

L-Arginine and S-Nitrosoglutathione Reduce Embolization in Humans

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Background—L-Arginine reduces platelet aggregation and adhesion in ex vivo studies, but there is no evidence as yet that it has a therapeutic effect on clinical end points. Doppler ultrasound can detect cerebral emboli noninvasively. Such embolic signals are common after carotid endarterectomy, and their frequency predicts risk of stroke recurrence. We used this situation to determine the antiplatelet efficacy of L-arginine and S-nitrosoglutathione (GSNO), a physiological nitric oxide donor with possible platelet specificity.

Methods and Results—Patients undergoing carotid endarterectomy were randomized in a double-blind manner between L-arginine (n=14), GSNO (n=14), or placebo (n=14) administered intravenously for 90 minutes, starting 30 minutes after skin closure. All patients were pretreated with aspirin and given heparin during surgery. Transcranial Doppler recordings were made from the ipsilateral middle cerebral artery for 4 hours after surgery, beginning 30 minutes after skin closure, and also at 6 and 24 hours. There were highly significant reductions in the number of Doppler embolic signals in the L-arginine and GSNO groups; first 4 hours, median (range) number of embolic signals, placebo 44.7 (6 to 778), L-arginine 9.5 (0 to 225), and GSNO 0.8 (0 to 8), both $P < 0.001$ versus control values. The reduction in the signals persisted at the 24-hour recording.

Conclusions—Intravenous L-arginine and GSNO attenuate Doppler embolic signals in humans. Modulation of the NO system with these agents may have applications in the treatment of thromboembolic disease. This study demonstrates the potential application of ultrasonic embolic signal detection to examine the efficacy of new antiplatelet agents in relatively small numbers of patients. (*Circulation*. 2001;103:2371-2375.)

Key Words: ultrasonics ■ drugs ■ platelets ■ inhibitors ■ carotid arteries

Nitric oxide (NO) is an important messenger molecule involved in a wide range of biological processes, including the regulation of hemostasis. NO is generated by the enzymatic oxidation of the semiessential amino acid L-arginine into L-citrulline through the action of NO synthase (NOS).¹ This biochemical system, which was first identified in the vascular endothelium,¹ is now known to exist in many other cells, including platelets.² Both in vitro and ex vivo studies have demonstrated that L-arginine reduces platelet aggregation. In an experimental model of balloon angioplasty, intravenous L-arginine inhibited platelet adherence to the denuded surface of the canine coronary artery.³ Both oral and intravenous L-arginine supplementation attenuates platelet aggregation ex vivo in healthy subjects^{4,5} and in hypercholesterolemic patients.⁶ Ex vivo platelet assays, however, are not always fully representative of the situation in vivo, and to date there is no evidence that L-arginine has a therapeutic effect on clinical markers such as risk of stroke or myocardial infarction or even asymptomatic cerebral embolization.

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Circulating solid emboli can be detected with the use of transcranial Doppler ultrasonography.⁷ This technique has been shown to be highly sensitive and specific for a variety of solid embolic materials in validation studies both in vitro and in animal models.^{8,9} Because of the increased frequency of embolic signals (ES) compared with stroke, they offer a surrogate end point that may be used to evaluate new antiplatelet and antithrombotic therapies.¹⁰ One situation in which ES are frequent is after carotid endarterectomy (CEA). During this procedure, the endothelium and inner media are removed, creating a highly thrombogenic surface on which platelet adherence and aggregation occur. Asymptomatic cerebral ES are common after CEA, and a high frequency during the early postoperative phase predicts risk of early stroke and transient ischemic attack.^{11,12}

We previously used this model to demonstrate that S-nitrosoglutathione (GSNO) reduces postoperative embolization in patients already treated with aspirin and heparin.

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TABLE 1. Patient Characteristics in the 3 Treatment Groups

	L-Arginine Group (n=14)	GSNO Group (n=14)	Placebo Group (n=14)
Male, n	9	6	9
Age, y (mean±SD)	67.5±6.9	69.7±9.7	67.3±9.0
Diabetes mellitus, n	2	4	1
Hypertension, n	11	10	9
Current smoker, n	11	13	13
Hypercholesterolemia, n	9	7	8
ES detected on preoperative TCD recording	2	4	3

TCD indicates transcranial Doppler ultrasonography. Hypertension is defined as systolic pressure >160 mm Hg or diastolic pressure >90 mm Hg or antihypertensive treatment. Hypercholesterolemia is defined as a fasting cholesterol level >6.0 mmol/L or receiving cholesterol-lowering therapy.

GSNO is a stable *S*-nitrosothiol from which NO is released by the action of enzymes associated with platelet membranes.¹³ In animals and humans, GSNO has significant antiplatelet action at doses that cause less hemodynamic effect than conventional NO donors.¹⁴ In our previous study, GSNO markedly reduced the rate of embolization when administered during CEA, starting from the induction of anesthesia until 2 hours after skin closure.¹⁵ This study was not double blind because of concerns over increased bleeding secondary to GSNO, although in fact this did not occur. In addition, some intraoperative hypotensive episodes occurred with administration of GSNO. It is possible that it may have similar therapeutic efficacy without this side effect if started immediately after surgery.

Therefore, in this double-blind randomized placebo-controlled study, we examined the hypothesis that L-arginine reduces asymptomatic embolization in humans when given immediately after CEA. We also determined the effectiveness of GSNO, administered after surgery, in the same setting.

Methods

We studied 42 patients who were undergoing CEA for symptomatic internal carotid artery stenosis of >70% determined angiographically by use of the European Carotid Surgery Trial method of measurement.¹⁶ Their baseline risk factor details are summarized in Table 1. The study was approved by the local hospital Ethical Committee, and written informed consent was obtained from all subjects. All patients were pretreated with aspirin 300 mg/d for ≥1 week before surgery and were given 5000 IU sodium heparin IV during the operation before carotid clamping. The aspirin was continued after surgery. Patients were randomly allocated to treatment with GSNO (n=14), L-arginine (n=14), or no additional treatment (normal saline, n=14) on a double-blind basis. The study medication was administered as an intravenous infusion starting 30 minutes after skin closure and continuing for 90 minutes. GSNO was infused at a rate of 2.2 nmol · kg⁻¹ · min⁻¹ and, if tolerated, increased to a rate of 4.4 nmol · kg⁻¹ · min⁻¹ after 10 minutes. In the L-arginine group, 30 g was administered in 150 mL normal saline IV. In the control group, 150 mL normal saline was infused IV.

A commercially available transcranial Doppler machine (TC 2020; EME/Nicolet Ltd) with a 2-MHz transducer was used to record from the ipsilateral middle cerebral artery (MCA). The MCA was identified via the transtemporal window, and a transducer was fixed in position with the use of a standard headset. A sample volume of 5 mm, a sweep speed of 5 seconds, and a mean (SD) recording depth

TABLE 2. Median (Range) Number of ES per Hour in the 3 Treatment Groups During the 3 Recording Periods After CEA

	0–4 Hours	6–7 Hours	24–25 Hours
GSNO	0.8 (0–8)*	0 (0)†	0 (0)‡
L-Arginine	9.5 (0–225)†	1.4 (0–5)§	0 (0–1)§
Control	44.7 (6–778)	21.1 (0–188)	5.1 (0–38)

* $P < 0.0001$; † $P < 0.005$; ‡ $P < 0.01$; § $P < 0.05$ vs control.

of 52.3 (2.5) mm (range, 48 to 58 mm) were used. Recordings of the raw Doppler signal were made to digital audiotape for offline analysis. In all patients, a 1-hour recording was made in the 24 hours preceding surgery. After surgery, a recording was made for 4 hours, beginning 30 minutes after skin closure, and also for 1 hour at both 6 and 24 hours after skin closure.

Analysis of recordings was performed by an observer (observer 1) who was blinded to the clinical details, time of recording, and study group. The Doppler signal was played into the same equipment for spectral analysis, and a 128-point fast Fourier transform was used, with a time-window overlap of >66%. ES were identified by their characteristic visual appearance and chirping sound according to recent consensus criteria.¹⁷ An intensity threshold of >7 dB was used because this has been shown to increase interobserver agreement without excessive loss of sensitivity.¹⁸ Intensity was determined from the color-coded intensity scale on the spectral display. The peak intensity of the ES and the intensity of the background spectra at the same frequency and part of the cardiac cycle from the preceding or subsequent cardiac cycle were determined. All possible ES detected were saved and then reviewed by a second experienced observer (observer 2); if both observers agreed that the signal was an ES, it was then included in subsequent analyses. Interobserver reproducibility in identifying ES was assessed by the 2 observers independently analyzing a separate recording that had been prepared from MCA recordings from 6 patients with carotid stenosis. Agreement was calculated with the use of the proportion of specific agreement, which estimates the probability that 1 observer will identify a specific ES if another observer has identified it, with a probability of 1 indicating complete agreement.¹⁸ Observer 1 detected 90 ES, and the agreement of observer 1 with observer 2 was 0.92. Observer 2 detected 89 ES, and the agreement with observer 1 was 0.90. The number of ES in each group was not normally distributed, and therefore, comparison between the numbers of ES detected in each group was performed with the Mann-Whitney *U* test for nonparametric data. For comparison of ES intensity, an unpaired *t* test was used.

Results

Baseline risk factor profiles of the patient groups are given in Table 1. There were no significant differences in these parameters between groups. There was no difference between the treatment arms in the proportion of patients with ES detected on the preoperative recording. All patients tolerated the full dose of the drugs administered with no clinically apparent side effects and no hypotensive episodes, defined as a fall in mean arterial pressure of >10 mm Hg.

There were highly significant reductions in the number of postoperative ES in both the L-arginine and GSNO groups compared with the control group, as shown in Table 2. In the placebo control group, the median (range) number of ES detected per hour during the initial 4-hour postoperative recording period was 44.7 (6 to 778) (Table 2). At 6 hours, this number had fallen to 21.1 (0 to 188). By 24 hours, it had fallen to 5.1 (0 to 38). GSNO resulted in a 98% reduction in the median number of ES in the first 4 hours compared with

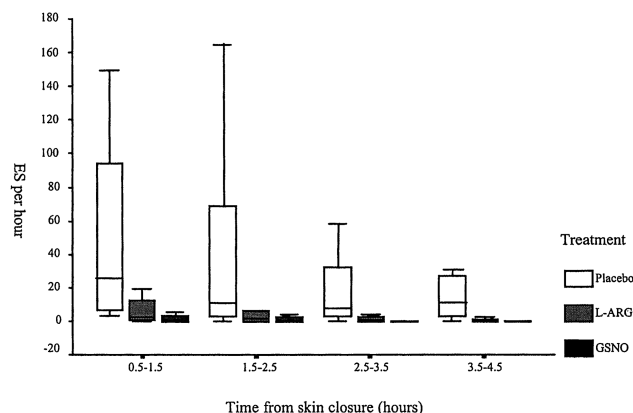


Figure 1. ES per hour vs time from skin closure according to treatment group. Solid line indicates median value; boxes, 25th to 75th percentile; and vertical lines, range.

a 79% reduction in the L-arginine-treated group. The breakdown of the first 4-hour period into hour-long segments is shown in Figure 1. Results for individual patients during the first postoperative hours are shown in Figure 2. The marked reduction in embolization persisted at both the 6- and 24-hour recording time points. There was no significant difference in the median (range) numbers of ES between the GSNO and L-arginine groups, although when the results from all the postoperative recordings were analyzed, there was a trend toward fewer ES with GSNO ($P=0.06$).

During the initial 4-hour postoperative recording, a total of 2502 ES were detected in the control group, compared with 529 in the L-arginine group and 44 in the GSNO group. Mean (SD) ES intensity was lower with GSNO [12.12 (2.38) dB] than with L-arginine [14.60 (4.41) dB, $P=0.002$] and was lower in both active arms than with placebo [16.07 (4.94) dB, $P<0.0001$ for both].

Five patients experienced perioperative ischemic events. Three of them had stroke within 24 hours after surgery in the ipsilateral internal carotid artery territory. The remaining 2 patients had ipsilateral transient ischemic attacks. There were no significant differences in frequency of stroke or transient ischemic attack between groups.

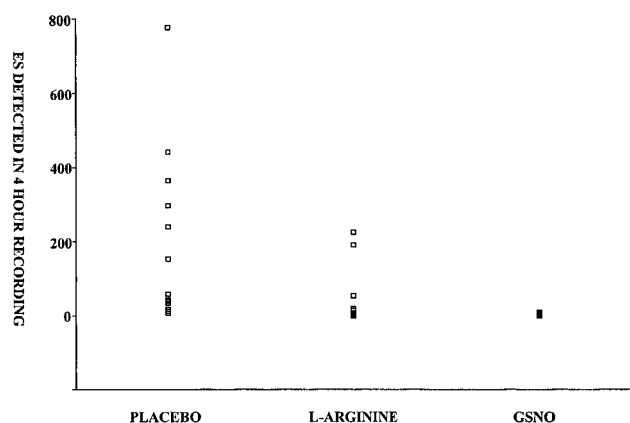


Figure 2. Scatter distribution of total ES detected in each patient during 4-hour postoperative recording according to treatment group.

Discussion

In this randomized double-blind placebo-controlled study, both L-arginine and GSNO, administered after surgery, resulted in a highly significant reduction in the frequency of ES after CEA in patients who were already treated with aspirin and heparin. Even though the drugs were administered for only 90 minutes, this reduction was maintained up to 24 hours after surgery. GSNO almost abolished ES, whereas L-arginine resulted in an $\approx 80\%$ reduction. All patients tolerated the full dose of the drugs with no clinically apparent side effects. ES intensity was lower in both active groups than in the control group. Assuming that embolus composition was similar in each group, our results are consistent with treatment resulting in smaller, as well as fewer, emboli.⁸ There was no significant difference between the L-arginine and GSNO groups, but there was a nonsignificant trend toward low rates of embolization with GSNO and lower ES mean intensity with GSNO.

Our data provide some of the first evidence that the antiplatelet effect of L-arginine has a therapeutic effect. Studies have demonstrated that both acute intravenous administration and chronic oral administration of L-arginine improve vascular function in patients with hypercholesterolemia,^{19,20} small-vessel disease,²¹ and stable angina pectoris²² and at sites of coronary artery stenosis.^{23,24} Previous *ex vivo* studies have shown that administration of L-arginine inhibits platelet aggregation in humans when given orally⁴ or intravenously,⁵ but its efficacy on thrombotic and thromboembolic clinical end points has not been demonstrated. Our study demonstrates that its acute administration resulted in a reduction in the frequency of ES in patients after CEA. A high frequency of ES in this setting has been shown to be an independent predictor of early postoperative stroke and risk of transient ischemic attack.¹¹ In addition, in patients with carotid stenosis, a number of studies have found the presence of asymptomatic ES to independently predict risk of future stroke.^{25,26}

Our study looked at the acute effect of a large dose of L-arginine in molar excess. Further studies are needed to determine whether oral supplementation with L-arginine also inhibits embolization. The oral route will be the only practical route if the drug is to have clinical use in the nonacute setting. Studies of vascular reactivity have demonstrated, however, that both oral and intravenous L-arginine can reverse impaired reactivity in patients with cardiovascular risk factors.^{19–24} We also looked at efficacy in only 1 setting, namely after CEA. During the procedure, endothelial denudation occurs, and this is a situation in which drugs acting on the NO system might be expected to be particularly effective. By use of the same technique of Doppler embolic signal detection, it should be possible to determine the effectiveness of L-arginine in other settings, such as active symptomatic carotid plaque and post carotid angioplasty.¹⁰

Intravenous administration of L-arginine at the same dose we gave (30 g) has been shown to result in a 10-fold increase in plasma L-arginine levels, and this was accompanied by a 32% inhibition of maximal platelet aggregation by ADP. Concurrent with this, platelet cGMP content increased by 43%, urinary cGMP increased by 65%, and urinary nitrate excretion increased by 78%. This is consistent with L-arginine

mediating its antiplatelet effect by enhancing NO formation and concomitantly, cGMP formation. Other methods in addition to an increased synthesis of NO due to increased availability of the substrate for NOS may play a role in the increased NO activity seen with L-arginine. Because intracellular levels of L-arginine exceed the K_m of NOS, administration of the substrate is unlikely to affect NO production.²⁷ Nevertheless, total cellular L-arginine concentration might not necessarily reflect the concentration in microdomains of the cell, for example, in the plasmalemmal caveolae, as the site of endothelial production of NO.²⁷ L-Arginine levels in endothelial cells can be modulated by the enzyme arginase, which degrades L-arginine to ornithine and urea.²⁸ It has been suggested that arginase, and not NOS, is the major pathway of L-arginine metabolism in endothelial cells.²⁹ Another possibility is that the vascular effects of L-arginine could be mediated by release of endogenous substances. L-Arginine stimulates insulin release from pancreatic β -cells. The hormone possesses vasoactive properties possibly mediated by endogenous NO release.³⁰ L-Arginine may also reverse the inhibitory effect of endogenous NOS antagonists, such as asymmetrical dimethyl arginine.³¹

In the present double-blind study, we have demonstrated that GSNO given after surgery markedly reduces the rate of embolization. In a previous study, GSNO given during surgery, starting from the induction of anesthesia and continuing until 2 hours after skin closure, reduced the rate of embolization, but some intraoperative hypotensive episodes were seen.¹⁵ These episodes might relate to GSNO itself, possibly interacting with anesthetic agents, but could be caused by other unrelated intraoperative events. No hypotensive episodes were seen in this study with postoperative administration. This platelet effect, despite a lack of effect on blood pressure, is consistent with GSNO having relative platelet specificity. Conventional NO donors given systemically in humans inhibit platelet aggregation, but only at doses that result in a hypotensive effect.³² Previous studies have also suggested a degree of platelet specificity. In human forearm studies, GSNO has significant antiplatelet action at doses that cause less hemodynamic effect than seen with conventional NO donors, when they are given at a dose resulting in a similar antiplatelet effect.¹⁴ We have previously shown that GSNO prevents platelet activation after coronary angioplasty, as determined by measurement of P-selectin and glycoprotein IIb/IIIa expression in coronary sinus blood.³³ GSNO also inhibits platelet activation in the setting of acute myocardial infarction and unstable angina, and hypotension observed with nitroglycerin was not seen in this study.³⁴ Although ex vivo platelet aggregation and flow cytometry studies provide an indication of antiplatelet activity, they are only indirect measures. No studies have assessed the effect of GSNO on such clinical end points as myocardial infarction and stroke. Our study provides further evidence of its effectiveness using a more clinically relevant surrogate end point, namely in vivo embolization. The effects we observed are likely to be due to an inhibition of platelet aggregation and adhesion. Inhibition of monocytes could also play a role, however, whereas an increase in local carotid flow might contribute to a reduction in platelet aggregate formation.

In summary, stroke in the immediate postoperative period is an important complication of carotid endarterectomy, and its pathogenesis is believed to be embolic in the majority of cases. Postoperative embolization continues despite conventional treatment with preoperative aspirin and intraoperative heparin. Treatment with both L-arginine and GSNO resulted in a marked reduction in this embolization. Such treatment approaches may reduce the postoperative stroke risk, although this now needs to be examined in larger studies using stroke as the end point. Our study also suggests that modulation of the NO system with these agents may have applications in the treatment of other types of thromboembolic disease.

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