Korean J Physiol Pharmacol
Vol 8: 1–5, February, 2004

Postischemic Treatment with Aminoguanidine Inhibits Peroxynitrite Production in the Rat Hippocampus Following Transient Forebrain Ischemia

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Transient forebrain ischemia results in the delayed neuronal death in the CA1 area of the hippocampus. The present study was performed to determine effects of aminoguanidine, a selective iNOS inhibitor, on the generation of peroxynitrite and delayed neuronal death occurring in the hippocampus following transient forebrain ischemia. Transient forebrain ischemia was produced in the conscious rats by four-vessel occlusion for 10 min. Treatment with aminoguanidine (100 mg/kg or 200 mg/kg, i.p.) or saline (0.4 ml/100 g, i.p.) was started 30 min following ischemia-reperfusion and the animals were then injected twice daily until 12 h before sacrifice. Immunohistochemical method was used to detect 3-nitrotyrosine, a marker of peroxynitrite production. Posttreatment of aminoguanidine (200 mg/kg) significantly attenuated the neuronal death in the hippocampal CA1 area 3 days, but not 7 days, after ischemia-reperfusion. 3-Nitrotyrosine immunoreactivity was enhanced in the hippocampal CA1 area 3 days after reperfusion, which was prevented by the treatment of aminoguanidine (100 mg/kg and 200 mg/kg). Our findings showed that (1) the generation of peroxynitrite in the hippocampal CA1 area 3 days after ischemia-reperfusion was dependent on the iNOS activity; (2) the postischemic delayed neuronal death was attenuated in the early phase through the prevention of peroxynitrite generation by an iNOS inhibitor.

Key Words: Transient forebrain ischemia, Aminoguanidine, Peroxynitrite, iNOS, Hippocampus, Rats

INTRODUCTION

Transient forebrain ischemia followed by reperfusion induces delayed neuronal death in the hippocampal CA1 region several days later (Pulsinelli & Brierley, 1979), and glutamate excitotoxicity and increased concentration of intracellular calcium have been known to be the underlying mechanisms (Alps et al, 1987; Deshpande et al, 1987). The increased intracellular calcium can accelerate the synthesis of nitric oxide (Olanow, 1993).

There have been a plethora of reports showing that nitric oxide is involved in neuronal death after ischemia- reperfusion. For example, the concentration of nitric oxide increases during ischemic and reperfusion periods (Kader et al, 1993; Kumura et al, 1996). However, it is unclear whether the inhibitor of nitric oxide synthases (NOSs) can protect neurons from ischemic insults. It was reported that 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase (nNOS), decreased the delayed neuronal death in the hippocampal CA1 region following ischemic injury (Chalimoniuk & Strosznajder, 1998; Nanri et al, 1998). On the other hand, Kirsch et al (1997) showed that treatment with NOS inhibitors didn't have any effects on neuronal damage after ischemic injury in cats. Furthermore, Zhang et al (1995) observed that pretreatment with NOS inhibitor further increased the extracellular concentration of glutamate during reperfusion period, suggesting that NOS inhibitors can aggravate excitotoxicity of ischemia and reperfusion. These results show that nitric oxide plays divergent roles in delayed neuronal death and repair process following ischemic injury.

Superoxide as well as nitric oxide is also increased after ischemic injury. These two free radicals nonenzymetically produce peroxynitrite (Beckman et al, 1990). The cytotoxicity of peroxynitrite includes nitrosylation of proteins, lipid peroxydation, DNA damage, and activation of poly ADP-ribose polymerase, resulting in depletion of energy (Szabo et al, 1996). Therefore, it is quite conceivable that increased production of peroxynitrite can be one of the important mechanisms involved in delayed neuronal death in the hippocampal CA1 region after transient forebrain ischemia.

In the present study, we questioned whether the inhibitor of inducible NOS (iNOS) could influence the production of peroxynitrite and delayed neuronal death in the hippocampus of ischemic rats. For this purpose, we intraperitoneally injected aminoguanidine, an iNOS inhibitor, starting from 30 min after reperfusion, and performed immunohistochemistry for 3-nitrotyrosine as an indicator of peroxynitrite generation.

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS.

METHODS

Animals and drug administration

Adult Sprague-Dawley male rats (250~300 g) were used in this study. Transient forebrain ischemia was induced by four-vessel occlusion and reperfusion, as previously described by Pulsinelli & Brierley (1979), with minor modifications (Choi et al, 2003). Briefly, the vertebral arteries were electrocauterized and, 24 h later, both common carotid arteries were occluded with miniature aneurysm clips for 10 min. Only animals with complete electroencephalogram (EEG) flattening upon vascular occlusion were classified as ischemic and used for the study. Rectal temperature was maintained at 37.5±0.3°C using heating lamp. Aminoguanidine (Sigma, MO, USA) was injected 30 min after reperfusion, every 12 h thereafter until 2.5 days to rats perfused three days after reperfusion, and 6.5 days to rats perfused 7 days after reperfusion. In sham operated rats, saline was administered at the same schedule. We assigned 6~9 rats to each group.

Tissue preparation

The rats were deeply anesthetized with pentobarbital (100 mg/kg) and perfused at 12 h after last aminoguanidine or saline injection with 4% paraformaldehyde (Merck, Germany). The brains were removed, postfixed in the same fixative for 4 h, and cryoprotected in 30% sucrose in 0.01 M PBS. The brains were rapidly frozen in liquid nitrogen and stored at −70°C.

Cresyl violet staining and microscopical analysis

Hippocampal coronal sections (20 μm thick) in 3.3 mm posterior to bregma were cut and mounted on gelatin-coated slides. The sections were hydrated in 100, 95, 90, 80, 70% ethanol, tap water for 5 min each and stained in 0.3% cresyl violet solution (Sigma) for 20 min. After decolorization in 95% acidic ethanol, the sections were dehydrated in xylene and mounted with canada balsam. The survived neurons within 250 μm of the hippocampal CA1 regions were then counted under microscope (X400: AHBS-514, Olympus, NY, USA) (Kirino et al, 1986). Values are expressed as mean±SD. Statistical evaluation of differences was performed using Student's t-test in experiments containing two groups, and ANOVA followed by *post hoc* Dunnett test in experiments containing three groups.

Immunohistochemisty

Immunohistochemistry for 3-nitrotyrosine was carried out in coronal section (20 μm thick). Briefly, free-floating sections were preincubated for 1 h in 0.01 M phosphate buffered saline (PBS) containing 10% normal goat serum (Vector Laboratories, CA, USA), and they were then incubated for 60 h at 4°C with anti-3-nitrotyrosine antibody (mouse monoclonal, 1 : 2000, Upstate Biotechnology, NY, USA) in preincubation solution. After PBS washing, the sections were incubated with secondary antibody (biotinylated horse anti-mouse; Vector Laboratories) for 1 h, followed by amplification with an avidin-biotin complex (Vector Laboratories) for 1 h. Subsequently, cells were visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide as substrates. The sections were mounted on gelatin-coated slides and rehydrated in 100, 95, 90, 80 and 70% ethanol followed by distilled water for 3 min. The sections were counterstained in hematoxylin solution (7.5 mg/ml, Sigma) for 2 min and decolorized in 1% hydrochloric acid. They were then dehydrated and mounted using canada balsam.

RESULTS

The effects of aminoguanidine on delayed neuronal death after transient forebrain ischemia

Fig. 1 shows that more neurons survived in the aminoguanidine-treated group 3 days after reperfusion than the saline-treated group. However, at 7 days after reperfusion, the distribution of survived cells was similar between the

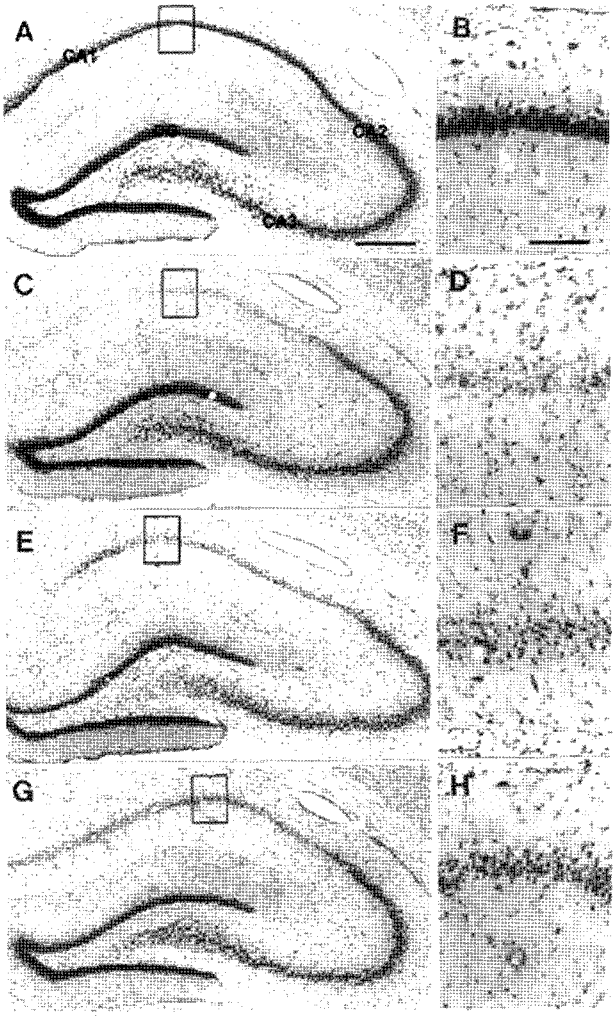

Fig. 1. Micrographs of the rat hippocampus stained with cresyl violet 3 days after transient forebrain ischemia of 10 min. A and B: a rat hippocampus 3 days after the sham operation. Post-treated with saline (C, D) or aminoguanidine of 100 mg/kg (E, F) or 200 mg/kg (G, H) after reperfusion. B, D, F and H show CA1 pyramidal layer at higher magnification. Scale bars, 200 μm (A, C, E, G) and 50 μm (B, D, F, H).

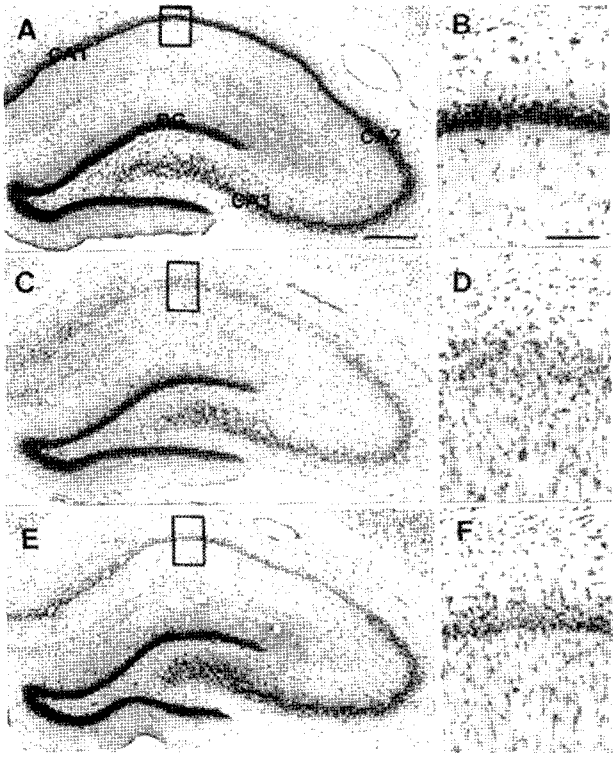

Fig. 2. Micrographs of the rat hippocampus stained with cresyl violet 7 days after transient forebrain ischemia of 10 min. A and B: a rat hippocampus 3 days after the sham operation. Post-treated with saline (C, D) or aminoguanidine (200 mg/kg: E, F) was post-treated after reperfusion. B, D and F show CA1 pyramidal layer at higher magnification. Scale bars, 200 μm (A, C, E) and 50 μm (B, D, F).

Table 1. Neuronal cell density per 0.25 mm linear length of the CA1 subfield after transient forebrain ischemia of 10 min

Treatment	Following reperfusion	
	3 days	7 days
Saline	1.7±1.5 (n=6)	1.4±1.5 (n=7)
AG (100 mg/kg)	17.0±19.1 (n=6)	ND
AG (200 mg/kg)	23.9±21.2* (n=9)	9.31±4.8 (n=7)

*$P<0.05$ vs. saline-treated group by *post hoc* Dunnett's test AG, aminoguanidine; ND, not determined: n, number of rats. The values are expressed as mean±SD.

two groups (Fig. 2). At 3 days after reperfusion, post-ischemic treatment with aminoguanidine (200 mg/kg) significantly increased the number of survived cells in the hippocampal CA1 following ischemic insult as compared with saline-treated group ($P<0.05$, Table 1). On the contrary, the number of survived cells was not significantly different between aminoguanidine (100 mg/kg)-treated and saline-treated groups. When the survived cells were counted 7 days after reperfusion, the numbers were 1.4± 1.5 and 9.3±14.8 in the saline- and aminoguanidine (200 mg/kg)-treated groups, respectively, and there was no significant difference between the two groups (Table 1).

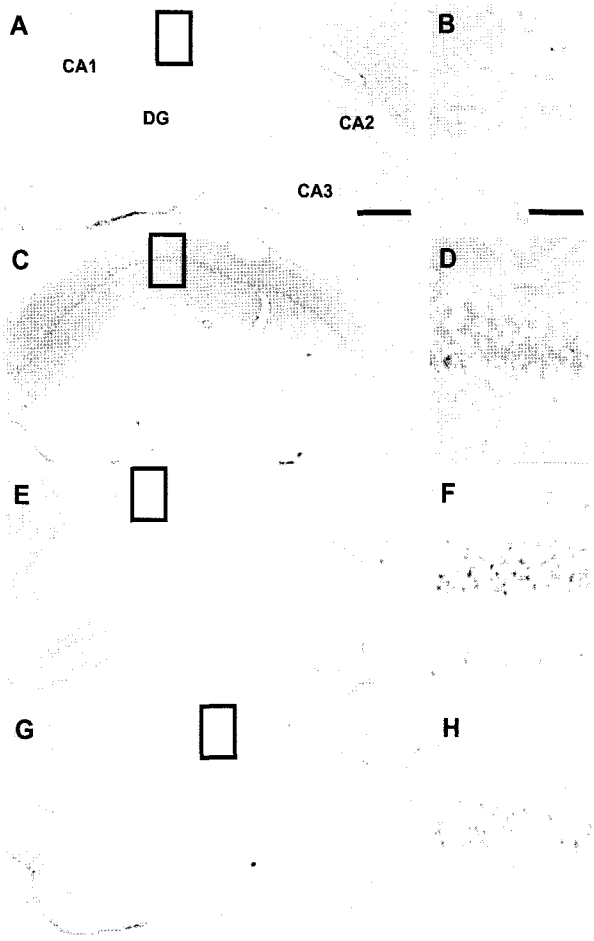

Fig. 3. Changes of 3-nitrotyrosine immunoreactivity in the hippocampus 3 days after transient forebrain ischemia. In a sham-operated rat (A, B), no significant nitrotyrosine immunoreactivity was detectable. In the saline-treated rat after ischemia of 10 min (C, D), immunoreactivity became prominent in the CA1 area. Post-treatment with aminoguanidine of 100 mg/kg (E, F) or 200 mg/kg (G, H) reduced the immunoreactivity to 3-nitrotyrosine. B, D, F and H show CA1 pyramidal layer at higher magnification. Scale bars, 200 μm (A, C, E, G) and 50 μm (B, D, F, H).

The effect of aminoguanidine on peroxynitrite production following transient forebrain ischemia

Immunoreactivity of 3-nitrotyrosine, a biochemical marker of peroxynitrite production, was increased in the hippocampal CA1 of saline-treated rats 3 days after reperfusion, but it was attenuated by the treatment with aminoguanidine (100 and 200 mg/kg) (Fig. 3).

DISCUSSION

One important strategy for the successful treatment of stroke is to protect neurons in penumbral area where the reversible damage occurs because neurons in ischemic core are damaged irreversibly (Hossmann, 1994). Therefore, in order to study the pathophysiology of ischemic injury and

identifying new drugs for the treatment of stroke, many researchers have used 4-vessel occlusion model, which induces reversible and delayed neuronal death. In the present study, we induced transient forebrain ischemia by 4-vessel occlusion, invented by Pulsinelli & Brierley (1979), with minor modifications. These include electrocauterization of the visible vertebral arteries between C1 and C2 vertebrae. In the present study, no rat died within 3 days after 10 min of forebrain ischemia and also no rat convulsed until the end of experiment. These results are similar to the results obtained by Pulsinelli & Brierley (1979).

Transient forebrain ischemia followed by reperfusion induces neuronal death prominently in the hippocampal CA1 region (Pulsinelli et al, 1982). Many reports have suggested that nitric oxide (NO) plays a significant role in delayed neuronal death after ischemic injury (Iadecola, 1997; Samdeni et al, 1997), and superoxide has also been known to increase significantly following ischemic insult (Kader et al, 1993; Kumura et al, 1996). Although NO can induce neurotoxicity by itself (Kuo & Schroeder, 1995), it can also react nonenzymetically with superoxide to produce peroxynitrite, which has more neurotoxicity (Beckman et al, 1990). In our previous study, we showed that nitrotyrosine immunoreactivity significantly increased in the hippocampal CA1 region 3 days after reperfusion (Kim & Kim, 2001). However, it is not certain whether the increased production of peroxynitrite contributes to the delayed neuronal death after ischemic insult or vice versa.

There are three isoforms of NOS, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), and it has been suggested that the expression of NOSs and their activities are regulated after ischemic insult in isoform-specific manners. Firstly, it has been known that nNOS rapidly increases following ischemic insult and plays a role in delayed neuronal death (Iadecola, 1997). Secondly, it is likely that ischemic injury induces sustained activation of eNOS, resulting in increased NO synthesis (Veltkamp et al, 2002). Because NO produced by eNOS maintains vasodilation and inhibits platelet aggregation, the activated eNOS is known to have neuroprotective effects against ischemic insult (Iadecola, 1997). Lastly, the expression of iNOS has been reported to begin to increase one to two days after focal ischemia (Fassbender et al, 2000). These observations strongly suggest a possibility that, 3 days after transient forebrain ischemia, the expression of iNOS is involved in the increased production of peroxynitrite, thereby resulting in the neuronal death.

Therefore, in order to explain this possibility, aminoguanidine, an iNOS inhibitor, was postischemically applied, and the neuronal death in the hippocampal CA1 region at 3 days after reperfusion, was found to be inhibited by aminoguanidine in a dose-dependent manner. However, there was no effect on neuronal survival examined at 7 days after reperfusion. These results, therefore, suggest that aminoguanidine has a partial neuroprotection activity against transient forebrain ischemia. Recently, Mori et al. (2001) reported that aminoguanidine reduced the deficits in long-term potentiation and learning ability after transient forebrain ischemia induced by 4-vessel occlusion. In addition, treatment with aminoguanidine starting 24 h after focal ischemia significantly reduced infarct volume in rats (Iadecola et al, 1995; Nagayama et al, 1998). Furthermore, aminoguanidine significantly inhibited ischemic damage and angiogenesis in the hippocampal CA1 and cerebral cortex of hypoxic rats (Niwa et al, 1999). Taken together, these results indicate that aminoguanidine can improve the neuropathologic changes following ischemic as well as hypoxic insults.

As shown above, while aminoguanidine exhibited only a partial neuroprotection against forebrain ischemia, its treatment blocked completely the increase of peroxynitrite production following ischemic insults. Therefore, in addition to peroxynitrite generation, sequential activation of several other mechanisms appears to be involved in the delayed neuronal death in the hippocampal CA1 area following ischemic insults. These mechanisms may include disruption of ionic gradient due to energy depletion (Hansen, 1985), glutamate-induced excitotoxicity (Choi, 1990), oxidative stress (Kinouchi et al, 1991; Kondo et al, 1997), inflammation (Lindsberg & Grau, 2003) and apoptosis (Villa et al, 2003).

In conclusion, we observed that aminoguanidine partially inhibited delayed neuronal death after transient forebrain ischemia. We also observed that, after transient forebrain ischemia, 1) the production of peroxynitrite is increased in the hippocampal CA1 region when neuronal death predominantly occurs; 2) postischemic aminoguanidine treatment inhibits the increase of peroxynitrite production in the hippocampal CA1 region.

ACKNOWLEDGEMENT

This work was supported by a grant No. R01-2002-000-00334-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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