Combined effect of L-arginine and superoxide dismutase on the spastic basilar artery after subarachnoid hemorrhage in dogs

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To investigate the function of nitric oxide (a major endothelium-derived relaxing factor) in cerebral arteries after subarachnoid hemorrhage (SAH) in vivo, several nitric oxide-related substances were administered to dogs that had undergone double SAH. These included L-arginine (a substrate for the formation of nitric oxide), N\(^{ \text{-monomethyl-L-arginine (L-NMMA), an analog of L-arginine that inhibits the formation of nitric oxide from L-arginine,}}\) and superoxide dismutase (SOD, which protects nitric oxide from oxidation by superoxide anion), which were given via intracisternal injection. The diameter of the basilar artery was assessed angiographically.

In intact dogs, intracisternal bolus injections of L-arginine (1, 10, or 100 \(\mu\)mol) produced a dose-dependent increase in the internal diameter of the basilar artery; conversely, L-NMMA reduced the diameter of the basilar artery from baseline in a dose-dependent manner. On Days 4 and 7, after two intracisternal injections of autologous blood, L-arginine produced transient vasodilation of the spastic basilar artery, whereas L-NMMA produced no significant vasodilatation. The vasodilator effect of L-arginine after SAH was stronger on Day 4 than on Day 7, but less than in intact dogs. Intracisternal injection of SOD, which caused no effect per se, enhanced the duration of the vasodilator effect of L-arginine on the basilar artery on Day 4 and both the magnitude and duration of that effect on Day 7.

Thus, the basal release of nitric oxide was impaired after SAH, but the ability to synthesize nitric oxide in the vascular wall was not abolished. The finding that the simultaneous injection of SOD enhanced and prolonged the vasodilation induced by sufficient exogenous L-arginine suggests that the inactivation of nitric oxide by superoxide anion contributes to the development of vasospasm.

**KEY WORDS** - nitric oxide - L-arginine - superoxide dismutase - angiography - subarachnoid hemorrhage - dog

**NITRIC oxide** or a closely related factor formed from L-arginine is strongly implicated as a major endothelium-derived relaxing factor (EDRF). Endogenous nitric oxide released from the endothelium induces relaxation after stimulation by such agents as acetylcholine, substance P, vasopressin, and others, but synthesis and release of nitric oxide in vascular tissue under basal conditions appear to be essential to the maintenance of basal tone in cerebral blood vessels. It is assumed that basal release of nitric oxide must continue in order to maintain basal vascular tone, because the half-life of nitric oxide is less than 10 seconds. Nitric oxide stimulates guanylate cyclase in smooth-muscle cells and leads to accumulation of cyclic guanosine monophosphate (cGMP); relaxation of smooth muscles follows. Nitric oxide is synthesized in nerve terminals innervating blood vessels, as well as in the endothelium, probably functioning not only as an EDRF but also as a neurotransmitter.

Morphological and functional damage to the endothelium, smooth muscle, and nerve terminals follows subarachnoid hemorrhage (SAH). Despite a variety of morphological findings, including folding and vacuolation of endothelial, loss of endothelial tight junction, necrosis of smooth muscle, infiltration of leukocytes or mastoid cells, and damage to adventitial nerves, functional alterations after SAH have been relatively consistent; that is, the response to EDRF was decreased or abolished in spastic vessels or in isolated vessels exposed to hemoglobin. The main cause of dysfunction of endothelium-dependent relaxa-
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**TABLE 1**

*Mean arterial blood pressure before and after intracisternal injection of saline, L-arginine, SOD, and L-NMMA in dogs undergoing experimental SAH*

<table>
<thead>
<tr>
<th>Treatment &amp; Time of Study</th>
<th>No. of Dogs</th>
<th>Control Values</th>
<th>Postinjection Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td></td>
<td></td>
<td>10 Min</td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>106 ± 14</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>L-arginine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>5</td>
<td>105 ± 12</td>
<td>108 ± 18</td>
</tr>
<tr>
<td>100 µmol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>109 ± 11</td>
<td>108 ± 18</td>
</tr>
<tr>
<td>SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4, 6000 U</td>
<td>4</td>
<td>105 ± 9</td>
<td>106 ± 7</td>
</tr>
<tr>
<td>Day 7, 6000 U</td>
<td>4</td>
<td>110 ± 10</td>
<td>114 ± 12</td>
</tr>
<tr>
<td>L-arginine + SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>3</td>
<td>115 ± 11</td>
<td>112 ± 14</td>
</tr>
<tr>
<td>6000 U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>107 ± 14</td>
<td>106 ± 12</td>
</tr>
<tr>
<td>SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4, 6000 U</td>
<td>3</td>
<td>104 ± 15</td>
<td>105 ± 19</td>
</tr>
<tr>
<td>L-NMMA 10 µmol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>4</td>
<td>107 ± 14</td>
<td>104 ± 11</td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>111 ± 11</td>
<td>110 ± 8</td>
</tr>
</tbody>
</table>

* SOD = superoxide dismutase; L-NMMA = N6-monomethyl-L-arginine; SAH = subarachnoid hemorrhage. Data are presented as mean ± standard error of the mean, in mm Hg. No significant change was seen in any group.

*Recombinant human SOD was a gift of Asahi Chemical Industry Co., Ltd., Tokyo, Japan; L-NMMA was obtained from Calbiochem, La Jolla, California. All other chemicals were reagent grade.

**Materials and Methods**

Mature mongrel dogs of both sexes, each weighing 8.5 to 14 kg, were used for the study. All procedures were performed with the animals under general anesthesia induced with intravenous pentobarbital (25 mg/kg). The dogs were intubated and respiration was controlled through tracheal tubes with room air delivered by a respirator. The ventilation rate (approximately 12 cycles/min) and tidal volume (20 ml/kg) were adjusted to maintain arterial blood gas levels and pH within normal physiological limits.

A catheter for angiography was inserted into the right vertebral artery just before the foramen of the transverse process of the C-6 vertebra. A second catheter was placed in the left femoral artery to monitor mean arterial blood pressure (Table 1). A control angiogram was obtained with 3 ml of 65% meglumine iothalamate at a fixed magnification before the injection of control or test solutions. Angiograms were then obtained at 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes after the test solutions had been injected into the cisterna magna through a No. 22 spinal needle. Physiological saline, 2.0 ml, was used as a control solution and the test substances (L-NMMA, L-arginine, or SOD) were dissolved in that volume of physiological saline just before injection.

Except for L-arginine (an alkaline amino acid) the pH of all test solutions was approximately the same as that of the control solution. The vasodilator effect of L-arginine solution neutralized with HCl does not differ from that of L-arginine per se.27 Solutions were injected into the cisterna magna only after the same volume of cerebrospinal fluid (CSF) had been gently withdrawn in order to maintain the intracranial pressure
at as constant a level as possible. All CSF collected was stored for subsequent assay of L-arginine concentration.

**Experimental SAH Technique**

After control angiography, the cisterna magna was penetrated with a No. 22 spinal needle and 5 ml CSF was withdrawn. An equal volume of fresh autologous blood was then injected into the cisterna magna, with the animal kept in a head-down position for 30 minutes to facilitate autologous blood coming into contact with the basilar artery. On Days 4 and 7 post-SAH, angiograms were obtained to determine the occurrence and degree of chronic cerebral vasospasm. Angiograms were then obtained periodically after injection of test solutions, as in the control group.

The internal diameters of the middle third of the basilar arteries were measured by means of a computerized image-analysis system. The data are expressed as a percentage of the diameter of the arterial segment before injection.

**Measurement of L-Arginine in CSF After SAH**

The concentration of L-arginine in CSF was measured before and after SAH. Before autologous blood or test solutions were injected intracisternally, CSF samples were withdrawn and kept at -80°C until assayed.

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean. Differences were analyzed by analysis of variance, followed by Fisher's protected least significant difference multiple-range test. Values of p less than 0.05 were considered statistically significant.

**Results**

**Effects of L-NMMA and L-Arginine in Intact Dogs**

The intracisternal injection of L-NMMA produced dose-dependent decreases in the internal diameter of the basilar artery on angiography, whereas L-arginine caused dose-dependent increases (Fig. 1). Administration of 10 μmol L-NMMA produced vasoconstriction that lasted more than 120 minutes, with a maximum decrease in diameter of 75.7% ± 7.4%; conversely, 100 μmol L-arginine caused significant dilation in the basilar artery for 45 minutes with a maximum increase of 138.0% ± 7.5%. The vasoconstriction induced by L-NMMA was partially and transiently reversed by administering a 10-fold higher concentration of L-arginine (Fig. 2).

† Macintosh IICx computer manufactured by Apple Computer, Inc., Cupertino, California; Image 1.27 software obtained from National Technical Information Service, Springfield, Virginia.
‡ Amino acid concentrations determined by SRL Industry Co., Tokyo, Japan.

**Effects of L-NMMA and L-Arginine in Dogs With Double SAH**

The intracisternal injection of autologous blood on Day 1 and Day 3 of the experiment reduced the mean diameter of the basilar arteries to 52.3% ± 2.3% compared to control on Day 4 and to 46.0% ± 1.7% on Day 7. Administration of L-NMMA into the cisterna magna on Days 4 and 7 failed to produce vasoconstriction in the spastic arteries (Fig. 3).

On Days 4 and 7 of the experiment, L-arginine (10 or 100 μmol) was injected intracisternally. The basilar arteries subsequently showed vasodilation on angiography (Fig. 4). The 100-μmol dose dilated the spastic basilar arteries from 58.3% ± 1.7% to 88.0% ± 3.2% compared to control on Day 4, but significant vaso-
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FIG. 2. Graph showing percentage change in diameter of the basilar artery in response to intracisternal injection of 100 μmol L-arginine 40 minutes after the injection of 10 μmol N⁵-monomethyl-L-arginine (L-NMMA) in intact dogs. The contractile effect of L-NMMA was partially and transiently reversed by the addition of L-arginine. The number of animals treated is indicated in parentheses.

FIG. 3. Graph showing percentage change in diameter of the basilar artery in response to intracisternal injection of 10 μmol N⁵-monomethyl-L-arginine (L-NMMA) on Days 4 and 7 in dogs undergoing experimental subarachnoid hemorrhage. Data are presented as mean ± standard error of the mean (vertical bars). The number of animals treated is indicated in parentheses.

Dilation persisted for only 20 minutes. On Day 7, the vasodilator response to L-arginine was less than that on Day 4, increasing from 52.4% ± 2.1% to 67.6% ± 4.2% compared to control and lasting less than 20 minutes.

Effects of SOD With L-Arginine

Although injection of 6000 U SOD alone did not produce any change in the internal diameter of the basilar arteries, SOD (600 or 6000 U) enhanced the vasodilation caused by 100 μmol L-arginine in a dose-dependent manner. The maximum vasodilation caused by L-arginine on Day 4 was not affected by the addition of SOD, but its efficacy was obviously prolonged. On Day 7, both the maximum vasodilation and the effective duration were significantly enhanced (Fig. 5). Typical vertebral angiograms are shown in Fig. 6.

Concentrations of L-Arginine in CSF After SAH

The mean concentration of L-arginine in CSF before the injection of autologous blood was 23.98 ± 3.05 nmol/liter. After the intracisternal injection of blood, this level was 86.08 ± 12.64 nmol/liter on Day 4 and 73.40 ± 21.95 nmol/liter on Day 7 (Fig. 7).

Discussion

Effects of L-Arginine, L-NMMA, and SOD

We found that a high concentration of L-arginine injected into the cisterna magna dilated spastic basilar
arteries on Days 4 and 7 of the experiment. In dogs with SAH, the magnitude of vasodilation induced by L-arginine and its effective duration were less than in control animals. In control animals, intracisternal injection of L-arginine induced significant, long-lasting dilation of the basilar arteries and partially reversed the vasoconstrictor response to L-NMMA for a short time. L-arginine, an alkaline amino acid, lacks vasodilator activity but elicits vasodilation mediated through the synthesis and release of nitric oxide by acting as a substrate for the formation of nitric oxide.\textsuperscript{5} Our evidence suggests that the ability to synthesize nitric oxide in the vascular wall is not abolished after SAH. In \textit{vitro} studies show that release of EDRF is not impaired in the spastic artery after SAH.\textsuperscript{17,18}

Although a high dose of L-NMMA reduced the diameter of the basilar artery in intact dogs to 75.7% of control, L-NMMA did not cause vasoconstriction of the basilar artery on Days 4 and 7. The vasodilation induced by vasopressin (which stimulates the release of nitric oxide) was completely absent on Day 4 and was still reduced on Day 7.\textsuperscript{17} The response to vasopressin on Day 4 did not improve even after simultaneous administration of SOD (data not shown), which suggests that after SAH nitric oxide no longer functions as a major modulator of basal vascular tone.

These results support the conclusion from other studies\textsuperscript{12,25} that reduced production of nitric oxide in the endothelium is a major cause of dysfunction of the nitric oxide-dependent mechanism following SAH and may be involved in the pathogenesis of cerebral vasospasm. Other factors, such as decreased transfer of nitric oxide or reduced responsiveness of smooth muscle to nitric oxide, may enhance the dysfunction.\textsuperscript{16}

The injection of SOD alone produced no significant alteration in the internal diameter of the basilar arteries on Days 4 and 7 of the experiment. This finding is consistent with the failure of SOD to protect monkeys against induced vasospasm.\textsuperscript{30} However, SOD strongly enhanced vasodilation when combined with L-arginine: doses of 600 and 6000 U showed a dose-dependent enhancement. The simultaneous administration of these two agents enhanced the maximum vasodilation on Day 7 and prolonged the duration of vasodilation on Days 4 and 7. A scavenger of free radicals, SOD increases the half-life of nitric oxide by inactivating superoxide anions.\textsuperscript{31,32} Superoxide anions, which are potent inactivators of nitric oxide,\textsuperscript{33} are produced in relation to autoxidation of oxyhemoglobin released from lysed erythrocytes\textsuperscript{34} or in relation to lipid peroxidation.\textsuperscript{32,33} Oxyhemoglobin, which penetrates the cerebral arterial walls after SAH and inhibits the activity of nitric oxide,\textsuperscript{16} is thought to be the main participant in the generation of oxygen radicals.\textsuperscript{35} Therefore, the combined administration of SOD and L-arginine may dilate a spastic artery by stimulating the synthesis and release of nitric oxide and then protecting it against oxidation. Our results suggest that after SAH the basal release of nitric oxide is impaired and any nitric oxide released is inactivated by superoxide anions.

Concentration of L-Arginine in CSF

The concentration of L-arginine in CSF in the present study rose considerably after SAH. All amino acids except taurine increase in human CSF after SAH.\textsuperscript{36,37} This increase may derive from proteolysis due to catabolic processes and from impaired clearance of the amino acids. In this study, despite the increase in L-arginine concentration in CSF, the ability of nitric oxide to maintain basal tone was abolished. A CSF concentration of L-arginine sufficient to permit formation of nitric oxide under normal conditions could not stimulate synthesis and release of nitric oxide in the spastic artery. The availability of L-arginine becomes a rate-limiting factor in the production of nitric oxide, assuming that the metabolic pathway in well preserved.

The fact that only a higher concentration of L-arginine
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FIG. 6. Representative vertebral angiograms showing the combined effects of 6000 U superoxide dismutase (SOD) and 100 μmol L-arginine in dogs undergoing experimental subarachnoid hemorrhage (SAH). A: Day 1, before intracistemal injection of autologous blood (SAH). B: Post-SAH Day 7 (before intracisternal injection of SOD and L-arginine). C: Ten minutes after intracisternal injection of SOD and L-arginine on Day 7. D: Thirty minutes after intracisternal injection of SOD and L-arginine on Day 7.

FIG. 7. Graph showing the change in concentration of L-arginine in cerebrospinal fluid after experimental subarachnoid hemorrhage. Data are presented as mean ± standard error of the mean (vertical bars). The number of animals in which measurements were taken is indicated in parentheses.

induced synthesis and release of nitric oxide in dogs undergoing experimental SAH suggests an impairment in the metabolic pathway mediated by nitric oxide synthase, probably due to decreased activity or sensitivity of nitric oxide synthase.

Biosynthetic Pathway of Nitric Oxide

In addition to production in the endothelium, a biosynthetic pathway for nitric oxide has been demonstrated immunohistochemically in parasympathetic nerve fibers innervating cerebral arteries from the sphenopalatine ganglion in rats. Colocalization of nitric oxide with vasoactive intestinal peptide was observed in some nerve fibers. A transient vasodilator response, inhibited by L-NMMA, following transmural electrical stimulation in cerebral arteries without endothelium suggests that nitric oxide functions as a neurotransmitter in the regulation of local cerebral blood flow under normal physiological conditions. Moreover, under some pathological conditions, nitric oxide may be produced by the inflammatory white cells that infiltrate the intimal or medial layer in spastic vessels or by smooth-muscle cells, mediated through newly produced nitric oxide synthase. In our in vivo study, it was not possible to determine whether L-arginine applied intracisternally could serve as a substrate for nitric oxide synthase in nerve terminals or in the pathological pathway. The depression of cGMP levels by hemoglobin was equivalent to the effect of endothelial denudation or to incubation with N^6^-nitro-L-arginine methyl ester, suggesting that the effect of hemoglobin after SAH is due to a specific action on endothelium rather than to interaction with a nitric oxide-like substance produced by the vascular smooth muscle or adventitial nerves.
Conclusions

We conclude that SAH impairs the basal release of nitric oxide that contributes to the maintenance of basal tone in the cerebral artery, although the system for producing nitric oxide is not abolished. Nitric oxide released from vascular tissue appears to be quickly oxidized or inactivated after SAH, probably by oxyhemoglobin released from lysed erythrocytes or by lipid peroxide from membrane components in the subarachnoid space. Intracerebral injection of a high concentration of L-arginine as a substrate for the formation of nitric oxide and of SOD to prevent oxidation of nitric oxide can dilate the spastic artery to a significant extent. These results encourage us to seek new therapeutic approaches that stimulate nitric oxide-dependent vasodilator mechanisms.

References

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