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Reversal of cerebral vasospasm via intravenous sodium nitrite after subarachnoid hemorrhage in primates:

Laboratory investigation

Ali Reza Fathi, M.D.^{1,2}, Ryszard M. Pluta, M.D., Ph.D.¹, Kamran D. Bakhtian, M.S.¹, Meng Qi, M.D.^{1,3}, and Russell R. Lonser, M.D.¹

¹Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland ²Department of Neurosurgery, Kantonsspital Aarau AG, Aarau, Switzerland ³Department of Neurosurgery, Jinling Hospital, School of Medicine, Nanjing University, Jiangsu Province, China

Abstract

Object—Subarachnoid hemorrhage (SAH)-induced vasospasm is a significant underlying cause of aneurysm rupture-related morbidity and death. While long-term intravenous infusion of sodium nitrite (NaNO₂) can prevent cerebral vasospasm after SAH, it is not known if the intravenous administration of this compound can reverse established SAH-induced vasospasm. To determine if the intravenous infusion of NaNO₂ can reverse established vasospasm, the authors infused primates with the compound after SAH-induced vasospasm was established.

Methods—Subarachnoid hemorrhage–induced vasospasm was created in 14 cynomolgus macaques via subarachnoid implantation of a 5-ml blood clot. On Day 7 after clot implantation, animals were randomized to either control (saline infusion, 5 monkeys) or treatment groups (intravenous NaNO₂ infusion at 300 µg/kg/hr for 3 hours [7 monkeys] or 8 hours [2 monkeys]). Arteriographic vessel diameter was blindly analyzed to determine the degree of vasospasm before, during, and after treatment. Nitric oxide metabolites (nitrite, nitrate, and S-nitrosothiols) were measured in whole blood and CSF.

Results—Moderate-to-severe vasospasm was present in all animals before treatment (control, 36.2% ± 8.8% [mean ± SD]; treatment, 45.5% ± 12.5%; $p = 0.9$). While saline infusion did not reduce vasospasm, NaNO₂ infusion significantly reduced the degree of vasospasm (26.9% ± 7.6%; $p = 0.008$). Reversal of the vasospasm lasted more than 2 hours after cessation of the infusion and could be maintained with a prolonged infusion. Nitrite (peak value, 3.7 ± 2.1 µmol/L), nitrate (18.2 ± 5.3 µmol/L), and S-nitrosothiols (33.4 ± 11.4 nmol/L) increased significantly in whole blood, and nitrite increased significantly in CSF.

Address correspondence to: Russell R. Lonser, M.D., Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 3D20, Bethesda, Maryland 20892-1414. lonser@ninds.nih.gov.

Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: Lonser, Fathi, Pluta. Acquisition of data: Fathi, Pluta, Bakhtian, Qi. Analysis and interpretation of data: Lonser, Fathi, Pluta, Bakhtian. Drafting the article: Fathi, Pluta. Critically revising the article: Lonser, Pluta, Bakhtian, Qi. Statistical analysis: Fathi, Pluta, Bakhtian. Administrative/technical/material support: Lonser, Pluta. Study supervision: Lonser, Pluta.

Conclusions—These findings indicate that the intravenous infusion of NaNO₂ can reverse SAH-induced vasospasm in primates. Further, these findings indicate that a similar treatment paradigm could be useful in reversing cerebral vasospasm after aneurysmal SAH.

Keywords

nitric oxide; sodium nitrite; subarachnoid hemorrhage; vasospasm; traumatic brain injury

There are approximately 27,000 new cases of aneurysm-related SAH every year in the US (10 cases/100,000 persons). Between 30% and 60% of patients who survive the initial aneurysm rupture will go on to experience vasospasm of the cerebral vasculature between Days 3 and 12 post-SAH.³¹ Despite optimized surgical and medical treatment, approximately 30% of these patients will suffer major neurological deficits^{4,13} related to vasospasm, with a mortality rate over 25% by 6 months after aneurysm rupture.¹⁶ Because of the lack of effective treatment paradigms, we need new therapeutic options that target the underlying pathophysiology of SAH-related cerebral vasospasm after aneurysm rupture and reverse its effects.

Emerging evidence indicates that NO plays a critical role in cerebral vasospasm development.^{19,28} Specifically, the reduced availability of endo- and perivascular NO has been shown to directly contribute to vasospasm development after aneurysm rupture.²¹ Nitric oxide replacement therapy using classic donors (sodium nitroprusside and nitroglycerin) in the experimental setting has been shown to prevent vasospasm. However, the high rate of significant adverse effects (significant drop in blood pressure, drug tolerance, and rebound phenomenon) associated with these agents has prevented their effective clinical use,²⁷ leading to the investigation of new NO donors and treatment paradigms.

The clinical and pharmacological properties of NaNO₂ indicate that it may be an ideal NO donor for the treatment of a variety of conditions responsive to NO replacement.² Recently, the intravenous infusion of NaNO₂ has been used to effectively dilate peripheral vasculature in the presence of deoxygenated hemoglobin.⁵ Further, recent data have shown that the infusion of NaNO₂ can be performed safely for prolonged periods without the side effects associated with other NO donors.^{19,20} While intravenous NaNO₂ can effectively prevent SAH-induced cerebral vasospasm in primates,¹⁹ it is not known if it can reverse established SAH-associated vasospasm.

To determine if intravenous NaNO₂ can reverse established cerebral vasospasm, we investigated the intravenous infusion of NaNO₂ for the treatment of established SAH-associated cerebral vasospasm in primates.

Methods

Animal Population

Fourteen cynomolgus macaques (3–12 kg) were included in the study. All animal experiments were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke at the NIH.

Anesthesia Protocol

General anesthesia was induced by the injection of ketamine (10 mg/kg) and xylazine (1 mg/kg) followed by tracheal intubation. Anesthesia was maintained by inhaled isoflurane (0.5%–1.0%).¹⁸ Blood pressure, heart rate, rectal body temperature, and end-tidal CO₂ were continuously monitored during anesthesia and drug infusion. Sodium thiopental (25 mg/kg) and cefazolin (500 mg) were injected at the beginning of cranial surgery.

Arteriographic Studies

For digital subtraction arteriography the right femoral artery was exposed and a 3-Fr catheter including guide-wire (F3 polyethylene, Cook Group Co.) was introduced into the right carotid artery under fluoroscopic guidance. One milliliter of contrast medium (Isovue-300, Bracco Diagnostics, Inc.) was injected, and arteriograms were acquired at a rate of 8 images per second. A ruler with a millimeter scale was placed at a standardized position by the head of each animal and was used for the calibration of every image. Three blinded examiners measured the area of the proximal 14 mm of the right MCA by using digital image analysis software (NIH Image J) 3 times, and the mean value was used for statistical analysis. The percent reduction in the ratio of the area measured on the serial arteriograms was compared with the ratio computed from the baseline arteriogram, defining the degree of vasospasm by the equation, diameter = area/length.

Subarachnoid Hemorrhage Clot Placement

The SAH model is described in detail elsewhere.^{7,19} Briefly, after creating a right frontotemporal craniectomy and opening the dura mater, the arachnoid of the sylvian fissure was dissected from the proximal 14 mm of the right MCA, distal internal carotid artery, and proximal anterior cerebral artery. Autologous blood was collected from the left femoral artery and allowed to clot for approximately 15 minutes. Five milliliters of clot was placed around the exposed arteries. After closing the dura and the wound, animals were returned to their cages for monitoring of clinical parameters including neurological deficits and postoperative course.

Sodium Nitrite

The NaNO₂ was obtained from the NIH Pharmacy (Clinical Center at the NIH), dissolved in 15 ml of phosphate-buffered saline, and filtered through a 0.2- μ m filter. The treatment dose was derived from the maximum tolerated dose in a healthy human volunteer phase I safety and toxicity study.²⁰

Experimental Design

Animals underwent arteriography 2 weeks before SAH to assess the baseline diameter of cerebral arteries and on post-SAH Day 7 to confirm the presence of cerebral vasospasm. Once cerebral vasospasm was confirmed, animals were randomly allocated to 2 groups (control and treatment), and the intravenous infusion of saline (control, 3.3 ml/hr) or NaNO₂ (treatment, 3.3 ml/hr [300 μ g/kg/hr]) was started immediately. The duration of the NaNO₂ infusion was either 3 hours (7 monkeys) or 8 hours (2 monkeys). Additional arteriograms were obtained immediately upon cessation of the infusion (14 animals) and at 2 (12

animals), 4 (1 animal), 6 (1 animal), and 8 hours (2 animals) after ceasing the infusion. Animals undergoing 8-hour NaNO₂ infusions had arteriograms at 3, 5, and 8 hours during the infusion.

Venous blood samples (1 ml) were collected at the time of craniectomy (14 animals); at the beginning of the infusion (14 animals); at 1.5 (14 animals) and 3 hours (14 animals) during the infusion; and at 2 (7 animals), 4 (2 animals), and 8 hours (2 animals) after ceasing the infusion. Both animals with prolonged infusions had additional blood collections at 5, 6.5, and 8 hours during the infusions. Cerebrospinal fluid samples were collected from the basal cisterns at the time of surgery and via sub-occipital puncture after the final arteriogram.

Animals were euthanized with an intravenous overdose of pentobarbital (90 mg/kg) while under general anesthesia and after the completion of arteriography and blood and CSF collections.

Nitric Oxide Metabolome Measurements

Two hundred fifty microliters of NO₂-stabilizing solution¹⁵ was added to the blood samples immediately after collection to stabilize the NO metabolome. Levels of NO₂, NO₃, and SNOs in whole blood and CSF were measured using a chemiluminescence technique (model 280i NO analyzer, Sievers Instruments), as described by MacArthur et al.¹⁵ Whole blood samples were treated with a 1:1 volume of ice-cold methanol and then were processed in a centrifuge at 4°C and 17,900 G for 5 minutes. Both the NO₂ and SNO concentrations were measured in acidic tri-iodide (I₃⁻) reagent. Forty microliters of supernatant was injected into the purge vessel for NO₂ measurement. To identify NO₂ versus SNO, samples were incubated with and without acidified sulfanilamide, and 200 µl was injected into the I₃⁻ reductant. The NO₃ was measured by reduction in 10 µl of vanadium (III) at 90°C. Cerebrospinal fluid supernatant was thawed and then processed in a centrifuge at 4°C and 17,900 G for 5 minutes, and pure supernatant was analyzed for NO₂, NO₃, and SNOs.

Statistical Analysis

All data were recorded in Excel (Microsoft Corp.), and statistical tests were computed using JMP software (SAS, Inc.). The degree of vasospasm, concentration levels of the NO metabolomes, and physiological parameters were assessed using 2-tailed t-tests and analysis of variance followed by 1-way ANOVA. Statistical significance was defined as $p < 0.05$. Values are expressed as the means $\pm$ SDs, unless indicated otherwise.

Results

Clinical Findings

Neurological deficits developed in none of the primates after subarachnoid MCA clot placement. Arteriography and intravenous infusion of saline (5 animals) or NaNO₂ (9 animals) were well tolerated in all of the animals. Blood pressure, heart rate, respiratory rate, body temperature, and end-tidal CO₂ remained unaffected in the saline and NaNO₂ groups over the 3- (Fig. 1) and 8-hour infusions.

Angiographic Findings

Moderate-to-severe vasospasm of the right MCA was confirmed in all 14 primates on Day 7 post-SAH (mean reduction in artery diameter, $42.17\% \pm 11.9\%$; $p < 0.005$; Fig. 2 upper) without significant difference between control ($36.2\% \pm 8.8\%$) and treatment groups ($45.5\% \pm 12.5\%$; $p > 0.05$). After 3 hours of NaNO_2 infusion, the degree of vasospasm in the right MCA was significantly reduced to $26.9\% \pm 7.6\%$ (mean increase in vessel diameter, $18.6\% \pm 11.5\%$; $p < 0.005$), while it remained unchanged after saline infusion ($37.1\% \pm 10.5\%$, 5 animals; $p > 0.05$). Two hours after cessation of the NaNO_2 infusion, the reduction in vasospasm persisted (mean increase in vessel diameter, $17.8\% \pm 10.9\%$; $p < 0.05$; Fig. 3). The NaNO_2 -induced vasodilation lasted 4 hours after cessation of the infusion (1 animal; Fig. 2 upper), but at 6 and 8 hours the vessels respasmed (mean reduction in artery diameter, $38.4\% \pm 8.3\%$, 2 animals). Continuous NaNO_2 infusion over 8 hours maintained the significantly reduced degree of vasospasm (mean reduction in artery diameter, $32.3\% \pm 5.3\%$ compared with $56.8\% \pm 23.1\%$ after SAH; mean increase in artery diameter, $24.5\% \pm 17.8\%$; Fig. 2 lower).

Metabolome Findings

Blood—Whole blood NO_2 , NO_3 , and SNO baseline levels on Day 7 after SAH did not differ between the control and treatment groups ($p > 0.05$). They remained unchanged during saline infusion (5 animals) and were significantly increased with NaNO_2 infusion. The NO_2 concentration increased 90 minutes after NaNO_2 infusion ($p < 0.05$) and peaked at 180 minutes at $3.7 \pm 2.1 \mu\text{mol/L}$ (7 animals; $p < 0.05$). Two hours after ceasing the NaNO_2 infusion, the NO_2 levels returned to baseline (7 animals; Fig. 4A) and remained unchanged at 4 and 8 hours (2 animals). The NO_3 levels increased after 90 minutes of infusion ($p < 0.05$) and peaked at 180 minutes at $18.2 \pm 5.3 \mu\text{mol/L}$ (7 animals; $p < 0.05$). The NO_3 levels then remained elevated 2 hours after ceasing the infusion ($p < 0.002$) and returned to baseline ($7.1 \pm 1.3 \mu\text{mol/L}$) at 4 and 8 hours (2 animals; Fig. 4B). The SNO concentrations increased after 90 minutes of infusion ($p < 0.05$) and reached a peak (7 animals; $33.4 \pm 11.4 \text{ nmol/L}$) after 180 minutes. Within 2 hours after ceasing the infusion, SNO levels returned to baseline (7 animals) and remained unchanged after 4 and 8 hours (2 animals; Fig. 4C). During the 8-hour NaNO_2 infusions, the concentration of all 3 metabolites remained elevated.

Cerebrospinal Fluid—Control and treatment groups showed no significant difference in CSF concentrations of NO_2 , NO_3 , and SNO at baseline ($p > 0.05$). On SAH Day 7, the NO_2 levels were significantly decreased ($p < 0.05$), and the NO_3 and SNO levels remained unchanged in the control group as compared with pre-SAH levels. The NO_2 levels in the 3-hour NaNO_2 infusion group with short observation periods increased significantly compared with the control group (from 1.7 ± 0.4 to $4.9 \pm 2.1 \mu\text{mol/L}$; $p < 0.005$; Fig. 5A), but the NO_3 levels in the same group were not significantly changed compared with controls. The NO_3 levels in CSF were significantly decreased in the treatment group (from 23.8 ± 6.2 to $7.5 \pm 4.4 \mu\text{mol/L}$; $p < 0.05$; Fig. 5B). The differences between SNO levels before and after treatment were not significant in either group ($p > 0.05$; Fig. 5C). All 3 metabolites increased in the CSF of the 2 primates that underwent prolonged 8-hour NaNO_2 infusions compared with the CSF concentrations at the time of SAH.

Discussion

Methods for Reversing Vasospasm

Currently available methods for reversing cerebral vasospasm after SAH include balloon angioplasty and/or the direct intraarterial administration of papaverine/nicardipine to the affected arteries, in addition to hemo-dynamic therapy.⁸ While these methods have been shown to reverse established cerebral vasospasm, the effects are often transient and/or ineffective.^{22,23} Balloon angioplasty has size limitations with regard to dilating smaller distal arteries, while arteries may be impossible to access due to the angioarchitecture in the distal and posterior circulation. Further, intraarterial papaverine infusion for established vasospasm does not improve outcome due to its transient effects.^{23,29} Moreover, complications, including thromboembolism, vessel rupture, reperfusion hemorrhage, arterial dissection, and bleeding from untreated aneurysms, have been associated with angioplasty (5%) and intraarterial papaverine infusion (9.9%).^{10,32} Additionally, papaverine infusions can result in an uncontrolled increase in intracranial pressure related to nonselective intracranial vasodilation and increased capacitance of the venous bed.³

Clinical Implications of NaNO₂ Infusion

Long-lasting intravenous infusion of NaNO₂ prevents vasospasm in a primate model.¹⁹ However, the prevention of vasospasm is of value only if patients are admitted before its onset and if NaNO₂ infusion is initiated at that time. As current methods for reversing vasospasm carry high risks and reduced effectiveness and can require significant logistical efforts, effective, safe, and simplified treatment options are needed. In this study, we have shown that the intravenous infusion of NaNO₂ at a dose of 300 µg/kg/hr (adapted from the maximum tolerated dose in a healthy human volunteer study)²⁰ is safe and reverses SAH-induced cerebral vasospasm in primates. The intravenous route of NaNO₂ administration simplifies treatment delivery compared with the available intraarterial endovascular treatment options.

The nearly 20% dilation of the SAH-induced spastic arteries during NaNO₂ infusion results in hemodynamic changes that may improve cerebral perfusion. It has been shown that vessel diameter has a strong correlation with cerebral blood flow.^{1,9} Using the Hagen-Poiseuille equation as an approximate estimation for flow, in which flow increases to the fourth power of the vessel diameter, a 70% increase in blood flow could result from the increase in arterial diameter (18.6%) seen in these primates. Nevertheless, cerebral blood flow is dependent on various factors that can be broadly divided into those affecting cerebral perfusion pressure and those affecting the radius of cerebral blood vessels. To further define cerebral blood flow, cerebral blood volume, and intracranial pressure changes related to NaNO₂, monitoring of these measures could be considered in future studies.

Prolonged observation in this study indicated that the therapeutic effect lasts for the duration of the infusion and approximately 4 hours after cessation of the infusion. Continuous infusion over 8 hours in 2 animals indicated that the therapeutic effect of NaNO₂ can be sustained if the infusion is not discontinued. Because it is safe and feasible to infuse NaNO₂

for prolonged periods of time (days),²⁰ continuous NaNO₂ infusion during spasms is possible and could provide continued therapeutic effect.

All animals tolerated the intravenous infusion of NaNO₂ well. We did not observe any relevant changes in heart rate, blood pressure, or respiratory rate in the primates under general anesthesia during the intravenous infusions of saline or NaNO₂. Previous studies in non-human primates¹⁹ and healthy human volunteers²⁰ using the same dose infused in this study, but over a much longer period, did not result in clinically relevant methemoglobinemia, another potential adverse effect of systemic NaNO₂. These findings indicate that intravenous NaNO₂ can be infused safely at clinically relevant doses.

Mechanism of NaNO₂ Effect

Deprivation of NO in the vicinity of brain vasculature has been established as a cause of cerebral vasospasm after SAH.^{12,21} The underlying mechanism includes endothelial NO synthase dysfunction,^{11,30} reduction of neuronal NO synthase, and scavenging of NO by hemoglobin.^{17,21} Sodium nitroprusside and nitroglycerin are NO donors that have been studied clinically and have shown limited success.^{24,25} Major drawbacks with both agents were also nonselective vasodilation, short-lasting effects with rebound phenomenon, drug tolerance, and side effects including systemic hypotension, nausea, vomiting, and increased intracranial pressure. The chemical properties and adverse effects of both drugs have excluded them from routine clinical application and have led to discoveries of new classes of NO donors like NONO-ates (chemical compounds that spontaneously release NO when exposed to normal pH fluid) and NaNO₂.¹⁴ Recently, the prolonged intravenous infusion of NaNO₂ has been shown to be a potent peripheral vasodilator via the release of NO in the presence of deoxygenated hemoglobin.⁵

Pharmacodynamic changes in NaNO₂ in the circulation can be measured by changes of the metabolome. The metabolome represents the complement of all the small-molecular-weight metabolites inside a sample of interest. The metabolome analyzed in the current study describes the dynamic changes in metabolites over short time lapses (seconds) during pharmacodynamic conversion of NaNO₂ to NO. Specifically, increased whole blood NO₂ concentration during infusion confirms the bioavailability of intravenous NaNO₂. To determine if NO was released from NaNO₂, we measured NO₃ and SNO concentrations in whole blood. Nitric oxide reacts with both oxyhemoglobin and deoxyhemoglobin, forming NO₃ in a nearly diffusion-limited reaction. Nitric oxide also reacts with intravascular circulating thiols forming SNO. Consistent with the reduction of NO₂ to NO, both NO₃ and SNO levels in whole blood were increased during infusion.^{2,6} Further, as verified in the CSF of these animals as well as in other studies, decreased NO₂ CSF levels after SAH reveal the important role that NO replacement therapy plays in treating vasospasm after SAH.^{12,19,26} The significant increase in CSF NO₂ concentration in the treatment group also confirms that intravenously delivered NaNO₂ penetrates into the CSF and that the NO₂ deficit after SAH can be reversed by NaNO₂ administration.

Sodium nitrite has a unique, selective, and local mechanism of NO release as compared with traditional NO donors because NO is released from NaNO₂ under acidic and hypoxic conditions in the presence of deoxyhemoglobin, acting in a local fashion as an on-demand

NO₂ reductase.² The conversion of NO₂ to NO and SNO is mediated by the reaction with deoxyhemoglobin. This reaction provides a means for hypoxia-regulated production of NO by erythrocytes, tissue heme proteins, and endothelium.² These conditions are present locally in the spastic vessels after SAH. Sodium nitrite infusions in healthy humans did not produce any clinical signs of increased intracranial pressure, such as headache or nausea, consistent with the lack of dilation in normal cerebral vasculature.²⁰ However, whether NaNO₂ is acting selectively on spastic intracranial vessels must be further confirmed in patients with SAH in future studies.

Potential Study Limitations

While the current study data provide direct insight into the potential therapeutic effects of NaNO₂, there are potential limitations of this animal model of SAH. Animal models do not reflect the entire clinical condition of patients with aneurysmal SAH. Cardiac dysfunction, hydrocephalus, and increased intracranial pressure, which can occur in patients with SAH, are not reflected in this model. Further, combinations of other pharmaceutical agents in patients with SAH, such as calcium channel blockers, could affect NaNO₂ pharmacodynamics and may require further investigation.

Conclusions

Intravenous infusion of NaNO₂ is safe and effective for reversing established cerebral vasospasm after SAH in a primate model. These findings indicate that this treatment strategy may be useful in reversing cerebral vasospasm after aneurysmal SAH.

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Abbreviations used in this paper

MCA	middle cerebral artery
NaNO₂	sodium nitrite
NIH	National Institutes of Health
NO	nitric oxide
NO₂	nitrite
NO₃	nitrate
SAH	subarachnoid hemorrhage
SNO	S-nitrosothiol.

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Fig. 1.

Graph demonstrating physiological parameters. There were no differences in mean arterial blood pressure ([MABP] 57.6 ± 13.3 mm Hg), mean heart rate ([HR] 147.6 ± 21.55 beats/minute), and mean respiratory rate ([RR] 30.3 ± 5.9 breaths/minute) during infusion between the control and treatment groups ($p > 0.05$).

Fig. 2.

Upper: Bar graph demonstrating the degree of vasospasm (%) of the right MCA on the baseline arteriogram (anteroposterior views) before and after the infusion of saline (gray bars) or NaNO₂ (black bars). Middle cerebral artery diameter on Day 7 post-SAH was not significantly different between the control and treatment groups. Saline did not affect the degree of vasospasm, whereas NaNO₂ infusion increased the diameter of the spastic vessel by 18.6%. Follow-up arteriograms after infusion cessation revealed that the therapeutic effect persisted for 4 hours. Vessel spasm returned after 6 (1 animal) and 8 hours (2

animals). **Lower:** Graph depicting arteriographic results of 2 animals with prolonged 8-hour NaNO₂ infusions. The average degree of vasospasm was higher in these animals than in the 3-hour infusion group. At 8 hours, the MCA was still dilated in comparison with the baseline diameter after SAH (mean increase in artery diameter, $24.5\% \pm 17.8\%$). n = number of animals; ns = not significant.

Fig. 3.

Representative arteriograms, anteroposterior views, of the right cerebral arteries of monkeys treated intravenously with NaNO_2 . **A:** Baseline arteriogram before SAH clot placement. **B:** Arteriogram on Day 7 after SAH and before infusion of NaNO_2 showing severe vasospasm of the right MCA. **C:** After 3 hours of continuous intravenous NaNO_2 infusion, the degree of vasospasm was significantly decreased. **D:** Two hours after ceasing the infusion, the therapeutic effect persisted. **E:** Eight hours after infusion, the spasm of the right MCA returned. *Arrows* indicate the right MCA.

Fig. 4.

Graphs demonstrating NO metabolome concentrations in whole blood. **A:** Significant increase in NO₂ demonstrates bioavailability of NaNO₂ after 3-hour intravenous infusion. **B:** Elevated NO₃ demonstrates hemoglobin-dependent oxidation of NO₂. **C:** An increase in SNOs indicates the release of NO from NO₂. In the 2 animals with continuous 8-hour infusions (*dotted line*), the NO metabolome remains elevated throughout the entire infusion. n = number of animals.

Fig. 5.

Bar graphs showing NO metabolome concentrations in CSF. **A:** Nitrite is significantly reduced after SAH in the control group. The significant increase in CSF NO₂ in the treatment group suggests penetration of NO₂ through the blood-CSF barrier. **B:** Nitrate. **C:** S-nitrosothiols. n = number of animals.