L-Arginine Infusion Promotes Nitric Oxide–Dependent Vasodilation, Increases Regional Cerebral Blood Flow, and Reduces Infarction Volume in the Rat

Eiharu Morikawa, MD; Michael A. Moskowitz, MD; Zhihong Huang, MD; Tazuka Yoshida, MD; Katsumi Irikura, MD; Turgay Dalkara, MD, PhD

Background and Purpose We previously reported that L-arginine administration increased pial vessel diameter by nitric oxide–dependent mechanisms, improved regional cerebral blood flow (rCBF) distal to middle cerebral artery (MCA) occlusion, and reduced infarction volume in spontaneously hypertensive rats when administered intraperitoneally before and after MCA occlusion. In this report we extend our findings (1) by examining the time course of L-arginine on rCBF and pial vessel diameter under basal conditions and on rCBF after MCA occlusion and (2) by reproducing the protective effect of L-arginine on infarct volume when given intravenously immediately after the onset of MCA occlusion in both normotensive and hypertensive models of focal cerebral ischemia.

Methods Changes in pial vessel diameter (closed cranial window) and rCBF (laser-Doppler flowmetry) were measured over time after L-arginine infusion into anesthetized Sprague-Dawley rats. rCBF was also measured distal to MCA occlusion in a brain region showing rCBF reductions within the range of 80% of baseline. The effects of infusing L-arginine (300 mg/kg for 10 minutes beginning 5 minutes after occlusion) were assessed on infarction volume in Sprague-Dawley rats after proximal MCA occlusion and in spontaneously hypertensive rats after common carotid artery plus distal MCA occlusion.

Results L-Arginine (300 mg/kg IV) elevated rCBF by 20% when measured in the dorsolateral cortex of Sprague-Dawley rats and caused L-nitroarginine-methyl ester–inhibitable increases in pial vessel diameter. L-Arginine (≥30 mg/kg IV) increased blood flow distal to MCA occlusion by 50%. These effects were sustained throughout the observation period (70 to 105 minutes). Changes in mean arterial blood pressure were not observed. L-Arginine (300 mg/kg IV) reduced infarction volume by 35% and 28% in Sprague-Dawley and spontaneously hypertensive rats, respectively, when examined 24 hours after vessel occlusion.

Conclusions These studies extend our previous findings by demonstrating that exogenous L-arginine induces sustained rCBF increases in normal brain as well as in a marginally perfused brain region distal to MCA occlusion. Our data in Sprague-Dawley rats support the conclusion that L-arginine–induced increases in rCBF can decrease infarction volume. We conclude that nitric oxide–mediated mechanisms increase rCBF and decrease infarction volume after MCA occlusion in both normotensive and hypertensive animals. (Stroke. 1994;25:429–435.)

Key Words • amino acids • cerebral blood flow • cerebral ischemia, focal • nitric oxide • rats

The semisessential amino acid L-arginine is a precursor for nitric oxide (NO), a potent vasodilator in a number of vascular beds. NO has been implicated in the pathophysiology of cerebral ischemia because it relaxes vascular smooth muscle, prevents platelet aggregation, and contributes to toxicity induced by N-methyl-D-aspartate (NMDA) receptor activation. Measures to reduce the availability of NO in cell culture decrease NMDA-mediated cell death. In some but not other studies, NO synthase inhibition reduces infarct volume after middle cerebral artery (MCA) occlusion. The explanation for the discrepancies remains obscure, and this has been reviewed recently.

We previously reported that L-arginine administered intraperitoneally reduces infarction volume when given before and after MCA occlusion and suggested that L-arginine–induced increases in regional cerebral blood flow (rCBF) mediate the response. In this report, we extend our experience on the effects of infusing L-arginine by documenting (1) blood flow and pial vessel diameter changes in normal brain with time, (2) time-dependent rCBF changes within marginally perfused tissue, and (3) infarction volume when the amino acid is administered intravenously and after MCA occlusion. Our data support the conclusion that exogenous L-arginine promotes the synthesis of NO or an NO-like compound that promotes vasodilation and increases rCBF and that these changes promote the reduction in infarction volume when the amino acid is administered after MCA occlusion.

Materials and Methods Sprague-Dawley (S-D) and spontaneously hypertensive rats (SHR) (Charles River Laboratories, Wilmington, Mass) were housed under diurnal lighting conditions and were allowed free access to food and water before experimentation. In all
experiments, (1) rats were intubated either transorally or through tracheostomy and ventilated to maintain normal arterial blood gases, (2) the right femoral artery was cannulated for continuous arterial blood pressure monitoring and to obtain measurements of pH, PaCO₂, PaO₂, hematocrit, and plasma glucose, (3) the right femoral vein was cannulated for drug infusion, and (4) body temperature was monitored by rectal probe and maintained at 37°C by a Homeothermic Blanket Control Unit (Harvard Apparatus).

Closed Cranial Window

Male S-D rats (weight, 280 to 340 g) were anesthetized (sodium pentobarbital, 50 mg/kg IP supplemented by 10 mg/kg IP every hour), paralyzed (pancuronium bromide 0.5 to 1.0 mg IV), and ventilated with O₂-supplemented room air. End-tidal PCO₂ was monitored continuously (Novametrix Medical Systems, Wallingford, Ct) and kept within the normocapnic range.

A closed cranial window was placed over the parietal cortex as described previously. The midline incision was made that exposed the skull to the occiput to the forehead. A left craniotomy (7x5 mm) was drilled using extreme care, after which the dura was incised and reflected. A metallic window (12.5 mm in diameter) equipped with three ports was placed over the exposed brain surface. The space under the window was filled with mock cerebrospinal fluid (CSF; Na⁺ 156.5 mM/L, K⁺ 2.95 mM/L, Ca²⁺ 2.5 mM/L, Mg²⁺ 1.33 mM/L, Cl⁻ 138.7 mM/L, HCO₃⁻ 24.6 mM/L, dextrose 66.5 mg/dL, and urea 40.2 mg/dL; equilibrated with gas containing 10% oxygen, 5% CO₂, and the balance nitrogen; pH 7.33 to 7.38). Intracranial pressure was maintained constant by adjusting the height of the outflow tube to approximately 6 cm H₂O.

Pial vessels (15 to 86 μm) were visualized using an intravital microscope (magnification ×200; Leitz, Germany). A television camera (Dage MTI Inc, CCD-72 series, Michigan City, Ind) attached to the microscope transposed the picture onto a video monitor (Dage MTI Inc). Vessel diameters were measured using a video system (VIA-100, Boeckler Instruments).

Protocol

Baseline measurements were obtained after 30 minutes of equilibration. L-Arginine (3 [n=3], 30 [n=5], or 300 [n=5] mg/kg) or D-arginine (300 mg/kg, n=4) was infused intravenously over 10 minutes, and diameters were recorded every 10 minutes for a total of 90 minutes. Changes in vessel calibers were expressed as percentage of baseline diameter. In separate groups of animals, the NO synthase inhibitor L-nitroarginine-methyl ester (L-NAME; Sigma Chemical Co, St Louis, Mo; 1 μM in mock CSF) was superfused under the window 20 minutes before L-arginine infusion (30 [n=3] and 300 [n=4] mg/kg).

**Laser-Doppler Flowmetry: Normal Regional Cerebral Blood Flow**

Male S-D rats were anesthetized with sodium pentobarbital as described above and were prepared for laser-Doppler flowmetry recording as described below. rCBF was monitored over the left parietal cortex (2 to 6 mm caudal from bregma and 2 to 6 mm from midline) in the same location as the pial vessels observed through the closed cranial window. However, probe placement over identifiable surface vessels was avoided. Measurements were made after L-arginine or saline infusion and expressed as percentage of baseline.

**Protocol**

L-Arginine (300 mg/kg, n=6) or a comparable volume of saline (n=7) was infused intravenously over 10 minutes. rCBF was recorded for 70 minutes.

**Table 1. Physiological Variables Before and After Vessel Diameter Measurements in Pentobarbital-Anesthetized Sprague-Dawley Rats**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>MABP, mm Hg</th>
<th>PaCO₂, mm Hg</th>
<th>PaO₂, mm Hg</th>
<th>pH</th>
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</thead>
<tbody>
<tr>
<td>L-Arg 3 mg/kg (3)</td>
<td>Before</td>
<td>108±6</td>
<td>33±1</td>
<td>124±10</td>
</tr>
<tr>
<td>After</td>
<td>108±7</td>
<td>32±1</td>
<td>132±14</td>
<td>7.40±0.01</td>
</tr>
<tr>
<td>L-Arg 30 mg/kg (5)</td>
<td>Before</td>
<td>101±5</td>
<td>35±1</td>
<td>139±17</td>
</tr>
<tr>
<td>After</td>
<td>102±2</td>
<td>35±1</td>
<td>133±12</td>
<td>7.40±0.01</td>
</tr>
<tr>
<td>L-Arg 300 mg/kg (5)</td>
<td>Before</td>
<td>101±2</td>
<td>34±1</td>
<td>118±16</td>
</tr>
<tr>
<td>After</td>
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<td>32±1</td>
<td>122±13</td>
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</tr>
<tr>
<td>d-Arg 300 mg/kg (7)</td>
<td>Before</td>
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<td>35±2</td>
<td>139±5</td>
</tr>
<tr>
<td>After</td>
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<td>35±2</td>
<td>156±6</td>
<td>7.32±0.01</td>
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<tr>
<td>L-Arg 30 and 300 mg/kg/L-NAME (7)</td>
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<td>32±1</td>
<td>152±25</td>
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<tr>
<td>After</td>
<td>114±3</td>
<td>33±2</td>
<td>143±15</td>
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</tbody>
</table>

Values are mean±SEM. MABP indicates mean arterial blood pressure; L-Arg, L-arginine; d-Arg, D-arginine; and L-NAME, L-nitroarginine-methyl ester.

**Laser-Doppler Flowmetry During Ischemia**

Male SHR were anesthetized with halothane (3% for induction and 1% for maintenance) plus 70% nitrous oxide/balance oxygen. Animals were placed in a stereotaxic frame, and a craniotomy (4 to 6 mm lateral and -2 mm to 1 mm rostral to bregma) was made within the MCA territory, which corresponds to a transitional zone between severely ischemic and mildly ischemic tissue. A paper-thin layer of the calvarium was left in place, thus avoiding epidural bleeding. The recording probe (0.8 mm in diameter, P-433) attached to a laser-Doppler flowmeter (BPM 403A, TSI Inc) was steadily advanced under stereotaxic control perpendicular to the cortical surface and away from large pial vessels. The probe touched but did not indent the surface.

Focal cerebral ischemia was accomplished by tandem right common carotid artery (CCA)/MCA occlusion after the application of a snare ligature and clip, respectively.

**Table 2. Physiological Variables Before and After Laser-Doppler Flowmetry in Pentobarbital-Anesthetized Sprague-Dawley Rats**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>MABP, mm Hg</th>
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<th>pH</th>
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<td>Before</td>
<td>107±4</td>
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<td>111±12</td>
</tr>
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<td>After</td>
<td>105±6</td>
<td>33±1</td>
<td>128±8</td>
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</table>

Values are mean±SEM. MABP indicates mean arterial blood pressure; L-Arg, L-arginine.
TABLE 3. Physiological Variables During Laser-Doppler Flowmetry In Halothane-Anesthetized Spontaneously Hypertensive Rats

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<th>Group (n)</th>
<th>MABP, mm Hg</th>
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<th>Paco₂, mm Hg</th>
<th>pH</th>
<th>GLU, mg/dL</th>
<th>Hct, %</th>
<th>BT, °C</th>
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</table>

Values are mean±SEM. MABP indicates mean arterial blood pressure; GLU, plasma glucose; Hct, hematocrit; BT, body temperature; TMT, temporalis muscle temperature; L-Arg, L-arginine; and D-Arg, D-arginine.

Protocol

L-Arginine (3 [n=4], 30 [n=6], or 300 [n=12] mg/kg), D-arginine (300 mg/kg, n=6), or an equivalent volume of saline (n=6) was infused over 10 minutes beginning 5 minutes after MCA occlusion. Preinfusion rCBF was recorded 2 and 5 minutes after MCA occlusion. The average of these two readings provides baseline values before infusion. rCBF was determined at 15-minute intervals for 105 minutes. Data are expressed as percent rCBF recorded after MCA occlusion.

Infarct Volume

Infarct volume was determined 24 hours after occlusion of either the proximal or distal MCA. In model 1, the right distal MCA plus ipsilateral CCA was ligated (tandem CCA/MCA occlusion) in SHR. In model 2, the right proximal MCA was occluded in S-D rats. Anesthesia was induced and maintained by halothane, 3% and 1%, respectively, plus 70% NO/balance oxygen. Blood pressure, arterial blood gases, and body temperature were monitored as described above. Models 1 and 2 were performed by separate investigators.

To occlude the MCA at its proximal or distal segments, the vessel was exposed as follows:14-16 A 1-cm skin incision was placed approximately midway between the right outer canthus and anterior pinna. Temporalis muscle was incised and retracted to expose the squamous bone (distal) or the infratemporal fossa (proximal). A craniotomy (3 mm in diameter) was created at the juncture of the zygoma and squamous bone (distal) or just anterior to the foramen ovale (proximal). The dura mater was opened with a fine curved needle. The MCA was occluded either by double ligation (10-0 monofilament) or by electrocauterization of the artery followed by transection. In model 1, the right CCA was ligated (4-0 silk) just before MCA occlusion. After surgery, the rats were returned to their cages, allowed free access to food and water, and given a single injection of cefazolin, 50 mg IM. The rats were killed by decapitation 24 hours later.

To measure infarct volume, brains were removed and placed in ice-cold saline for 10 minutes and sectioned coronally into seven 2-mm slices in a rodent brain matrix. Slices were placed in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (Sigma) at room temperature for 30 minutes, followed by 10% formalin overnight.14 The area of infarction, outlined in white, was measured (Bioquant IV image analysis system) on the posterior surface of each section, and infarct volume was calculated by numeric integration of the sequential areas.

Protocol

Rats received L-arginine (300 mg/kg) by intravenous infusion over 10 minutes beginning 5 minutes after MCA occlusion and again intraperitoneally 1 hour after MCA occlusion.
Regional Cerebral Blood Flow in Normotensive Sprague-Dawley Rats

Pial Vessel Diameter in Normotensive Sprague-Dawley Rats

Baseline vessel diameters were comparable for the groups receiving 30, 300 mg/kg of l-arginine or 300 mg/kg of d-arginine (43±3, 49±4, 40±5, and 44±1 μm, respectively). L-Arginine, 30 and 300 mg/kg, increased pial vessel diameter significantly, reaching a maximum of 121±6% of baseline. Pial vessel diameter increased shortly after infusion but became significant 20 minutes after infusion in both groups and was sustained throughout the 90-minute observation period (Fig 1). A lower dose of L-arginine (3 mg/kg) or D-arginine (300 mg/kg) did not dilate pial vessels.

Baseline diameters for the groups receiving L-arginine with or without L-NAME were comparable (36±5 and 40±5 μm for 30 and 300 mg/kg l-arginine plus L-NAME groups, respectively). L-NAME (1 μmol/L) superfusion did not affect vessel diameter (35±5 and 40±5 μm for 30 and 300 mg/kg l-arginine plus L-NAME groups, respectively). When measured 30 minutes after L-arginine infusion, L-NAME (1 μmol/L) attenuated the dilator response from 115±3% to 104±2% (30 mg/kg) and from 115±4% to 106±2% (300 mg/kg) (Fig 2).

Regional Cerebral Blood Flow in Normotensive Sprague-Dawley Rats

L-Arginine (300 mg/kg IV) infusion increased rCBF in left parietal cortex in S-D rats, and the effect was sustained for the 70-minute duration of recording (Fig 3). The maximum response, achieved 10 minutes after starting infusion, reached 120±3% of baseline. Lower dosages were not tested.

Regional Cerebral Blood Flow During Focal Ischemia

Regional cerebral blood flow immediately after CCA/MCA occlusion was 20±5, 18±3, 20±5, 22±3, and 21±5% of preischemic rCBF for saline, 3, 30, and 300 mg/kg of l-arginine, and 300 mg/kg of d-arginine, respectively. These reductions in dorsolateral cortex are comparable to published data after tandem CCA/MCA occlusion.13 After L-arginine infusion, blood flow increased. A significant overall difference among groups was detected by repeated-measures ANOVA with baseline as a covariate (P=.03). Significant differences were determined between saline and 30 mg/kg L-arginine (P<.01) and between saline and 300 mg/kg L-arginine (P=.01) by Fisher's protected least-squares difference test. Although the increased rCBF after L-arginine was sustained for the entire recording period, there were no further changes after the first 10 minutes (Fig 4).

Infarct Volume

Treatment initiated 5 minutes after occlusion decreased infarct volume by 35% in normotensive S-D rats subjected to proximal MCA occlusion and by 28% in SHR with tandem MCA/CCA occlusion. The infarct volumes were 154±9 mm³ (n=12; SHR) and 231±13 mm³ (n=17; S-D) for the saline-treated control groups and were 111±12 mm³ (n=10; P<.05) and 150±12 mm³ (n=14; P<.05) for the L-arginine groups, respectively (Table 5).

Discussion

Our studies demonstrate that L-arginine causes dose-dependent and sustained increases in pial arteriolar diameter and rCBF in normal anesthetized rats. The threshold for both was 30 mg/kg, and the effects lasted...
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FIG 2. Bar graphs show that L-nitroarginine-methyl ester (L-NAME) (1 μmol/L) superfusion blocked L-arginine-induced dilation of pial vessels when tested 10 to 30 minutes after amino acid infusion (filled column). Increases after L-arginine infused at 30 mg/kg (top) and 300 mg/kg (bottom) (open column) were blocked by L-NAME (1 μmol/L) superfused 20 minutes before L-arginine. The enzyme inhibitor did not affect baseline diameter. Error bars denote SEM. *P<.05 compared with baseline control; +P<.05 compared with L-arginine by unpaired Student's t test.

Fig 3. Bar graph shows that L-arginine 300 mg/kg IV increases regional cerebral blood flow within dorsolateral cortex (fine-hatched column) in normal Sprague-Dawley rats compared with saline (open column). Maximum response was achieved 10 minutes after infusion and sustained without further increases for the 70-minute study. Error bars denote SEM. *P<.05 compared with baseline control by one-way ANOVA followed by Fisher's protected least-squares difference test.

Fig 4. Bar graph shows that L-arginine 30 mg/kg IV (coarse-hatched column, n=6) or 300 mg/kg IV (fine-hatched column, n=12) increases regional cerebral blood flow (rCBF) within dorsolateral ischemic cortex during middle cerebral artery (MCA) occlusion in spontaneously hypertensive rats compared with saline (filled column, n=6). The infusion continued for 10 minutes and began 5 minutes after MCA occlusion. One hundred percent rCBF refers to rCBF just after the onset of MCA occlusion and was approximately 20% of the preischemic value. Significant increases in rCBF were not observed after L-arginine 3 mg/kg (open column, n=4) or n-arginine 300 mg/kg (cross-hatched column, n=6). Error bars denote SEM.

for the entire observation period. A possible role for NO was suggested by the potency of the L- but not the D-amino acid isomer (Fig 1) and by the inhibition of the vasodilator response by L-NAME (Fig 2). We speculate that the sustained effects of a 10-minute infusion relate to continued NO production from L-arginine sequestered in endothelial cells, and/or perhaps NO sequestered in plasma combined to proteins as an S-nitroso adduct.17 Significant decreases in infarction size were also demonstrated after intravenously administered L-arginine by two independent investigators in both hypertensive and normotensive models of focal ischemia. These findings extend our previously published work documenting that intraperitoneally administered amino acid decreases infarction size when administered before and after MCA occlusion.

Consistent with findings in normal brain, the rCBF response to L-arginine was present in ischemic brain distal to an occluded MCA. Flow increases were observed within 10 minutes after MCA occlusion and sustained for the full 105 minutes of recording. In these experiments, rCBF increased within dorsolateral cortex from approximately 20% to values of approximately 30% of baseline. We did not observe decreases or increases in systemic arterial blood pressure after administering the amino acid during the entire recording period.

The importance of blood flow to tissue survival is underscored by preliminary data showing that L-arginine-induced flow changes are accompanied by recovery in electrocorticogram activity.18 This recovery, detected by a glass microelectrode placed in cortical gray matter below the laser flow probe, followed shortly after blood flow increases (5 minutes) within the ischemic penumbra to approximately 30%. In this paradigm, increases in blood flow always anticipated recovery in the amplitude of the near-silent electroencephalogram and never vice versa. Hence, the effects of L-arginine on functional recovery are probably mediated via flow
increases and not via direct effects on tissue metabolism. Preliminary findings using dynamic susceptibility contrast magnetic resonance imaging are in agreement with the above.\(^1^9\) In those experiments, L-arginine infusion was accompanied by increases in indexes of both cerebral blood volume and flow within the ischemic SHR brain after MCA/CCA occlusion as well as by an apparent decrease in the volume of unperfused tissue.

We remain intrigued by the apparent specificity of the response to the cerebrovasculature (at the dosages tested) and by the suggestion that within this compartment, NO synthase remains unsaturated with substrate.\(^1^0\) Indeed, in most tissues including brain, NO synthase is reportedly saturated. Intracellular free L-arginine levels are approximately 300 to 800 \(\mu\)mol/L,\(^2^0,2^1\) whereas the \(K_m\) for substrate is approximately 2.9\(^2^2\) and 2 \(\mu\)mol/L\(^2^3\) within bovine aortic endothelial and rat cerebellar cells, respectively. Free L-arginine levels may be more difficult to assess within component cells of brain because glia tend to sequester the amino acid,\(^2^4\) and endothelial cells possess a selective uptake and transport mechanism.\(^2^5\)

We believe that blood vessels remain the most likely site for the conversion of L-arginine because (1) pial vessel dilatation can be blocked by topical \(\text{L-NNAME}\) at concentrations and time intervals that are insufficient to reduce tissue NO synthase activity,\(^2^6\) (2) L-arginine may not accumulate within brain at early time points because it does not readily cross the blood-brain barrier, and (3) the L-arginine--induced blood flow response develops before recovery of the electroencephalographic amplitude (see above), suggesting that it probably reflects events other than those due to enhanced neuronal activity. Nevertheless, we cannot completely rule out the participation of glial cells or perivascular fibers at this time.\(^2^7\)

There are several disadvantages to the use of L-arginine in ischemia. Synthesis of NO is dependent on a complex enzymatic reaction that may not be sustained within ischemic tissue, as found recently for brain NO synthase activity (and NO levels).\(^2^8,2^9\) In fact, time-dependent decreases in NO synthase activity may account for our preliminary findings showing that L-arginine treatment begun at 1 hour after MCA occlusion failed to increase blood flow or reduce infarct size (E.M., M.A.M., Z.H., T.Y., K.I., T.D., unpublished data, 1993).

Taken together, these findings suggest that NO synthesis within brain possesses features that may be unique and suggest novel strategies for improving blood flow to the brain.

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Mechanism of Effect of L-Arginine on Infarct Size

The role of nitric oxide (NO) during cerebral ischemia is an area of intense investigation. The accompanying article by Morikawa et al provides additional evidence that acute administration of L-arginine increases cerebral blood flow and reduces volume of cerebral infarction after occlusion of the middle cerebral artery in rats. This effect appears to occur by an NO-dependent mechanism. Thus, elevation of NO levels before or immediately after the onset of cerebral ischemia may be beneficial (Morikawa et al and References 1 and 2), presumably by increasing blood flow. In some models of cerebral ischemia, however, NO may become toxic within hours to days, perhaps as the result of induction of NO synthase and overproduction of NO (for review, see Reference 3).

Many issues remain to be resolved before the role of NO during cerebral ischemia is understood. Current inconsistencies in the literature regarding NO and cerebral ischemia may be explained by use of different experimental models of ischemia (permanent or temporary, middle cerebral artery occlusion or transient forebrain ischemia), use of different doses, duration or agent to inhibit NO synthase, and perhaps species differences. The area of research is exciting and complex and offers hope for novel approaches to treatment of cerebral ischemia.

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