Increased cerebral blood flow but no reversal or prevention of vasospasm in response to L-arginine infusion after subarachnoid hemorrhage

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Object. The reduction in the level of nitric oxide (NO) is a purported mechanism of delayed vasospasm after subarachnoid hemorrhage (SAH). Evidence in support of a causative role for NO includes the disappearance of nitric oxide synthase (NOS) from the adventitia of vessels in spasm, the destruction of NO by hemoglobin released from the clot into the subarachnoid space, and reversal of vasospasm by intracarotid NO. The authors sought to establish whether administration of L-arginine, the substrate of the NO-producing enzyme NOS, would reverse and/or prevent vasospasm in a primate model of SAH.

Methods. The study was composed of two sets of experiments: one in which L-arginine was infused over a brief period into the carotid artery of monkeys with vasospasm, and the other in which L-arginine was intravenously infused into monkeys over a longer period of time starting at onset of SAH. In the short-term infusion experiment, the effect of a 3-minute intracarotid infusion of L-arginine (intracarotid concentration 10^{-4} M) on the degree of vasospasm of the right middle cerebral artery (MCA) and on regional cerebral blood flow (rCBF) was examined in five cynomolgus monkeys. In the long-term infusion experiment, the effect of a 14-day intravenous infusion of saline (control group, five animals) or L-arginine (10^{-4} M; six animals) on the occurrence and degree of cerebral vasospasm was examined in monkeys. The degree of vasospasm in all experiments was assessed by cerebral arteriography, which was performed preoperatively and on postoperative Days 7 (short and long-term infusion experiments) and 14 (long-term infusion experiment). In the long-term infusion experiment, plasma levels of L-arginine were measured at these times in the monkeys to confirm L-arginine availability.

Vasospasm was not affected by the intracarotid infusion of L-arginine (shown by the reduction in the right MCA area on an anteroposterior arteriogram compared with preoperative values). However, intracarotid L-arginine infusion increased rCBF by 21% (p < 0.015; PCO2 38–42 mm Hg) in all vasospastic monkeys compared with rCBF measured during the saline infusions. In the long-term infusion experiment, vasospasm of the right MCA occurred with similar intensity with or without continuous intravenous administration of L-arginine on Day 7 and had resolved by Day 14. The mean plasma L-arginine level increased during infusion from 12.7 ± 4 µg/ml on Day 0 to 21.9 ± 13.1 µg/ml on Day 7 and was 18.5 ± 3.1 µg/ml on Day 14 (p < 0.05).

Conclusions. Brief intracarotid and continuous intravenous infusion of L-arginine did not influence the incidence or degree of cerebral vasospasm. After SAH, intracarotid infusion of L-arginine markedly increased rCBF in a primate model of SAH. These findings discourage the use of L-arginine as a treatment for vasospasm after SAH.

Key Words • nitric oxide • subarachnoid hemorrhage • vasospasm • cerebral blood flow • L-arginine

Increased NO available in the arterial wall may result in development of delayed cerebral vasospasm after SAH.14 This hypothesis is supported by the loss of nNOS in the vessel wall,45 by the high affinity of NO to oxyhemoglobin,14,19,33,56 a purported factor responsible for vasospasm after SAH,26,42 and by the reversal and prevention30 of vasospasm with intracarotid delivery of NO. It has also been established that intravascular administration of L-arginine, the substrate for the NO-producing enzyme NOS, results in vasodilatation6,7,34,37 and increased CBF2,4,11,14,16,34,48,57 Furthermore, intracisternal administration of L-arginine attenuates vasospasm of the basilar artery in dogs.13,22 The mechanism by which L-arginine induces these effects is unclear but may involve an increase in NO production by the upregulated eNOS52,41,28,48,55 or iNOS19,35,36 that is present after SAH occurs in macrophages,15,19,30 reactive astrocytes,26,36,46 and smooth-muscle cells stimulated by hemoglobin.50 We hypothesized that intravascular administration of L-arginine might increase production of NO in the arterial wall or its vicinity, and we sought to determine whether parenteral delivery of L-arginine would reverse and/or prevent vasospasm in a primate model of SAH.
Materials and Methods

Experimental Design

The effect of parenteral administration of L-arginine on the diameter of the right MCA and on rCBF after placement of an autologous clot around the right MCA was examined in two experimental settings. In the short-term infusion group, five cynomolgus monkeys with delayed cerebral vasospasm of the right MCA (confirmed arteriographically on Day 7 after SAH) were tested during a continuous 3-minute intracarotid infusion of saline, followed by cerebral arteriography. Thirty minutes later, L-arginine was infused into the ICA in the same animals for 3 minutes. This infusion was immediately followed by cerebral arteriography. After an additional 30 minutes, two new L-arginine infusions, separated by 30 minutes, were administered. After the third intracarotid infusion of L-arginine, the final arteriogram was obtained. All infusions were performed under controlled physiological conditions (PCO\(_2\) 38–42 mm Hg). Arterial blood pressure was monitored using a transducer located in the left femoral artery, and end expiratory PCO\(_2\) was controlled by the ventilator rate. Cortical rCBF in the distribution of the right MCA was measured using a thermomonitoring probe placed under the dura in the right MCA territory during the infusions.\(^5\)

In the long-term infusion group, 11 cynomolgus monkeys were randomly allocated to one of two groups after onset of SAH. The monkeys received continuous intravenous infusion of either normal saline (control group, five animals) or L-arginine (10^{-3} M, six animals) for 14 days beginning 24 hours after SAH occurred. Cerebral arteriography was performed before the onset of SAH and on Days 7 and 14 post-SAH. The monkeys were later killed.

All animals were tested while in a state of general anesthesia induced by 0.5% isoflurane/pancuronium; their systemic arterial blood pressure and end expiratory PCO\(_2\) were continuously monitored.

Model of Cerebral Vasospasm

After induction of general anesthesia, all monkeys underwent right frontotemporal craniectomy with dissection of the arachnoid of the sylvian fissure. The techniques are fully described elsewhere.\(^6\) The right MCA was exposed and 5 ml of preclotted arterial blood collected from the left femoral artery was placed around the artery. The dura was closed watertight and the wound was closed in layers. Each monkey was extubated after recovery of the gag reflex.

Arteriographic Studies

To assess vasospasm, cerebral arteriography was performed preoperatively and on Days 7 (short- and long-term infusion experiments) and 14 (long-term infusion experiment) after surgery.\(^6\) The monkeys were anesthetized by an intramuscular injection of ketamine (10 mg/kg) and xylazine (Rompun, 1 mg/kg). A femoral artery cutdown was performed under aseptic conditions and a No. 3 French polyethylene catheter was advanced, with the aid of a fluoroscope, to the right ICA. Contrast medium (0.75 ml of 60% Conray) was injected by hand. Subtraction images of the AP projections were acquired. Arteriographically demonstrated vasospasm was quantified relative to each animal’s baseline arteriogram.\(^4\) For grading vasospasm, the proximal 14-mm segment of the right MCA observed on both preoperative and postoperative AP arteriograms was measured using a computerized image analysis system, as previously described.\(^6\) Vasospasm was classified on the basis of a comparison between the pre- and postoperative image measurements of the area of the right MCA observed in the AP view (11–25% reduction = mild vasospasm, 26–50% = moderate vasospasm, and > 50% = severe vasospasm).\(^4\)

Cerebral Blood Flow Measurement

After induction of general anesthesia, the short-term infusion group underwent a small right-sided parietal craniectomy. After the dura was opened, a CBF probe was slipped between the dura and the brain and placed over a region perfused by the right MCA.\(^5\) The position of the probe was confirmed by a lateral skull x-ray film and by changes in CBF induced by an intracarotid bolus injection of saline. Regional CBF was measured continuously (one measurement every 4.5 seconds) before, during, and after intracarotid infusion of saline or L-arginine. To compare rCBF among groups of monkeys, CBF ratios were used; these were obtained for each monkey by normalizing the CBF measured during the saline and L-arginine infusions to the mean CBF of that monkey at a PCO\(_2\) of 40 mm Hg immediately before starting the infusion.\(^10\) Heart rate, blood pressure, rCBF, and PCO\(_2\) were allowed to return to steady state (baseline) for at least 20 minutes between infusions.

Preparation and Delivery of L-Arginine

In the short-term infusion group, L-arginine was delivered directly, by an infusion pump, at the rate of 1.1 ml/minute through an intracarotid catheter placed in the ICA. The L-arginine solution was prepared to achieve an intracarotid concentration of 10^{-5} M (calculated by assuming an average CBF in a monkey of 50 ml/100 g minute, brain weight of 80 g, and perfusion of two-thirds of the cerebral hemisphere by the ICA; the l-arginine concentration in the solution was 10^{-3} M).\(^5\) In the long-term infusion group, L-arginine (10^{-3} M) was delivered using a D-infusor pump (20 ml/24 hours) through an indwelling catheter in the femoral vein. The pump, placed in a jacket on the back of the monkey, was changed every 24 hours. Saline was infused using the same method in the control group.

Levels of L-Arginine

The L-arginine levels in plasma samples, which were collected for the long-term infusion experiment at surgery and during postoperative arteriography, were measured using high-performance liquid chromatography. One hundred microliters of plasma was mixed with 100 \mu l of 0.1 M hydrochloric acid containing 2 \mu g of l-methionine sulfone. The mixture was filtered and centrifuged in a fixed-angle centrifuge for 20 minutes at 4000 G. The filtrate (40 \mu l) was placed in a prewashed 15 x 100-mm borosilicate glass culture tube; prewashed with 6 M hydrochloric acid, water, and 100% ethanol to remove impurities; and dried in a nitrogen atmosphere at 35°C for 60 minutes. Samples were neutralized and derivatized. The dried residue was kept at 70°C until reconstituted in 95 \mu l of PICO-TAG diluent. Ten-microliter aliquots were analyzed. High-performance liquid chromatography conditions and quantification were similar to those described by the manufacturer of the PICO-TAG system for analysis of free amino acids.

Statistical Analysis

For statistical analysis of data, we used ANOVA, the paired t-test, and Wilcoxon’s signed-rank test. Significance was accepted at a probability value less than 0.05. The animal protocol was reviewed by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee and met the National Institutes of Health guidelines for animal care.

Sources of Supplies and Equipment

Cerebral blood flow was monitored using a Saber thermomonitoring probe available from Flowtronix (Phoenix, AZ). The O-Arm fluoroscope was obtained from OEC Medical Systems, Inc. (Salt Lake City, UT) and the Conray contrast material from Mallinckrodt Medical, Inc. (St. Louis, MO). Areas of the MCA observed on preoperative and postoperative AP arteriograms were measured using the Image (version 1.61) computerized image analysis system developed by Wayne S. Rashbund, National Institutes of Health, Bethesda, MD). The infusion pump used to deliver L-arginine in the acute infusion experiment was obtained from Harvard Apparatus, Inc. (So. Natick, MA). The D-infusor pump used in the chronic infusion experiment was obtained from Disetronic (Minneapolis, MN). The L-methionine sulfone (M 876) was purchased from Sigma Chemical Co. (St. Louis, MO) and the Amicon No. 4104 Centrifree filters from Millipore Corp. (Bedford, MA). The filtrate was dried using the Multivap unit manufactured by Organon Inc. (Berlin, MA). The PICO-TAG diluent (No. 88119) by R. M. Pluta, et al.
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and the PICO-TAG system were obtained from Waters Corp. (Milford, MA). Statistical analysis was performed with the aid of the Statview+Graphics software program, available from Abacus Concepts, Inc. (Berkeley, CA).

Results

Short-Term Infusion Experiment

Seven days after SAH occurred, vasospasm of the right MCA developed in all five monkeys. Because we found no difference in the degree of vasospasm observed on the first and last arteriograms obtained after L-arginine infusions, only the results of the last arteriogram are presented. On arteriography performed immediately after intracarotid infusion of saline, the mean reduction in the right MCA area on the AP view was 44\% compared with the preoperative area (PCO$_2$ 38–42 mm Hg). The vessel AP area did not change in response to intracarotid infusion of saline and L-arginine. The values presented are the means ± standard deviation of the CBF ratio during saline or L-arginine infusion in the same animals.

Concurrent with this increase in rCBF, cerebrovascular resistance (mean arterial blood pressure/CBF) decreased in the infused ICA from the preinfusion value of 1.64 ± 0.11 mm Hg/ml/100 g per minute (p < 0.001). There was no change in systemic mean arterial blood pressure during intracarotid L-arginine infusion compared with saline infusion (L-arginine group, 86 ± 13 mm Hg; saline group, 83 ± 13 mm Hg).

Fig. 1. Bar graph depicting relative changes in rCBF in response to intracarotid infusion of saline (five animals) or L-arginine (six animals) in a primate model of vasospasm. Infusion was made through a catheter placed in the ICA. Regional CBF increased during intracarotid infusion of L-arginine (* p < 0.015, paired t-test). Regional CBF was measured continuously (one measurement every 4.5 seconds) before, during, and after intracarotid infusion of saline and L-arginine. The values presented are the means ± standard deviation of the CBF ratio during saline or L-arginine infusion in the same animals.

Fig. 2. Bar graph showing degrees of vasospasm on Days 7 and 14 post-SAH in groups that received continuous infusion of saline (five animals) or L-arginine (six animals). We found no significant difference in the degree of vasospasm between the saline and L-arginine groups on Day 7 or Day 14. Values represent the percent decrease in the proximal 14-mm segment of the right MCA (RMCA) on postoperative AP arteriograms obtained after onset of SAH compared with the values obtained before SAH. The values presented are the means ± standard error of the mean of the decrease measured by three researchers who measured the vessel diameters in a blinded fashion.

Long-Term Infusion Experiment

Moderate-to-severe vasospasm of the right MCA developed in all monkeys in the intravenous saline and L-arginine infusion groups on Day 7. The mean reduction in the right MCA AP area was 45 ± 4% in the control group and 36 ± 14% in the L-arginine group. The difference in the reduction in areas of the proximal segment of the right MCA between the control group (five animals) and the L-arginine group (six animals) was not significant (p = 0.16; Fig. 2). Vasospasm resolved, as is the normal course in this model, on Day 14 (the mean reduction in the right MCA area on the AP view was 3 ± 13% in the saline group and 2 ± 12% in the L-arginine group). There was no difference in the right MCA area between the pre-SAH observation and that made on post-SAH Day 14 (p = 0.2; Fig. 2). Also, the difference in the degree of vasospasm on post-SAH Day 14 between the control and L-arginine groups was not significant (p = 0.4). The plasma L-arginine level (12.7 ± 4 μg/ml on Day 0, before intracarotid infusion of L-arginine) increased to 21.9 ± 13.1 μg/ml on Day 7 of the infusion (p < 0.05) and was 18.5 ± 3.1 μg/ml on Day 14 (p < 0.03; Fig. 3). Plasma L-citrulline, a byproduct of NO production from L-arginine, was unchanged on Days 7 and 14 (Fig. 3).

Discussion

Continuous release of NO from endothelial cells is necessary to maintain resting cerebrovascular tone and basal CBF. After SAH occurs, hemoglobin released from the clot binds NO, reducing the amount of NO available in the arterial wall. It may also destroy ni-
must be responsible for using L-arginine for the NO pro-
cisternally directly at the side of the vasospasm.13,22,51
demonstrate the standard deviation of the mean.
spasm.8,45,51,56 Supporting this hypothesis are the reduced
availability in the arterial wall is a putative cause of vaso-
rupting vasodilation caused by NO. This decrease in NO
levels of cGMP, the second messenger for biological ac-
tivity of NO,19 in the wall of cerebral arteries exposed to
blood23,25 and the reversal1 and prevention of vasospasm
after an intracisternal infusion of NO.44 In the primate mod-
el of SAH, vasospasm resolves approximately 14 days af-
after onset of SAH. Production of NO from yet unidentified
sources may explain this natural resolution of vasospasm.
Administration of L-arginine, a substrate for NO pro-
duction,12 increases NO production by endothelial cells in vitro,7,28 dilates vessels,6,7,23,34,35,37 and increases CBF in vivo.1,13,14,44,46,48,49 Subsequently, L-arginine has been pro-
posed as a potential therapy for delayed cerebral vaso-
spasm.13,22,51,53 We sought to establish whether intracisternal
or intravenous infusion of L-arginine reverses or prevents
vasospasm in a primate model of SAH.

Short-Term Intracisternal Infusion of L-Arginine

Despite promising results in other models of SAH,13,22,51 short-term ICA administration of L-arginine did not re-
verse vasospasm in our study. This discrepancy with prior
studies may be due to a difference in L-arginine adminis-
tration. In prior studies L-arginine was introduced intra-
cisternally directly at the side of the vasospasm.13,22,51
Because endothelial cells produce NO on exposure to
L-arginine and because the endothelial cells of the vessels
were in contact with the plasma containing the increased
levels of L-arginine in our experiment, another cell type
must be responsible for using L-arginine for the NO pro-
duction that reversed vasospasm in previous studies.

Increased rCBF to the area supplied by the artery in
spasm following short-term intracisternal administration of
L-arginine, which induces NO release from endothelial
cells,28,37 supports earlier in vitro and in vivo observations
that L-arginine dilates cerebral vessels and increases
CBF.6,13,22,34,35,37,48,49 The mechanism of this increase in
rCBF is not clear;34 however, our findings and earlier ob-
servations15 suggest that it is the small-resistance arteri-
oles, rather than the conductive vessels of the brain that
dilate in response to the high plasma levels of L-argi-
nine. These findings also provide further evidence that
NO, probably synthesized by eNOS, is an important medi-
ator of rCBF regulation in primates.20,53

Long-Term Intravenous Infusion of L-Arginine

In the long-term infusion experiment, a continuous in-
crease in L-arginine plasma levels did not alter the degree,
incidence, or time course of delayed cerebral vasospasm
after SAH. We propose several explanations for the lack
of effect of L-arginine on vasospasm. First, the level of
L-arginine in plasma may not be a limiting factor in NO
production by eNOS. Levels of L-arginine normally pres-
ent in plasma may already saturate either the cellular
uptake system for L-arginine or binding of L-arginine by
endothelial cells (eNOS), 2 and neurons (nNOS).3,46,58 Nitric oxide
that is produced by NOS from L-arginine by any of these
cells in the brain contains NOS, including endothelial cells
(eNOS and iNOS),55,60 macrophages,15,38 reactive astro-
cytes,9,36 smooth-muscle cells (iNOS),3,44,59 activated astro-
cytes (eNOS),2 and neurons (nNOS).40,45,46,49 Nitric oxide
that is produced by NOS from L-arginine by any of these
cells binds to the heme moiety of soluble guanylate cy-
clase and promotes synthesis of cGMP.14,18,31,42 Increased
cGMP levels in smooth-muscle cells lead to sequestration
of Ca++ from a myosin–actin complex, relaxation of the
muscle cell, and vasodilation.14,31,42 Thus, NO that is pro-
duced by any of the cells containing NO and that diffus-
esto smooth-muscle cells in the cerebral arterial wall will
produce a vasodilatory effect.

If we assume that production of NO is associated with
resolution of vasospasm, the cells responsible for produc-
ing NO have not been identified. Endothelial cells are a

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Fig. 3. Graph depicting levels of L-arginine and L-citrulline in
plasma (μg/ml) during long-term intravenous infusion of L-argi-
nine in six monkeys. Plasma L-arginine levels increased in re-
sponse to the continuous intravenous infusion (* p < 0.05 on Day
7, ANOVA; ** p < 0.03 on Day 14, ANOVA); however, there was no change in plasma L-citrulline levels (p = 0.7). Error bars
represent the standard deviation of the mean.

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candidate because eNOS activity remains present in the endothelium during vasospasm after SAH.45 Intravascular administration of L-arginine should increase endothelial production of NO33 and, consequently, relieve the spasm. Furthermore, because L-arginine does not readily cross the blood-brain barrier,44 this effect is limited to endothelial cells. However, systemic administration of L-arginine did not reverse or prevent vasospasm. Thus, endothelial cells are an unlikely candidate. Neurons are an even more unlikely source of NO. Neuronal NOS is not present in the adventitia of the artery in spasm and its activity does not return even when vasospasm resolves.35 Furthermore, the short half-life30 of NO, coupled with the lack of nNOS-positive neurons in proximity to the spastic vessel,36 suggests that nitroxdergic neurons of the brain are also unlikely to be responsible for resolution of vasospasm by NO production. The same limitations exclude iNOS and eNOS in reactive astrocytes2,9,36 as a source of NO. Thus, iNOS present in macrophages that migrate to the arterial wall after SAH,17,46,59 or iNOS expressed by smooth-muscle cells in response to hemin40 released from the subarachnoid clot may provide NO to resolve vasospasm.

Conclusions

Intracarotid (short-lasting) and intravenous (long-lasting) infusion of L-arginine did not influence the incidence or degree of vasospasm. On the other hand, intracarotid infusion of L-arginine markedly increased rCBF in a primate model of SAH. These findings discourage the use of L-arginine as a treatment for vasospasm after onset of SAH.

References


