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The Protective Effect of Aminoguanidine on Cerebral Ischemic Damage in the Rat Brain

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Summary

The NADPH-diaphorase (NADPH-d) histochemical technique is commonly used to localize the nitric oxide (NO) produced by the enzyme nitric oxide synthase (NOS) in neural tissue. The expression of inducible nitric oxide synthase (iNOS) is induced in the late stage of cerebral ischemia, and NO produced by iNOS contributes to the delay in recovery from brain neuronal damage. The present study was performed to investigate whether the increase in nitric oxide production *via* inducible nitric oxide synthase was suppressed by the administration of aminoguanidine, a selective iNOS inhibitor, as it follows a decrease of NADPH-diaphorase activity (a marker for NOS) after four-vessel occlusion used as an ischemic model. The administration of aminoguanidine (100 mg/kg i.p., twice per day up to 3 days immediately after the ischemic insult) reduced the number of NADPH-diaphorase positive cells to control levels. Our results indicated that aminoguanidine suppressed NADPH-diaphorase activity, and also decreased the number of NADPH-diaphorase positive cells in the CA1 region of the hippocampus following ischemic brain injury.

Key words

Cerebral ischemia • Hippocampus • NADPH-diaphorase • Nitric oxide synthase • Aminoguanidine

Introduction

Nitric oxide (NO) is a gaseous, ubiquitous neurotransmitter with multiple functions in the central nervous system. NO is produced from arginine by three distinct forms of nitric oxide synthase (NOS). Neuronal NOS (nNOS) is a calcium-dependent, constitutive neuronal enzyme. During ischemia, nNOS is upregulated and the produced NO reacts with reactive oxygen species (ROS) to produce radicals that have deleterious effects on neuronal survival (Iadecola *et al.* 1997, Samdani *et al.* 1997, Stagliano *et al.* 1997, Ashwal *et al.* 1998). Endothelial NOS (eNOS) is a calcium-dependent constitutive enzyme, which is cerebroprotective under ischemia conditions due to a possible vasodilatation that improves cerebral perfusion (Faraci and Heistad 1998, Forstermann *et al.* 1998, Samdani *et al.* 1997). Inducible NOS (iNOS) is a calcium-independent enzyme, which is upregulated under various stress conditions and has deleterious effects on neuronal survival in ischemia because it causes mitochondrial and cellular dysfunction (Dalkara and Moskowitz 1994, Iadecola *et al.* 1997, Samdani *et al.* 1997). All isoforms require homologous subunit dimerization and association with the cofactors calmodulin, flavin-mononucleotide (FMN), and β-nicotinamide adenine dinucleotide phosphate (NADP)

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as well as heme tetrahydrobiopterin (BH_4) to be active (Baek *et al.* 1993). During ischemia, extracellular levels of excitatory amino acids rise and cause the opening of ligand-gated ion channels with attendant increases in calcium and sodium flux. Higher intracellular levels of calcium lead to enhanced eNOS and nNOS enzymatic activity *via* a calcium-activated calmodulin mechanism (Tymianski and Tator 1996). The delayed increase in tissue NO levels could be ascribed to the robust and sustained NO production by the iNOS isoform as part of an inflammatory reaction (Iadecola *et al.* 1995, 1996). Inducible NOS activity was significantly enhanced at 24 h in the striatum and at 48 h in the cortex after severe transient focal cerebral ischemia in rats (Lerouet *et al.* 2002). The iNOS enzymatic activity reaches a maximum at 48 h after ischemia and returns to baseline values within 7 days (Iadecola *et al.* 1995). Aminoguanidine (AG), a selective iNOS inhibitor, when administered between 24 and 96 h after ischemia, attenuates post-ischemic iNOS activity and reduces the size of the infarcted locus, suggesting that NO produced by iNOS contributes to the development of tissue damage (Iadecola *et al*. 1994). When AG is administered up to 48 h after middle cerebral artery occlusion (MCAO), it does not reduce the volume of injury. However, if the administration of AG is prolonged beyond 48 h, a reduction in the extent of the injury is observed (Zhang and Iadecola 1998). In cerebral ischemia AG has been reported to have neuroprotective effects in the infarcted area (Zhang and Iadecola 1998, Nagayama *et al.* 1998, Takizawa *et al.* 1999, Fassbender *et al.* 2000, Mori *et al.* 2001, Sugimoto and Iadecola 2002). The administration of AG did not substantially modify arterial pressure, arterial blood gases, pH, hematocrit, plasma glucose, or rectal temperature (Zhang and Iadecola 1998). The protective effect of AG is time-dependent and occurs only when the drug is administered for longer than two days, starting after the induction of ischemia. This may be because iNOS enzymatic activity develops more than 24 h after MCAO (Iadecola *et al*. 1995).

In contrast to the above AG effects on focal cerebral ischemia induced by occlusion of the middle cerebral artery, there are no studies that have examined the effects of AG on transient forebrain ischemia induced by four-vessel occlusion (4-VO). Only Mori *et al.* (2001) have reported that pretreatment with AG prevented the impairment of learning behavior and hippocampal long-term potentiation following transient ischemia.

In the present study, we studied the effects of AG on NADPH-diaphorase activity by histochemistry that provides a simple method for localizing this novel messenger system because the localization of NOS is absolutely coincident with NADPH-diaphorase activity (Dawson *et al.* 1991, Bredt and Snyder 1992).

Methods

The experiments conformed with the Slovak Law for Animal Protection No. 115/1995 and were approved by the Institutional Ethical Committee for animal research. Male Wistar rats weighing 250-300 g (9-10 weeks of age) were used for this study. The animals were fed *ad libitum* until the day of experiment. Ischemia was induced using the four-vessel occlusion (4-VO) model of Pulsinelli and Brierley (1979) with modifications (Schmidt-Kastner *et al*. 1989). Under ketamine (100 mg/kg body wt i.p.) and xylazine (15 mg/kg body wt i.p.) anesthesia, the first incision was made in the dorsal midline of the neck, and both vertebral arteries were electrocauterized with a monopolar coagulator through the alar foramen of the first cervical vertebra. A second incision was made in the ventral cervical midline, and both common carotid arteries were exposed. A silk thread was loosely placed around each artery without interrupting carotid blood flow, and each incision was closed with a single suture. The animals were allowed to recover from anesthesia. Next day, under light fluorothane anesthesia, both common carotid arteries were reexposed and occluded with aneurysmal clips to induce forebrain ischemia. The severity of ischemia was judged by neurological investigation. Normothermic conditions were monitored using a thermistor in the ear and maintained at approx. 37.0 °C by a feedback-controlled heating lamp and pad. After 20 min of bilateral carotid occlusion, blood flow was restored by releasing the clips. The following criteria for forebrain ischemia were used: loss of the righting reflex, paw extension, mydriasis, and in some cases even the loss of EEG activity. The rats that became unresponsive and had lost the righting reflex during bilateral carotid artery occlusion but showed no seizure during and after ischemia were used for the experiment. Only such animals are considered to have met the criteria for adequate ischemia (Pulsinelli *et al.* 1982). Sham-operated controls (n=6 at each time point) were treated similarly to the ischemic group, but neither of the common carotid arteries was occluded. Intact control rats were also studied (n=4). One group of animals (n=6) was

administered AG at a dose of 100 mg/kg i.p. immediately after the occlusion and then twice per day for three consecutive days. After the recirculation periods (3, 7 or 14 days), the animals were decapitated, the brains were removed and the hippocampus was dissected and homogenized in 10 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 % vol/vol Triton X-100 (Teflon-glass homogenizer, 1.500 rpm) and the homogenates were centrifuged at 1000 x*g* for 10 min. The pellet was discarded and the supernatant was re-centrifuged at 17500 x*g* for 25 min. Aliquots of the resulting supernatant fraction were taken for NADPH-diaphorase assay and protein determination.

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

NADPH-diaphorase activity was assayed spectrophotometrically by measuring the reduction of 0.5 mM nitroblue tetrazolium with 1 mM βNADPH in 0.3 ml of 50 mM Tris-HCl buffer, pH 7.5, at 37 °C for 8 min. The reaction was stopped with 0.3 ml of 100 mM sulphuric acid and the absorbance of the formazan was determined at its isobestic wavelength, 595 nm (Davies *et al*. 1985, Kuonen *et al*. 1988). Protein quantities were adjusted to obtain absorbance readings from the linear portion of the absorbance curve (<0.8 OD). Diaphorase activity determined in the absence of added NADPH (less than one third of the total diaphorase activity) was always subtracted from the total NADPH diaphorase activity.

Statistical significance of differences between groups was analyzed using a one-way ANOVA followed by a *post-hoc* Duncan's test. $P<0.05$ was considered to be significant.

For histological analysis, after the recirculation periods, the rats (n=6 per group) were deeply anesthetized and fixed by intracardial perfusion with 4 % paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4. The brains were then removed and incubated with phosphate-buffered 4 % paraformaldehyde for 24 h at 4 ºC. After postfixation, coronal 30 μm thick sections were incubated for 3 h at 37 ºC in a solution containing: 1 mM Nitroblue tetrazolium, 5 mM β-NADPH, 0.3 % Triton X-100 dissolved in 0.1 M phosphate buffer (pH 7.4) (Vincent and Kimura 1992, Valtschanoff *et al.* 1993). Then the specimens were rinsed in 0.1 M phosphate buffer, mounted on slides, air-dried overnight and coverslipped with DPX.

Quantification of NADPH-diaphorase positive cells as well as the number of surviving neurons in CA 1 region of hippocampus was counted from photographs obtained by microscope Olympus model BX51 with a digital camera system DP50. Graphical analysis was performed by UTHSCSA software Image Tool.

Results

Since nitric oxide is known to be toxic for neurons and to mediate some of the effects of ischemia, we decided to determine whether transient forebrain ischemia might change the pattern of nitric oxide production *in vivo*. In this model, a 20-min period of ischemia followed by reperfusion results in a selective loss of the CA1 pyramidal neurons after a certain delay. There is clear histological evidence of neural degeneration by three days, which is consistent with our histological observations (data not shown). Figure 1 shows quantitative cell counts of normal-appearing neurons per high-power field. If compared to ischemic group without AG administration, the presence of AG significantly increased the number of pyramidal neurons in the CA1 of the hippocampus.

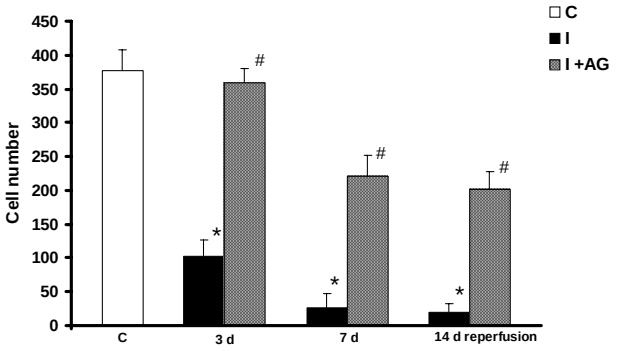

Fig. 1. The effect of aminoguanidine on the survival of the hippocampal pyramidal neurons after 3, 7 and 14 days of reperfusion. Values given as means ± S.D. represent 10 measurements of the number of surviving cells per millimeter in the middle of the linear part of the CA1 region of the hippocampus per animal (n=6). C – sham-operated animals; I – 20 min of ischemia; I + AG – ischemic animals given aminoguanidine; * $p<0.05$ compared to control; # $p<0.05$ compared to ischemia.

In the normal hippocampus, the pyramidal neurons were generally invisible by NADPH-diaphorase staining. Scattered neurons that were intensely stained for NADPH-diaphorase in their cytoplasm were seen in all subregions of the hippocampus (Fig. 2 Aa). Three days after ischemia, NADPH-diaphorase staining slightly increased in the pyramidal neurons of the CA1 region (Fig. 2 Bb). By seven days after ischemia NADPH-

diaphorase stained cells appeared in the area flanking the CA1 neurons as well as in several other areas of the hippocampus (Fig. 2 Cc). Both the intensity of NADPH-diaphorase staining, and the number and size of the stained cells increased dramatically from day 7 to day 14 (Fig. 2 Dd). These NADPH-diaphorase positive cells tended to be concentrated in the regions flanking the pyramidal cell layer. By day 7 or 14 (Fig. 2 Cc and Dd), the diaphorase staining of the CA1 pyramidal cell layer had disappeared. Most CA1 neurons died by this time, the number of stained microvessels further increased and reactive glial cells were intensely stained in the CA1 subfield. In the animals administered AG, the NADPH-diaphorase staining of the CA1 neurons already returned to control levels after 3 days of reperfusion (Fig. 3).

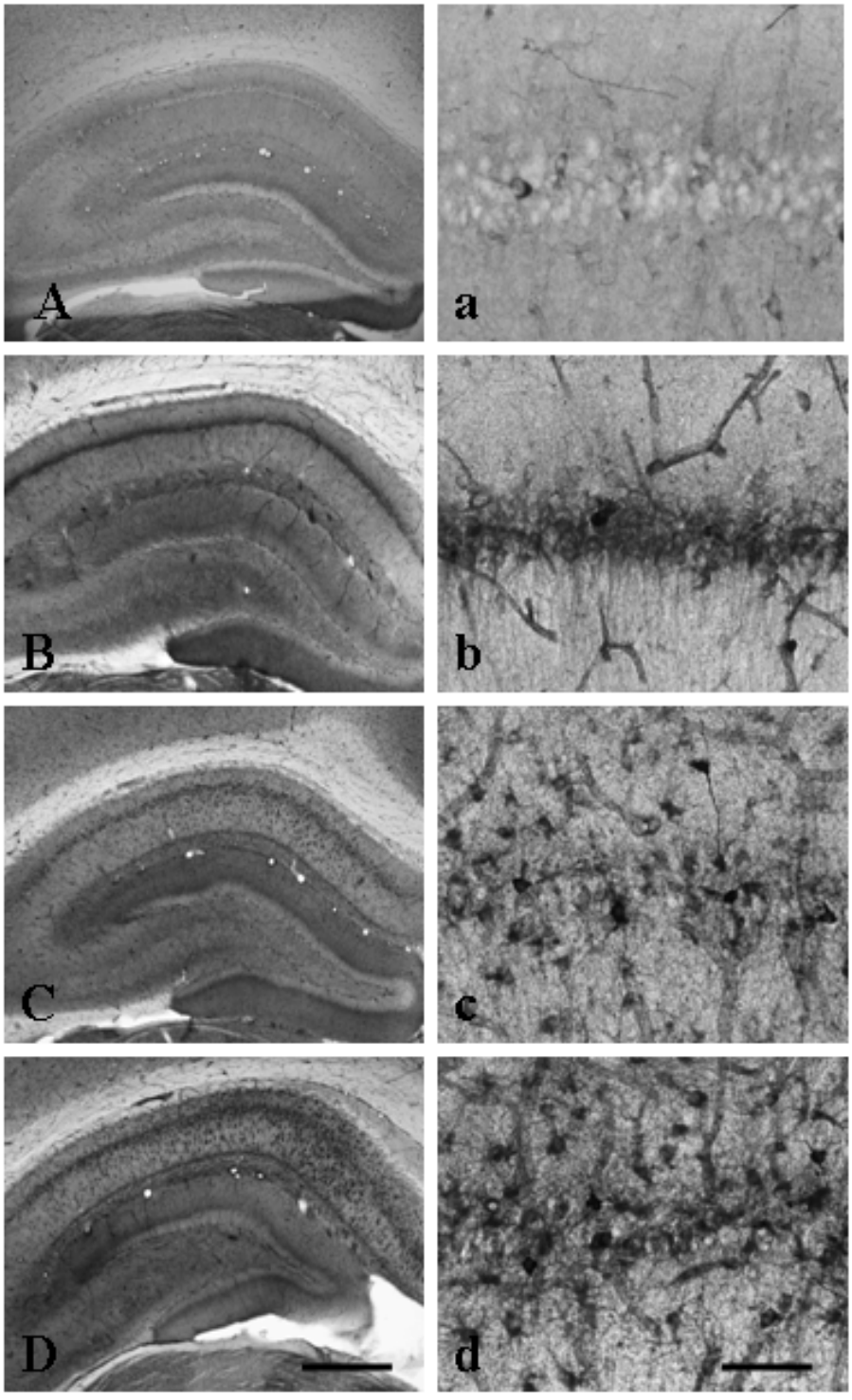

Fig. 2. NADPH-diaphorase histochemistry in the hippocampus (A, B, C, D) and the CA1 region (a, b, c, d) of a sham-operated rat (Aa) and rats subjected to 20 min of forebrain ischemia followed by 3 days (Bb), 7 days (Cc) and 14 days (Dd) of reperfusion. Scale bar: 400 μm (A, B, C, D) and 40 μm (a, b, c, d).

In the ischemic animals, the number of NADPH-diaphorase positive staining neurons in the hippocampal CA1 region was significantly increased compared to the controls. The administration of AG significantly decreased the number of NADPH-diaphorase positive cells compared to the ischemic groups (Fig. 4)

In the hippocampus, a twofold increase of the NADPH-diaphorase activity was found after 20 min ischemia and this increase persisted during 3 and 7 days of reperfusion. The administration of AG, a selective iNOS inhibitor (100 mg/kg i.p. twice per day up to 3 days immediately after ischemia), blocked the increase of NADPH-diaphorase activity after ischemia and this activity remained at control levels during the whole period of reperfusion (Fig. 5)

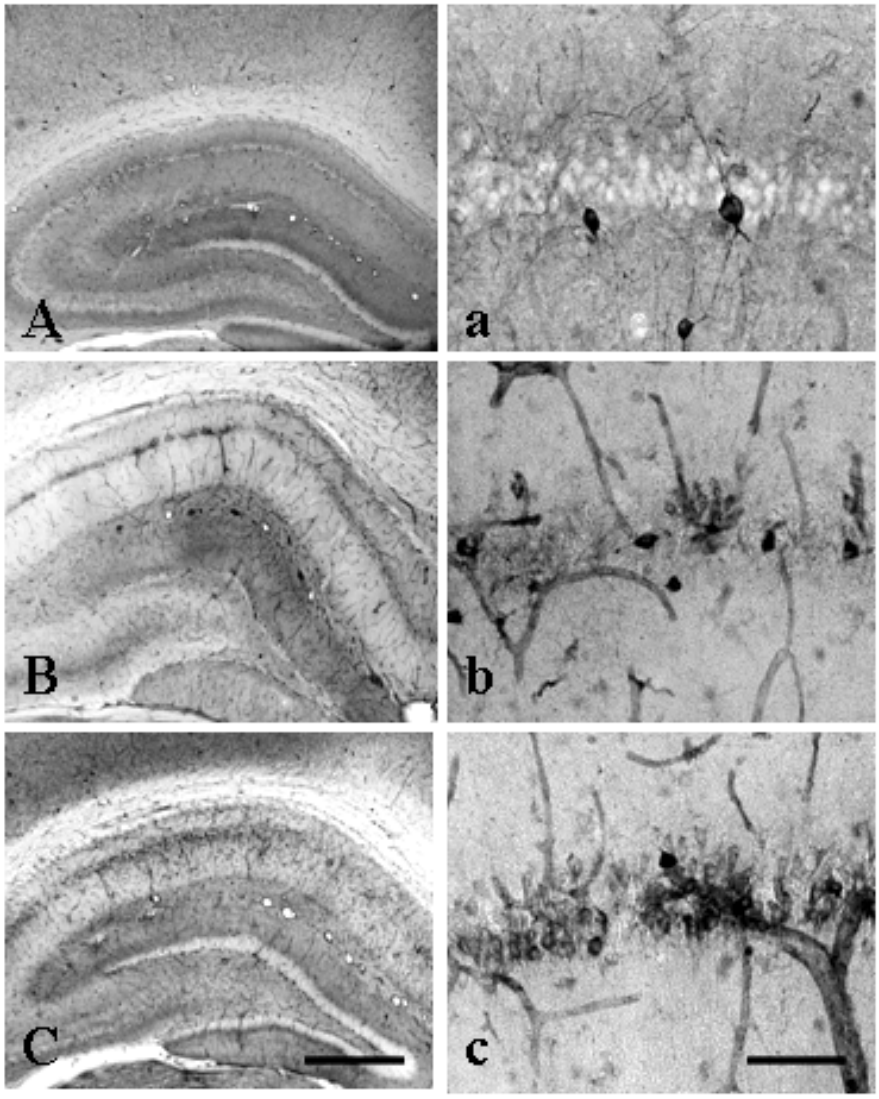

Fig. 3. NADPH-diaphorase histochemistry in the hippocampus CA 1 region of a rat subjected to 20 min of forebrain ischemia and the administration of amino-guanidine (100 mg/kg i.p. twice per day for up to 3 days) followed by 3 days (Aa), 7 days (Bb) and 14 days (Cc) of reperfusion. Scale bar: 400 μm (A, B, C) and 40 μm (a, b, c).

Discussion

The present study showed that transient forebrain ischemia temporarily induces NADPH-diaphorase activity in CA1 pyramidal neurons, which are normally only lightly stained. Increased NADPH-diaphorase activity was observed following ischemia and 3 days of reperfusion. NADPH-diaphorase activity was seen in reactive glial cells in the damaged CA1 region of the animals subjected to 20 min of ischemia and 7 or 14 days of reperfusion. The administration of AG twice per day up to 3 days after four-vessel occlusion served as a neuroprotective procedure. Treatment with AG starting immediately after the occlusion decreased the number of NADPH-diaphorase positive neurons in CA1 pyramidal neurons to control levels. AG also attenuated the progression of tissue damage, which suggests that AG may be protective against irreversible damage.

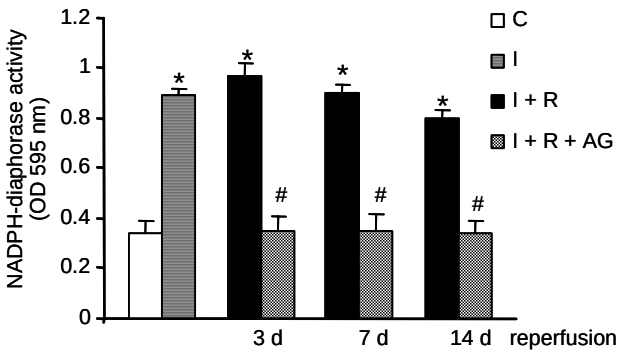

Fig. 4. The number of NADPH-diaphorase staining positive neurons in the hippocampal CA1 region. Neurons were counted per high-power field (400x) in the CA1 region, along a predetermined number of grid areas (~ 1 mm^2). Values given as means ± S.D. represent 10 measurements in the middle of the linear part of the CA1 hippocampal region per animal (n=6). C – sham-operated animals; I – 20 min of ischemia; I + AG – ischemic animals given aminoguanidine; * $p<0.05$ compared to control; # $p<0.05$ compared to ischemia.

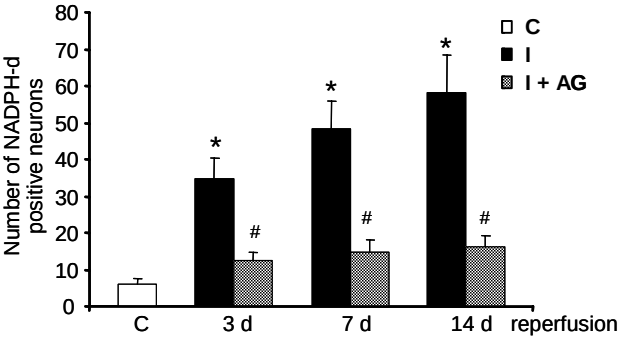

Fig. 5. The effect of aminoguanidine on NADPH-diaphorase activity measured spectrophotometrically (optical density 595 nm) in the hippocampus after 20 min of ischemia and 3, 7, or 14 days of reperfusion. Data given as means ± S.D. are normalized to 100 μg protein (original absorbance values were obtained from the linear portion of the absorbance curve, i.e. O.D. < 0.8); n=6 each performed in triplicate. C – sham-operated animals; I – 20 min of ischemia; I + AG – ischemic animals given aminoguanidine; * $p<0.05$ compared to control; # $p<0.05$ compared to ischemia.

The neuroprotective effect of AG suggests that NO formed through iNOS may play an injurious role in ischemia and that iNOS may also contribute to neuronal damage in ischemia through the production of peroxynitrite (Niwa *et al.* 1999). The administration of AG is beneficial to the postischemic brain, significantly reducing ischemic neuronal damage. This effect of aminoguanidine underscores the therapeutic potential of iNOS inhibitors for the treatment of ischemic stroke. Current treatment modalities advocate the combination of interventions aimed at re-establishing blood flow with neuroprotective therapies directed at increasing the resistance of the brain to ischemia. These therapeutic modalities are effective only if they are introduced within a few hours after cerebral ischemia (Marshall and Mohr 1993). Neuroprotection by AG has been ascribed primarily to its inhibitory effect on the inducible isoform of nitric oxide synthase (Iadecola *et al.* 1995, Lecanu *et al.* 1998). Previous studies have implicated NO as a contributor to neuronal damage early in stroke, i.e. during acute ischemic events (Dalkara and Moskowitz 1994, Siesjö *et al.* 1995). iNOS is not implicated in NO-mediated cytotoxicity during this early acute phase of ischemia as this isoform is ordinarily expressed in cells only subsequent to the induction by immune system effectors (Nathan and Xie 1994). Studies using a rodent model have indicated that inflammation occurring during reperfusion may trigger induction of iNOS within the ischemic territory, suggesting that NO produced by this isoform may contribute to post-ischemic tissue damage (Iadecola *et al.* 1996, Zhang *et al.* 1996).

High levels of NO generated by iNOS account for much of the neuronal damage in the late post-ischemic phase of MCAO and AG was neuroprotective in this system (Cash *et al.* 2001). This does not preclude a role of AG as a neuroprotective agent *via* its ability to inhibit iNOS, but other targets for the neuroprotective actions of AG must be present since AG treatment at 1 or 2 h after the ischemia led to a significant reduction in lesion volumes at 24 h after the occlusion (Cockroft *et al.* 1996). Because iNOS induction normally requires at least 8 to 12 h, AG-induced neuroprotection observed here is likely to have been exerted on a target other than iNOS. AG is known to act on a variety of other cellular metabolic pathways and some of these actions may be relevant in biochemical events during ischemia (Cash *et al.* 2001). AG inhibits polyamine metabolizing enzymes polyamine oxidase and diamine oxidase which evidently are targets for the neuroprotective effects of AG (Ivanova *et al.* 1998). Free radical scavenging is another mechanism by which AG may lead to neuroprotection (Yildiz *et al.* 1998).

We studied the pathological changes in the rat hippocampus by histochemistry after 20 min of transient forebrain ischemia, which resulted in selective delayed death of the CA1 pyramidal cells. NADPH-diaphorase activity increased after ischemia, and these changes were observed three days after ischemia and became more prominent from one week to two weeks after ischemia. This increase of iNOS expression, and presumably NO production may play a role in the process of delayed

neuronal death after ischemic damage. The administration of AG significantly reduced the pathological changes in CA1 pyramidal cells and also reduced the number of NADPH-diaphorase positive neurons compared to the ischemic groups.

In conclusion, we found that the administration of aminoguanidine, a selective iNOS inhibitor, attenuates the development of NADPH-diaphorase activity and reduces brain tissue damage from four-vessel occlusion in rats.

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Reprint requests

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