Pharmacologically Augmented S-Nitrosylated Hemoglobin Improves Recovery From Murine Subarachnoid Hemorrhage

Huaxin Sheng, MD; James D. Reynolds, PhD; Richard L. Auten, MD; Ivan T. Demchenko, PhD; Claude A. Piantadosi, MD; Jonathan S. Stamler, MD; David S. Warner, MD

Background and Purpose—S-nitrosylated hemoglobin (S-nitrosohemoglobin) has been implicated in the delivery of O₂ to tissues through the regulation of microvascular blood flow. This study tested the hypothesis that enhancement of S-nitrosylated hemoglobin by ethyl nitrite inhalation improves outcome after experimental subarachnoid hemorrhage (SAH).

Methods—A preliminary dosing study identified 20 ppm ethyl nitrite as a concentration that produced a 4-fold increase in S-nitrosylated hemoglobin concentration with no increase in methemoglobin. Mice were subjected to endovascular perforation of the right anterior cerebral artery and were treated with 20 ppm ethyl nitrite in air, or air alone for 72 hours, after which neurologic function, cerebral vessel diameter, brain water content, cortical tissue PO₂, and parenchymal red blood cell flow velocity were measured.

Results—At 72 hours after hemorrhage, air- and ethyl nitrite–exposed mice had similarly sized blood clots. Ethyl nitrite improved neurologic score and rotarod performance; abated SAH-induced constrictions in the ipsilateral anterior, middle cerebral, and internal carotid arteries; and prevented an increase in ipsilateral brain water content. Ethyl nitrite inhalation increased red blood cell flow velocity and cortical tissue PO₂ in the ipsilateral cortex with no effect on systemic blood pressure.

Conclusions—Targeted S-nitrosylation of hemoglobin improved outcome parameters, including vessel diameter, tissue blood flow, cortical tissue PO₂, and neurologic function in a murine SAH model. Augmenting endogenous PO₂-dependent delivery of NO bioactivity to selectively dilate the compromised cerebral vasculature has significant clinical potential in the treatment of SAH. (Stroke. 2011;42:471-476.)

Key Words: brain ■ mouse ■ subarachnoid hemorrhage ■ S-nitrosylated hemoglobin ■ ethyl nitrite

Red blood cells (RBCs) regulate tissue O₂ delivery by using hemoglobin (Hb) as both an O₂ sensor and a transducer of NO vasodilator activity to match local tissue blood flow to that region’s O₂ requirements. Impairment of this microcirculatory interrelationship may occur in pathophysiologic conditions, including subarachnoid hemorrhage (SAH).

After SAH, delayed narrowing of vessels impairs delivery of O₂ and nutrients to brain tissue. This delayed arteriopathy is due, at least in part, to local disruption of NO bioactivity. Addressing this disruption is problematic. Systemic administration of nonspecific vasoactive agents has shown limited efficacy due to dose-limiting arterial hypotension. Central (directed) administration of NO donors has been reported to be beneficial in some, but not other, applications and is complicated by the need for invasive access. Nonetheless, affected cerebral vessels appear to maintain vasoreactivity, so a different course of action may be augmentation of the body’s innate ability to selectively increase blood flow to areas of low O₂ tension.

Increasing the circulating pool of physiologic NO bioactivity (that is, S-nitrosothiols, including S-nitrosylated hemoglobin, SNO-Hb) could selectively improve flow to focal ischemic brain tissue without altering flow to other tissue beds. This does not involve the generation of free NO, which is rapidly metabolized by Hb. Instead, hypoxic vasodilation results from a series of transnitrosylation reactions when NO bioactivity is released by the RBC. Accumulating evidence suggests that a small S-nitrosothiol, S-nitrosoglutathione, which is derived from RBC SNO-Hb, subserves hypoxic regulation of O₂ delivery.

The goal of the present study was to test the hypothesis that augmentation of SNO-Hb improves outcome after exper-
mental SAH. We reasoned that increased vessel diameter and enhancement of cortical tissue \( \text{PO}_{2} \) (tPO2) in the affected cortex would be reflected in improved neurologic outcome. The experiments were conducted in mice and utilized ethyl nitrite (ENO), a selective nitrosylating agent that preferentially forms SNO-Hb\(^{12,13} \) and other nitrosylated thiols on exposure to blood (the other reaction product is ethanol). ENO has not previously been tested as a therapy for a focal pathologic condition such as SAH, but it has shown benefit in a disparate collection of disorders characterized by disruptions in \( \text{O}_{2} \) delivery, including pulmonary hypertension\(^{13} \) and laparoscopic surgery.\(^{14} \)

Materials and Methods

The Duke University institutional animal care and use committee approved all aspects of the study design. Experiments were conducted on male C57Bl/6J mice (20 to 25 g; The Jackson Laboratory, Bar Harbor, ME). Gas exposures occurred within a 5.5-L acrylic box kept at room temperature and normal atmospheric pressure. ENO (Custom Gas Solutions, Durham, NC) was blended with \( \text{N}_{2} \) to the desired concentration at the time of delivery. In this SAH model, cerebral vasospasm has been reported to peak 72 hours after SAH.\(^{18} \) We therefore examined mice at this recovery interval in the following studies.

Dose Finding and NO Measurements

Mice (n=5 per group) were exposed to ENO (0, 20, 40, or 80 ppm) in air for 72 hours. Mice were then anesthetized, and arterial blood (0.5 mL) was sampled via cardiac puncture for determination of total \( \text{Hb} \) and methemoglobin with a Gem Premier 3000 co-oximeter (Instrumentation Laboratory, Lexington, MA). In a separate set of mice, the 72-hour 20-ppm ENO exposure was repeated to measure final blood SNO-Hb and RBC NO concentrations (that is, FeNO and SNO) by Hg-coupled photolysis/chemiluminescence.\(^{16,17} \) On the basis of the methemoglobin and SNO-Hb values obtained from these experiments and evidence that 10 ppm ENO attenuates lipopolysaccharide-induced lung inflammation,\(^{18} \) we elected to further study 20 ppm (0.002% atm) ENO.

Post-SAH Neurologic Function, Vessel Diameter, and Brain Edema

The following experiments were conducted with animals randomly assigned to experimental groups. SAH was induced in isoflurane-anesthetized, mechanically ventilated mice by endovascular perforation of the anterior cerebral artery (ACA) with a 5-0 nylon monofilm suture, according to previously reported procedures.\(^{19,20} \) Pericranial temperature was maintained at 37.0 ±0.2°C. After ACA perforation, the mice were awakened. At 60 minutes after SAH, mice were moved to the exposure chamber for 72 hours. Fresh gas inflow was 21% \( \text{O}_{2} \) with (n=20) or without (n=23) 20 ppm ENO, balanced with \( \text{N}_{2} \). In a separate experiment, sham mice were subjected to all procedures (except ACA perforation). Mice were then anesthetized with orotracheal isoflurane and mechanically ventilated with 30% \( \text{O}_{2} \) balance \( \text{N}_{2} \). A femoral artery was cannulated. A transcranial laser Doppler flow (LDF) probe was positioned over the ventral cerebral cortex. Mean arterial pressure (MAP) and LDF were continuously measured for a 30-minute baseline interval and for 60 minutes after onset of ENO (20 ppm) treatment.

Acute Effects of ENO on Brain tPO\(_2\) 72 Hours After SAH

Brain tPO\(_2\) was measured according to a previously described polarographic method.\(^{24} \) Mice were exposed to SAH or sham surgery (n=6 per group) and treated with air during 72 hours of recovery. Mice were then anesthetized with 1% isoflurane in 30% \( \text{O}_{2} \) balance \( \text{N}_{2} \). A femoral artery was cannulated. A NaCl reference electrode was clipped to the tail, and a 10-\( \mu \)m-diameter Pt microelectrode was inserted 1 mm into the cortex. The system was calibrated in artificial cerebrospinal fluid at 37°C equilibrated with 0, 8, or 21% \( \text{O}_{2} \). MAP, LDF, and tPO\(_2\) were recorded every 5 minutes. After 30 minutes of stabilization, 20 ppm ENO was added to the inspired gas mixture. Measurements continued for 60 minutes. Arterial blood gas/glucose values were then measured.

Statistical Analysis

Power calculations with this model indicated that 20 mice per experimental group would have an 80% power to detect a >30% difference in MCA diameter.\(^{20} \) Nonparametric values (neurologic score, hemorrhage size, and tissue optical density) were compared by the Mann–Whitney \( U \) statistic and are reported as median ±interquartile range. Physiologic values, rotted latency, vessel diameter, and brain water content were compared with the unpaired Student’s \( t \) test. The paired Student’s \( t \) test was used to compare baseline values of MAP, LDF, and tPO\(_2\) versus those at 60 minutes after ENO exposure onset. Methemoglobin and Hb concentrations were compared by 1-way ANOVA. Parametric values are reported as mean±SD.

Results

Blood Parameters

There were no differences among groups for total Hb (0 ppm=14±1, 20 ppm=15±1, 40 ppm=14±1, 80 ppm=14±1

Time-Dependent ENO Effects on Cortical Blood Flow Velocity and Systemic Blood Pressure

Mice were subjected to SAH and exposed to air for 24 or 72 hours or to sham surgery (all procedures except ACA perforation). Mice (n=3 per group) were then anesthetized with orotracheal isoflurane and mechanically ventilated with 30% \( \text{O}_{2} \) balance \( \text{N}_{2} \). A femoral artery was cannulated. A transcranial laser Doppler flow (LDF) probe was positioned over the ventral cerebral cortex. Mean arterial pressure (MAP) and LDF were continuously measured for a 30-minute baseline interval and for 60 minutes after onset of ENO (20 ppm) treatment.

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g/dL; $P=0.08$). Methemoglobin was increased by 40 and 80 ppm ENO (0 ppm $P=0.08$, 20 ppm $P=0.2$, 40 ppm $P=0.2$, 80 ppm $P=0.12$; main effect $P<0.001$). ENO (20 ppm) increased SNO-Hb and total RBC NO (Figure 1).

**Post-SAH Neurologic Function and Vessel Diameter**

Three SAH mice died during the 72-hour recovery interval (1 from the ENO group and 2 from the air-only group). This was likely due to intracranial hypertension. There was no intergroup difference in body weight change from baseline (SAH-air $P=0.25$, ENO $P=0.25$). SAH grades were similar (Air=$4.36$, SAH-ENO=$2.11$, $P=0.25$). SAH was grade 4. All mice were treated with either air (n=21) or 20 ppm ENO (n=19) for 72 hours after SAH. ENO improved neurologic score (A) ($P=0.009$) and rotarod latency to fail (B) ($P=0.003$). Open circles indicate individual mouse values. Horizontal bars indicate median values for neurologic score and mean values for rotarod. There was no effect of ENO on sham (n=5) performance ($P=0.25$, respectively).

ENO improved 72-hour post-SAH neurologic scores ($P=0.009$, Figure 2A) and rotarod performance ($P=0.003$, Figure 2B). Right ACA, MCA, and ICA diameters were greater in the ENO group ($P=0.003$, $P=0.04$, $P=0.0001$). Open circles indicate individual mouse values. Horizontal bars indicate mean values. There was no effect of ENO on vessel diameters in shams ($P=0.85$).

**Edema, LDF, MAP, and Cortical tPO2**

ENO decreased cerebral edema in the hemisphere ipsilateral to the hemorrhage (Figure 4). In anesthetized shams, 20 ppm ENO inhalation did not alter MAP or LDF (Figure 5A). Mice were allowed to survive 24 or 72 hours after SAH in air. At 24 hours, the ENO LDF response was episodic, indicating an unstable effect of ENO at this stage of disease progression (Figure 5B). Although MAP remained constant, acute ENO administration...
rapidly and consistently improved LDF at 72 hours (Figure 5C), indicating that the postinjury vasculature can still respond to an increase in SNO-Hb. Another set of SAH and sham mice recovered for 72 hours in air. Acute ENO inhalation increased both LDF and cortical tPO₂ (Figure 6), with no effect on MAP. At completion of the experiment, pH=7.36±0.07, PaCO₂=35±7 mm Hg, PaO₂=163±34 mm Hg, and glucose=169±49 mg/dL.

Discussion

Inhaled ENO improved neurologic deficits attributable to experimental SAH. This was associated with greater vessel diameter, decreased brain edema, and improved LDF and tPO₂, but ENO had no effect on MAP. ENO increases tissue oxygenation selectively in the ischemic brain and suggests that SNO-Hb provides a route to regulate microvascular blood flow.

The release of NO bioactivity from SNO-Hb is regulated allosterically by O₂ saturation: NO bioactivity is liberated preferentially in environments that favor O₂ offloading. Decreased tPO₂ occurs in clinical SAH. After SAH, tPO₂ values observed in this experiment were increased to a viable range by ENO (for example, 32±10 mm Hg). A likely
The NO moiety from solvent.\(^1\) In the deoxy state, the cysteine \(-\text{nitrosylated}\) and causes the \(\text{SNO-Hb}\) concentrations.

Clinically relevant attribute of pharmacologically increased \(\text{SNO-Hb}\) concentrations in animals. This hypoxic selectivity provides perhaps the most consistent with the normal \(\text{tPO}_2\) values in sham-operated animals. This hypoxic selectivity provides perhaps the most clinically relevant attribute of pharmacologically increased \(\text{SNO-Hb}\) concentrations.

A target for \(\text{S-nitrosylation}\) is the \(\beta\)93 cysteine thiol in \(\text{Hb}\). The extent to which \(\text{Hb}\) is \(\text{S-nitrosylated}\) is dependent on the \(\text{Hb}\) oxygenation state.\(^{1,2}\) Oxyhemoglobin is readily nitrosylated and causes the \(\text{S-nitrosothiol}\) to face inward, protecting the NO moiety from solvent.\(^1\) In the deoxy state, the cysteine residue is allosterically rotated outward into the blood phase, thereby enabling \(\text{SNO-Hb}\) to transnitrosylate other moieties. Thus, \(\text{SNO-Hb}\) provides a hypoxia-activated source of NO bioactivity that constitutes a basis for increased delivery of \(\text{O}_2\) to a hypoxic region.

There have been brief human exposures to \(\text{ENO}\). Adverse effects were not observed. Pharmacologic enhancement of \(\text{SNO-Hb}\) provides a novel strategy for \(\text{SAH}\) therapeutic intervention. Optimal \(\text{ENO}\) dosing strategies, the presence of sustained efficacy in long-term outcome analyses, direct comparison with established treatments, and confirmation of purported molecular mechanisms of action require continued investigation.

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**Disclosures**

Duke University has filed a use patent application for \(\text{ENO}\) in \(\text{SAH}\). Individual authors have no conflicts of interest to disclose.

**References**


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