Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage

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Inadecola, Costantino, Fangyi Zhang, and Xiaohong Xu. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am. J. Physiol. 268 (Regulatory Integrative Comp. Physiol. 37): R286–R292, 1995.—We sought to determine whether expression of the inducible, calcium-independent isoform of nitric oxide synthase (iNOS) contributes to the tissue damage produced by focal cerebral ischemia. The middle cerebral artery was occluded in halothane-anesthetized spontaneously hypertensive rats. Twenty-four hours later rats received intraperitoneal injections of the iNOS inhibitor amino-guanidine (100 mg/kg twice per day; n = 10) or of amino-guanidine + L-arginine (300 mg/kg four times per day; n = 7), amino-guanidine + L-arginine (n = 7), arginine alone (n = 6), or vehicle (n = 9). Drugs were administered for 3 consecutive days. Infarct volume was determined by image analysis in thionin stained brain sections 4 days after induction of ischemia. Administration of amino-guanidine reduced infarct volume by 33 ± 4% (P < 0.05). Aminoguanidine did not affect resting cerebral blood flow or the cerebrovascular vasodilation elicited by hypercapnia, as determined by laser-Doppler flowmetry (n = 4). We conclude that amino-guanidine attenuates NOS activity in the area of infarction and reduces the volume of the infarct produced by middle cerebral artery occlusion. The findings demonstrate that iNOS expression after focal cerebral ischemia contributes to the late stage of tissue damage and raise the possibility that iNOS inhibitors could be a valuable tool in the management of ischemic stroke.

aminoguanidine; cerebral blood flow; laser-Doppler flowmetry; nitric oxide synthase assay; rat

METHODS

Methods for MCA occlusion, for determination of infarct size, and for measurement of NOS activity have been described in detail in previous publications (11, 12, 28) and will only be summarized.

General Surgical Procedures

Studies were conducted on 53 male spontaneously hypertensive rats (Harland) weighing 300–400 g. Under halothane anesthesia (induction 5%, maintenance 1%, in 100% oxygen), the left femoral artery was cannulated and rats were placed on a stereotactic frame (Kopf). Body temperature was maintained at 37 ± 0.5°C by a thermostatically controlled infrared lamp. The arterial catheter was connected to a pressure transducer.
for recording of mean arterial pressure and heart rate. Plasma glucose was measured by a glucose analyzer (Beckman). Blood gases were not measured because it was previously determined that arterial PCO₂, PO₂, and pH are stable during procedures for MCA occlusion in spontaneously breathing rats (28). After completion of the surgical procedures the arterial catheter was tunneled under the skin and exteriorized at the level of the tail. The catheter was used for recording of arterial pressure and for determination of plasma glucose and hematocrit at different times after MCA occlusion.

MCA Oclusion and Measurement of Infarct Volume

Procedures for MCA occlusion and for determination of infarct volume are identical to those published previously (28) and will only be summarized. Briefly, a 3- to 4-mm hole was drilled at a site superior and lateral to the left foramen ovale to expose the left MCA. The MCA was elevated and cauterized medial to the inferior border of the inferior cerebral vein (28). Rats were allowed to recover and returned to their cages.

Ninety-six hours after MCA occlusion, rats were killed, and their brains were removed. The cerebellum was dissected and frozen in liquid nitrogen for assay of NOS activity. The forebrain was frozen in cooled isopentane (−30°C). Coronal brain sections (thickness 30 μm) were serially cut in a cryostat, collected at 500-μm intervals, and stained with thionin. As described in detail elsewhere (28), infarct volume was determined using an image analyzer (MCID, Imaging Research). Infarct volume in cerebral cortex was corrected for shrinkage according to the method of Lin, as previously described (see Ref. 29).

In some experiments the area of neocortical infarction was removed and assayed for NOS activity (11). A 4-mm-thick coronal brain slice was cut at the level of the optic chiasm, and the infarcted cortex was dissected using the corpus callosum as a ventral landmark. The medial border of the cortical infarct was identified visually. The cortex below the rhinal fissure was not sampled. The homotopic region of the contralateral cortex was also dissected. Samples were frozen in liquid nitrogen for later analysis.

NOS Assay

NOS catalytic activity was measured using the assay of Bredt and Snyder (see Refs. 11 and 12 for references). Brain samples were homogenized in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) containing 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol, and 0.32 M sucrose at 23,000 revolutions/min for 30 s (Polytron PT3000, Brinkmann). The homogenate was centrifuged at 20,000 g for 15 min. Enzyme activity was assayed in triplicate aliquots of cytosol (150 μg protein). For determination of calcium-dependent (constitutive) NOS activity (cNOS), samples were incubated for 45 min (37°C) with a buffer (pH 7.4) containing 20 mM HEPES, 0.5 mM EGTA, 1 mM dithiothreitol, 0.32 M sucrose, 0.5 mM CaCl₂ (1 μM free Ca²⁺), 200 μM NADPH, 1 μM L-arginine, and 1 μCi/ml L-[3H]arginine (specific activity 65 Ci/mmol). The reaction was stopped by adding 2 ml of ice-cold 20 mM HEPES containing 2 mM EDTA (pH 5.5). Samples were applied to Dowex AG50W-X8 (Na⁺ form) columns to remove L-[3H]arginine. Columns were then washed with 2 ml of water, and L-[3H]citrulline was quantified in the flow-through fraction using a liquid scintillation spectrophotometer (Beckman, LS 6000). The level of L-[3H]citrulline was computed after subtracting the blank value, which represents nonspecific radioactivity in the absence of enzyme. For determination of calcium-independent (inducible) NOS activity (iNOS), the incubation buffer also included the cofactors tetrahydrobiopterin (THB; 50 μM), flavin mononucleotide (1 μM), and FAD (1 μM) but was devoid of calcium. In previous experiments we observed that THB is essential for assaying calcium-independent NOS activity (11). Therefore THB was added only to the reaction mixture for the assay of calcium-independent NOS activity in brain homogenates.

Experimental Protocol

**Effect of aminoguanidine on infarct size.** The MCA was occluded as described above, and treatments were started 24 h later. The rationale for beginning treatment at this time is that iNOS enzymatic activity develops more than 24 h after MCA occlusion (11). Drugs were given for 3 consecutive days. In one group of rats (n = 10), aminoguanidine hemisulfate (Sigma; 100 mg/kg in 1 ml of saline) was administered intraperitoneally twice a day, at 1000 and 1800. A second group of rats (n = 7) was treated with both aminoguanidine (100 mg/kg at 1000 and 1800) and L-arginine (Sigma; 300 mg/kg in 1 ml of saline at 0800, 1000, 1500, and 1800). A third group of rats (n = 7) was treated with aminoguanidine and D-arginine (Sigma; 300 mg/kg) according to the same administration schedule used for aminoguanidine + L-arginine. A fourth group of rats (n = 6) was treated with L-arginine alone (300 mg/kg at 0800, 1000, 1500, and 1800), and a fifth group of rats (n = 9) was treated with saline (vehicle; 1 ml at 1000 and 1800). The pH of the solutions injected was adjusted to 7.

Arterial pressure, rectal temperature, and plasma glucose were measured before each aminoguanidine administration and 1, 3, and 6 h after. In the groups of rats that received aminoguanidine + L-arginine, aminoguanidine + D-arginine, or L-arginine alone, arterial pressure, temperature, and glucose were measured before 1 h after each administration. Arterial hematocrit was measured before injection and 24, 48, 72, and 96 h after MCA occlusion. Ninety-six hours after MCA occlusion rats were killed for determination of infarct size and for measurement of cerebellar NOS activity.

**Effect of aminoguanidine on NOS activity.** In these experiments rats were treated either with vehicle (n = 5) or with aminoguanidine (100 mg/kg) (n = 5), starting 24 h after MCA occlusion according to the administration schedules described above. Rats were killed 96 h after MCA occlusion, and the infarcted cortex and the cortex contralateral to the stroke were removed and frozen for assay of NOS activity.

**Effect of aminoguanidine on cerebral blood flow.** Rats (n = 4) were anesthetized with halothane, placed on the stereotaxic frame, and artificially ventilated. Arterial pressure was recorded and arterial PCO₂, PO₂, and pH were measured using a blood gas analyzer (9). Cerebral blood flow was monitored in the frontoparietal cortex using a laser-Doppler flowmeter as previously described (9). Aminoguanidine was administered intraperitoneally (100 mg/kg), and arterial pressure and cerebral blood flow were recorded continuously. Hypercapnia was induced by introducing 5% CO₂ into the circuit of the ventilator (9).

Data Analysis

Data presented in the text, Table 1, and Figs. 1–3 are expressed as means ± SE. Comparisons among multiple groups were statistically evaluated by the analysis of variance and the Tukey-Kramer modification of Tukey’s test (Systat). Comparisons between two groups were evaluated by the Student’s t-test. Differences were considered significant for P < 0.05.
Fig. 1. Effect of administration of saline (vehicle), aminoguanidine (AG), or L-arginine (L-Arg) on mean arterial pressure (MAP; A), plasma glucose (Glut; B), and rectal temperature (Temp; C). Note that AG or L-Arg did not affect these variables \( P > 0.05 \) analysis of variance (ANOVA) and Tukey’s test. MAP, Glut, and Temp did not differ also in rats receiving AG + L-Arg or AG + D-Arg \( P > 0.05 \); data not shown. MCAO, middle cerebral artery occlusion.

RESULTS

Effect of Aminoguanidine and/or Arginine on Infarct Volume and Cerebellar cNOS Activity

In rats receiving vehicle after MCA occlusion, arterial pressure, plasma glucose, and rectal temperature remained stable over the 96-h survival time and did not differ from those of rats treated with aminoguanidine, aminoguanidine + L-arginine, aminoguanidine + D-arginine, or L-arginine alone \( P > 0.05 \) analysis of variance and Tukey’s test (Fig. 1). No significant differences in hematocrit were observed among treatment groups over the 96-h survival period \( P > 0.05 \). In vehicle-treated rats \( n = 9 \), MCA occlusion produced infarction involving almost exclusively the cerebral cortex (Table 1). Treatment with aminoguanidine \( n = 10 \) reduced the size of infarct significantly \( P < 0.05 \) (Table 1; Fig. 2A). In neocortex, the reduction averaged \( 33 \pm 4\% \) (mean \pm SE; \( P < 0.05 \)). The tissue rescued from infarction was located at the border zone between the territories of the middle and anterior cerebral arteries and was most marked in the posterior regions of the infarct (Fig. 2B). Coadministration of aminoguanidine and L-arginine abolished the reduction in infarct size \( n = 7; P > 0.05 \) from vehicle and aminoguanidine alone), whereas coadministration of aminoguanidine and D-arginine did not counteract the effect of aminoguanidine \( n = 7; P > 0.05 \) (Table 1; Fig. 2A). Treatment with L-arginine alone did not affect the size of the infarct \( n = 6; P > 0.05 \) from vehicle) (Table 1; Fig. 2A). The volume of tissue swelling did not differ among the treatment groups studied \( P > 0.05 \); Table 1). Similarly, calcium-dependent NOS activity in the cerebellum did not differ among groups [e.g., vehicle \( 4,611 \pm 186\),

Table 1. Effect of aminoguanidine and/or L-arginine on volume of infarct produced by middle cerebral artery occlusion in rats

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>AG</th>
<th>AG + L-Arg</th>
<th>AG + D-Arg</th>
<th>L-Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct volume, mm³</td>
<td>187 ± 5</td>
<td>145 ± 8*</td>
<td>180 ± 11</td>
<td>156 ± 7*</td>
<td>193 ± 8</td>
</tr>
<tr>
<td>Neocortical infarct</td>
<td>179 ± 4</td>
<td>139 ± 7*</td>
<td>176 ± 11</td>
<td>145 ± 8*</td>
<td>184 ± 8</td>
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<tr>
<td>Swelling</td>
<td>59 ± 5</td>
<td>60 ± 4</td>
<td>50 ± 5</td>
<td>56 ± 3</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>Neocortical infarct (corrected)</td>
<td>120 ± 6</td>
<td>80 ± 5*</td>
<td>126 ± 8</td>
<td>89 ± 6*</td>
<td>129 ± 5</td>
</tr>
<tr>
<td>Striatal infarct</td>
<td>6 ± 2</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \) = no. of rats/group. AG, aminoguanidine; D-Arg, D-arginine; L-Arg, L-arginine. See text for administration schedule. *\( P < 0.05 \) vs. vehicle, AG + L-Arg, and L-Arg groups (analysis of variance and Tukey’s test).
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Fig. 2. Effect of administration of AG, with or without L-Arg or D-Arg, and of L-Arg alone on size of infarct produced by MCA occlusion in spontaneously hypertensive rats. Rats were killed 96 h after MCA occlusion. See text for schedule of administration of drugs. A: AG (n = 10) reduced infarct size (*P < 0.05 vs. vehicle; ANOVA and Tukey's test), an effect that was counteracted by L-Arg but not D-Arg (P > 0.05 vs. vehicle; n = 7/group). L-Arg alone (n = 6) did not affect infarct size (P > 0.05). B: rostrocaudal distribution of area of infarct plotted as a function of distance from interaural line. The greatest reductions in cross-sectional area of infarct occurred in the posterior region of lesion (*P < 0.05 vs. vehicle and AG + L-Arg; +P < 0.05 vs. vehicle only).

Effect of Aminoguanidine on NOS Activity in the Area of Infarction

In these experiments we sought to determine whether the reduction in infarct size afforded by aminoguanidine was associated with inhibition of iNOS enzymatic activity in the area of infarction. In sham-operated rats (n = 5), neocortical calcium-independent NOS activity was 51 ± 10 dpm·mg protein⁻¹·min⁻¹ in the sham-operated side and 47 ± 10 dpm·mg protein⁻¹·min⁻¹ contralaterally (P > 0.05). Ninety-six hours after MCA occlusion (n = 5), calcium-independent NOS activity was markedly increased in the area of infarction (P < 0.001; t-test; Fig. 3). In contrast, calcium-dependent NOS activity was substantially reduced, a finding reflecting loss of NOS neurons in the core of the infarct (11). Treatment with aminoguanidine, according to the same protocol used for studies on infarct size, attenuated calcium-independent NOS activity in the infarcted tissue (P < 0.001) without influencing calcium-dependent NOS activity (P > 0.05 from vehicle) (Fig. 2).

Cerebrovascular Effects of Aminoguanidine

To determine whether the reduction in infarct size by aminoguanidine was a consequence of a cerebrovascular action of this drug, we studied the effect of aminoguanidine on resting cerebral blood flow and on the cerebrovasodilation elicited by hypercapnia. Aminoguanidine (100 mg/kg ip; n = 4) did not affect resting cerebral blood flow (before aminoguanidine, 17.0 ± 0.6 perfusion units; 30 min after aminoguanidine, 16.5 ± 0.5 perfusion units; P > 0.05) or the vasodilation to hypercapnia (Pco₂ = 55 ± 1 mmHg; cerebral blood flow increase

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before aminoguanidine, 129 ± 5%; 30 min after aminoguanidine, 142 ± 7%; P > 0.05).

DISCUSSION

We have demonstrated that administration of the iNOS inhibitor aminoguanidine, 24 h after MCA occlusion, attenuates calcium independent arginine to citrulline conversion in the area of infarction and reduces ischemic brain damage. The protective effect of aminoguanidine is substantial and is antagonized by the NO precursor L-arginine but not by the inactive isomer D-arginine. L-Arginine does not affect infarct size when administered alone. The findings provide the first demonstration that inhibition of iNOS activity after focal ischemia reduces tissue damage and indicate that NO production by iNOS participates in cerebral ischemic damage.

The reduction in infarct size afforded by aminoguanidine cannot be a consequence of effects on arterial pressure, body temperature, or plasma glucose because these variables were monitored and did not differ among the various treatment groups. Similarly, the effect of aminoguanidine is unlikely to be a consequence of hemodilution or hemoconcentration because the arterial hematocrit did not differ among groups. Furthermore, the protective effect of aminoguanidine cannot result from hemodynamic actions of this drug resulting in increased cerebral blood flow to the ischemic territory because 1) we have demonstrated here that aminoguanidine does not influence resting cerebral blood flow or the increases in flow evoked by hypercapnia, and 2) aminoguanidine was administered 24 h after MCA occlusion, at a time when maneuvers to restore flow to the ischemic territory are not effective in reducing infarct size (21).

In addition to inhibiting NOS, aminoguanidine has also other pharmacological properties. These include inhibition of formation of advanced glycosylation end-products, inhibition of the histamine-inactivating enzyme diamine oxidase, and increase in the angiotensin II-mediated release of prostacyclins (4, 5, 20; see Ref. 8 for discussion). It is, however, unlikely that these actions are responsible for the effect of aminoguanidine on cerebral ischemic damage. This is because the reduction in infarct size afforded by aminoguanidine is antagonized by L-arginine but not by D-arginine, indicating that the protective effect is mediated through the L-arginine-NO pathways.

Several lines of evidence indicate that, in the present study, the inhibitory effect of aminoguanidine on NOS is restricted to the inducible (calcium-independent) isoform of the enzyme. First, in rats in which the effect of aminoguanidine on infarct volume was studied, cerebellar calcium-dependent NOS activity was not inhibited by aminoguanidine, indicating that brain cNOS was not affected by this drug. Second, aminoguanidine attenuated the calcium-independent NOS activity developing in the infarct but did not reduce calcium-dependent NOS activity in the contralateral cerebellar cortex. This finding also indicates that the inhibitory effect of aminoguanidine was specific for iNOS. Third, aminoguanidine did not elevate arterial pressure, suggesting that this drug did not inhibit endothelial eNOS in systemic vessels. Fourth, aminoguanidine did not affect resting cerebral blood flow or the reactivity of the cerebral circulation to hypercapnia, providing further evidence that cerebral endothelial and/or neuronal eNOS was not influenced by aminoguanidine. Taken together, these observations provide convincing evidence that the effect of aminoguanidine on cerebral ischemic damage does not result from inhibition of the neuronal and/or endothelial isoforms of NOS.

NO is synthesized by NOS, an enzyme of which several isoforms have been identified (19). Two distinct constitutively expressed isoforms of NOS are expressed, one in selected neurons and another in endothelial cells (19). An inducible isoform of NOS is expressed in a wide variety of cells after exposure to endotoxins and cytokines (17). The mechanisms regulating activation of eNOS and iNOS are substantially different. Neuronal and endothelial eNOS produce NO transiently during increases in intracellular calcium, a process thought to be mediated by the binding of calmodulin to the enzyme (19). In contrast to eNOS, iNOS is continuously active, probably because calmodulin is tightly bound to the enzyme even at low calcium concentrations (2). Therefore, whereas eNOS produces NO transiently and in small amounts, iNOS produces NO continuously and in large quantities (19, 17).

High concentrations of NO are cytotoxic. The mechanisms of the cytotoxicity of NO involve several aspects of cellular function. NO, via its nitrosyl derivatives, produces energy failure by inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, by deregulating poly(ADP-ribose) synthetase, and by inhibiting key mitochondrial enzymes (25, 30). NO leads to inhibition of ribonucleotide reductase, the rate-limiting enzyme for DNA replication, and to DNA breakdown (see Refs. 17 and 25 for references). Furthermore, NO reacts with superoxide ions to generate peroxynitrite, a stable oxidant that leads to production of toxic free radicals (26). These effects are thought to be responsible for the well-known toxicity of NO in several cell systems (17, 25). Therefore, after cerebral ischemia, continuous production of NO by iNOS may aggravate the metabolic state of the ischemic tissue, thereby contributing to brain damage. This hypothesis is supported by our finding that focal ischemia leads to the development of substantial calcium-independent NOS activity in the infarcted tissue and that the iNOS inhibitor aminoguanidine attenuates the calcium-independent enzymatic activity and reduces tissue damage. Therefore, the findings of the present study strongly suggest that focal ischemia results in iNOS expression in the affected brain and that production of large amounts of NO by iNOS contributes to the tissue damage.

Because iNOS enzymatic activity develops more than 24 h after MCA occlusion, NO production by iNOS cannot be responsible for the damage that occurs in the acute phase of cerebral ischemia. Volumetric measurements of the ischemic lesion, identified by the area of pallor in stained sections at low magnification, indicate
that the volume of the lesion is fully developed 24 h after induction of ischemia (13). However, recent investigations in which a detailed histological analysis was conducted in different sectors of the lesion have shown that the evolution of the damage is temporally and spatially heterogeneous (3). Whereas areas in the center of the ischemic region exhibit clear evidence of neural damage 8 h after induction of ischemia, areas surrounding the ischemic core develop damage at 24–72 h (3). These areas may overlap with the so-called ischemic penumbra. Therefore, neuronal death in the postischemic brain continues for days at the periphery of the lesion. In our study the brain tissue rescued from infarction by delayed treatment with aminoguanidine was located at the border of the infarct. The data suggest that aminoguanidine may act on peripheral areas in which the damage develops relatively late and that may correspond to the ischemic penumbra. Therefore, the present study indicates that NO produced by iNOS may contribute to the damage that occurs in the areas surrounding the ischemic core in the late stages of cerebral ischemia.

The mechanisms of iNOS expression after focal cerebral ischemia remain to be elucidated. In several cell systems iNOS is induced by endotoxins and cytokines (17). These immunomodulators, or the intracellular transcription factors that they induce, may interact with response elements in the iNOS promoter and initiate transcription (27). After focal cerebral ischemia there is substantial production of cytokines (14, 24). It is therefore conceivable that cytokines induce iNOS expression in cells in the area of infarction. iNOS induction could occur in endothelial cells, smooth muscle cells, astrocytes, and microglia, as well as in polymorphonuclear cells and macrophages invading the area of infarction (17, 18, 23). Further studies will be required to establish the molecular mechanisms and the cellular site of iNOS expression after MCA occlusion.

The histopathology of the ischemic lesion varies in the different animal models of cerebral ischemia (see Refs. 6 and 7 for discussion). If iNOS expression occurs in infiltrating cells, the degree of iNOS expression is likely to be more marked in experimental settings in which the inflammatory infiltrate is more pronounced. Therefore, it would be important to study the effects of aminoguanidine on tissue damage in other models of cerebral ischemia and under experimental conditions that enhance or diminish the inflammatory reaction in the infarct.

Aminoguanidine reduces infarct size when administered 24 h after MCA occlusion. This feature of the 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 reestablishing blood flow with neuroprotective therapies directed at increasing the resistance of the brain to ischemia (15). These therapeutic modalities are effective only if they are instituted within a few hours after cerebral ischemia (15). The results of the present study suggest that inhibition of iNOS activity may be effective when instituted 24 h after ischemia. Therefore, iNOS inhibition may represent a novel therapeutic avenue for the management of ischemic stroke.

In conclusion, we have demonstrated that administration of the iNOS inhibitor aminoguanidine, 24 h after MCA occlusion, attenuates the expression of iNOS activity in the postischemic brain and reduces the extent of the brain damage associated with focal ischemia. The findings indicate that NO production by iNOS contributes to the late stages of focal cerebral ischemic damage and raise the possibility that iNOS inhibitors could be a valuable tool in the management of cerebral ischemia in stroke patients.

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