moskowitz, M.A., 1996. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J. Cereb. Blood Flow Metab. 16, 981–987.

Iadecola, C., 1997. Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci. 20, 132–139.

Iadecola, C., Xu, X., Zhang, F., El-Fakahany, E.E., Ross, M.E., 1995. Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia. J. Cereb. Blood Flow Metab. 15, 52–59.

Kader, A., Frazzini, V.I., Solomon, R.A., Trifiletti, R.R., 1993. Nitric oxide production during focal cerebral ischemia in rats. Stroke 24, 1709–1716.

Malinski, T., Bailey, F., Zhang, Z.G., Chopp, M., 1993. Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J. Cereb. Blood Flow Metab. 13, 355–358.

Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M., Currie, M.G., 1993. A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. 214, 11–16.

Muramatsu, K., Fujimoto, I., Togari, H., Fukuda, A., Nishino, H., Wada, Y., 1996. The expression of inducible nitric oxide synthase mRNA in the neonatal rat brain after hypoxic–ischemic insult. Acta Neonatol. Jpn. 32, 634–636.

Rice, J.E., Vannuci, R.C., Brierly, J.B., 1991. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann. Neurol. 9, 131–141.

Salter, M., Duffy, C., Garthwaite, J., Strijbos, P.J.L., 1996. Ex vivo measurement of brain tissue nitrite and nitrate accurately reflects nitric oxide synthase activity in vivo. J. Neurochem. 66, 1683–1690.

Shibata, M., Araki, N., Hamada, J., Sasaki, T., Shimazu, K., Fukuuchi, Y., 1996. Brain nitrite production during global ischemia and reperfusion: An in vivo microdialysis study. Brain Res. 734, 86–90.

Southan, G.J., Szabo, C., 1996. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem. Pharmacol. 51, 383–394.

Trifiletti, R.R., 1992. Neuroprotective effects of N^G-nitro-L-arginine in focal stroke in the 7-day old rat. Eur. J. Pharmacol. 218, 197–198.

Vannuci, R.C., 1990. Experimental biology of cerebral hypoxia–ischemia: Relation to perinatal brain damage. Pediatr. Res. 27, 317–326.

Yoshida, T., Limmroth, V., Irikura, K., Moskowitz, M.A., 1994. The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. J. Cereb. Blood Flow Metab. 14, 924–929.

Zhang, Z.G., Chopp, M., Gautam, S., Zaloga, C., Zhang, R.L., Schmidt, H.H.H.W., Pollock, J.S., Försterman, U., 1994. Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. Brain Res. 654, 85–95.

Zhang, F., Casey, R.M., Ross, E., Iadecola, C., 1996. Aminoguanidine ameliorates and L-arginine worsens brain damage from intraluminal middle cerebral artery occlusion. Stroke 27, 317–323.