Beneficial Effects of Nitric Oxide on Outcomes after Cardiac Arrest and Cardiopulmonary Resuscitation in Hypothermia-treated Mice

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ABSTRACT

Background: Therapeutic hypothermia (TH) improves neurological outcomes after cardiac arrest (CA) and cardiopulmonary resuscitation (CPR). Although nitric oxide prevents organ injury induced by ischemia and reperfusion, role of nitric oxide during TH after CPR remains unclear. In this article, the authors examined the impact of endogenous nitric oxide synthesis on the beneficial effects of hypothermia after CA/CPR. The authors also examined whether or not inhaled nitric oxide during hypothermia further improves outcomes after CA/CPR in mice treated with TH.

Methods: Wild-type mice and mice deficient for nitric oxide synthase 3 (NOS3−/−) were subjected to CA at 37°C and then resuscitated with chest compression. Body temperature was maintained at 37°C (normothermia) or reduced to 33°C (TH) for 24 h after resuscitation. Mice breathed air or air mixed with nitric oxide at 10, 20, 40, 60, or 80 ppm during hypothermia. To evaluate brain injury and cerebral blood flow, magnetic resonance imaging was performed in wild-type mice after CA/CPR.

Results: Hypothermia up-regulated the NOS3-dependent signaling in the brain (n = 6 to 7). Deficiency of NOS3 abolished the beneficial effects of hypothermia after CA/CPR (n = 5 to 6). Breathing nitric oxide at 40 ppm improved survival rate in hypothermia-treated NOS3−/− mice (n = 6) after CA/CPR compared with NOS3−/− mice that were treated with hypothermia alone (n = 6; P < 0.05). Breathing nitric oxide at 40 (n = 9) or 60 (n = 9) ppm markedly improved survival rates in TH-treated wild-type mice (n = 51) (both P < 0.05 vs. TH-treated wild-type mice). Inhaled nitric oxide during TH (n = 7) prevented brain injury compared with TH alone (n = 7) without affecting cerebral blood flow after CA/CPR (n = 6).

Conclusions: NOS3 is required for the beneficial effects of TH. Inhaled nitric oxide during TH remains beneficial and further improves outcomes after CA/CPR. Nitric oxide breathing exerts protective effects after CA/CPR even when TH is ineffective due to impaired endogenous nitric oxide production. (Anesthesiology 2014; 120:880-9)

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Sudden cardiac arrest (CA) is a leading cause of death worldwide.1 Out-of-hospital CA claims the lives of an estimated 310,000 Americans each year.2 Despite advances in cardiopulmonary resuscitation (CPR) methods, only approximately 10% of adults treated for out-of-hospital CA survive to hospital discharge, and up to 60% of survivors have moderate to severe cognitive deficits 3 months after resuscitation.3 Most of the post-CA mortality and morbidity are caused by global ischemic brain injury.4 To date, no pharmacological agent is available to improve the outcome after CA/CPR.

Although therapeutic hypothermia (TH) confers significant neuroprotective effects when applied for 12 to 24 h after ventricular fibrillation–induced out-of-hospital CA in adults, TH has been shown to benefit, at most, 20% of victims in whom return of spontaneous circulation (ROSC) is achieved.5,6 It is unknown why many patients do not benefit from TH after out-of-hospital CA. Elucidating the mechanisms responsible for the protective effects of TH will enable not only optimization of TH but also development of other novel therapeutic strategies to improve the outcome after CA/CPR.

Nitric oxide is produced by nitric oxide synthases (such as NOS1, NOS2, and NOS3). One of the primary targets of nitric oxide is soluble guanylate cyclase, which is activated by nitric oxide to generate the second messenger cyclic guanosine monophosphate (cGMP). cGMP activates cGMP-dependent protein kinase G which phosphorylates a number of proteins including vasodilator-stimulated...
phosphoprotein (VASP). Nitric oxide exerts several effects that would be expected to attenuate ischemia–reperfusion (I/R) injury. Studies using mice genetically deficient for NOS3 have demonstrated that NOS3 reduces I/R injury in multiple organs including brain and heart. We recently reported that deficiency of NOS3 or the α1 subunit of soluble guanylate cyclase worsened outcomes after CA/CPR, whereas cardiomyocyte-specific overexpression of NOS3 rescued NOS3-deficient mice from myocardial and neurological dysfunction and death after CA/CPR. However, we did not previously investigate whether NOS3 has protective roles after CA/CPR when animals are treated with TH. Specifically, whether or not the beneficial effects of TH require NOS3 remains to be determined.

Although originally developed as a selective pulmonary vasodilator, inhaled nitric oxide has been shown to have systemic effects in a variety of preclinical and clinical studies without causing systemic vasodilation. For example, inhaled nitric oxide attenuates myocardial I/R injury in mice and swine, and it reduces hepatic I/R injury in patients undergoing liver transplantation. We recently reported that inhaled nitric oxide improved the survival rate after CA/CPR in mice. However, we have not studied whether or not nitric oxide breathing further improves outcomes after CA/CPR in mice that are also treated with TH.

The primary goal of this study was to determine whether inhaled nitric oxide remains beneficial in mice treated with TH after CA/CPR. We also sought to examine the hypothesis that endogenous nitric oxide synthesis by NOS3 is required for the beneficial effects of TH after CA/CPR.

Materials and Methods

Animals

After obtaining approval from the Massachusetts General Hospital Subcommittee on Research Animal Care (Boston, Massachusetts), we studied 2- to 3-month-old and weight-matched male C57BL/6J wild-type (WT) and NOS3-deficient (NOS3−/−, B6.129P2-Nos3 tm1Unc/J) mice on a C57BL/6J background.

Animal Preparation

Mice were intubated, mechanically ventilated (mini-vent; Harvard Apparatus, Holliston, MA), and instrumented during anesthesia as previously described. Arterial blood pressure was measured via left femoral arterial line. A microcatheter (PE-10; Becton Dickinson, Franklin Lakes, NJ) was inserted into the left femoral vein for drug and fluid administration. Blood pressure and needle-probe electrocardiogram monitoring data were recorded and analyzed with the use of a computer-based data acquisition system.

Murine CPR Model

Cardiac arrest and CPR in mice were performed as previously described with some minor modifications. Two different protocols were used in this study.

**Protocol 1.** To determine whether or not the beneficial effects of hypothermia require NOS3 after CA/CPR, WT mice were subjected to 7.5 min of CA, whereas NOS3−/− mice were subjected to 6.5 min of CA using the protocol previously described. After CA, chest compressions were delivered with a finger at a rate of 300 to 350 per minute with resumption of mechanical ventilation (FiO2 = 1.0) and continuous i.v. infusion of epinephrine. Core body temperature was maintained at 37°C by a warming lamp after ROSC for 1 h and then mice were kept at a room temperature to allow spontaneous hypothermia (approximately 33°C). In subgroups of NOS3−/− mice, we examined the effects of nitric oxide inhalation at 40 ppm mixed in air starting at 1 h after ROSC and continued for 24 or 48 h in custom-made chambers via an INOVent, NO Delivery System (IKARIA, Hampton, NJ), as previously described.

**Protocol 2.** To examine the effects of nitric oxide inhalation in TH-treated mice, WT mice were subjected to a prolonged CA of 8 min at 37°C. After 8 min of CA, chest compressions were delivered at a rate of 300 per minute with a novel mouse CPR device. The mouse CPR device is consist of a controller and an air-driven piston chest compressor and enables chest compression with a uniform rate and force. The core body temperature measured by esophageal temperature probe was maintained at 37°C using a warming lamp for 30 min after ROSC, whereupon TH was induced by application of a small ice pack. The core body temperature was then maintained at 33°C for the first 24 h after CA/CPR and documented using a telemeter system (TA10TA-F20; DSI, St. Paul, MN) in a subgroup of mice. Similarly, blood pressure was measured continuously using a telemeter system (TA11PA-C10; DSI) in another group of mice that were treated with TH or maintained at normothermia after CA/CPR. Mice breathed nitric oxide at 10, 20, 40, 60, or 80 ppm starting 30 min after ROSC and continued for 24 h. In subgroups of mice, effects of nitric oxide inhalation at 40 ppm starting at 2 or 6 h after ROSC and continued for 24 h were also examined. Medical-grade nitric oxide (INOMAX: NO 800 ppm, balance nitrogen; IKARIA) gas was mixed with 100% oxygen between 30 and 60 min after ROSC. Thereafter, mice breathed nitric oxide mixed with air in custom-made chambers.

Measurement of Protein Levels and Phosphorylation

Cerebral cortex tissue were obtained from mice 3 h after sham operation or CA/CPR and treated with hypothermia or maintained at normothermia after CA/CPR. Tissue homogenates were centrifuged and the supernatant proteins were fractionated for 1 h and then mice were kept at a room temperature to allow spontaneous hypothermia (approximately 33°C). In subgroups of mice, effects of nitric oxide inhalation at 40 ppm starting at 2 or 6 h after ROSC and continued for 24 h were also examined. Medical-grade nitric oxide (INOMAX: NO 800 ppm, balance nitrogen; IKARIA) gas was mixed with 100% oxygen between 30 and 60 min after ROSC. Thereafter, mice breathed nitric oxide mixed with air in custom-made chambers.
BD Biosciences, San Jose, CA), phospho NOS3 at Ser\(^{1177}\) (1:1,000), phospho NOS3 at Thr\(^{495}\) (1:5,000; BD Biosciences), total (1:1,000) and phospho Akt at Ser\(^{473}\) (1:1,000) and Ser\(^{308}\) (1:1,000), total (1:1,000) and phospho 5'-prime-AMP-activated protein kinase \(\alpha\) at Thr\(^{72}\) (1:1,000), total (1:1,000) and phospho VASP at Ser\(^{239}\) (1:1,000), heat shock protein 90 (1:10,000), and \(\beta\)-tubulin (1:5,000). Bound antibody was detected with a horseradish peroxidase–linked antibody directed against rabbit IgG (1:5,000) or mouse IgG (1:20,000; Thermo-Pierce, Rockford, IL) and was visualized using chemiluminescence with Lumigen TMA-6 (Lumigen, Inc., Southfield, MI) or Immobilon Western (Millipore, Billerica, MA).

**Measurement of Nitrite Levels**
Nitrite levels in serum and brain homogenates were determined by the tri-iodide-based liquid-phase chemiluminescence assay (Sievers 280i Nitric Oxide Analyzer; General Electric Company, Boulder, CO), as described previously.17

**Acquisition and Analysis of Magnetic Resonance Imaging**
Magnetic resonance imaging was performed 24 h after CA/CPR in WT mice that were treated with TH alone or TH combined with inhaled nitric oxide at 40 ppm. Imaging used a 9.4-tesla magnet and a four-channel phased array receiver coil inside a volume radio frequency transmitter (Bruker BioSpin Corporation, Billerica, MA). All scans were acquired at an isotropic in-plane resolution of 150 \(\mu\)m with 400-\(\mu\)m coronal slices that covered the brain from olfactory bulb to cerebellum. To search for focal lesions of the type that should appear bright on T2-weighted images, we used fast spin-echo imaging with eight echoes per excitation and an effective echo time of 60 ms. In addition, we acquired multiple images with stepped echo time values (10, 30, and 50 ms) using a conventional spin-echo sequence to enable calculation of regional T2 values.

**Cerebral Blood Flow Measurement after CA/CPR**
Cerebral blood flow (CBF) after successful CPR in WT mice was measured by continuous arterial spin labeling (ASL) with magnetic resonance imaging as described previously.18 CBF were measured in WT mice in the following order: baseline (20 min), inhaled nitric oxide (40 ppm, 10 min), baseline (20 min), inhaled carbon dioxide (\(\mathrm{CO}_2\), 10 min), and baseline (10 min). We used carbon dioxide (\(\mathrm{CO}_2\), 7%; oxygen, 21%, and balance nitrogen) as a positive control because carbon dioxide is known to increase CBF based on a previous report.19

**Statistical Analysis**
All data are expressed as mean ± SD. Data were analyzed using unpaired \(t\) test or one-way ANOVA with a Bonferroni, post hoc test. Differences in survival rates were analyzed with log-rank test. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA) with two-tailed hypothesis. \(P\) values less than 0.05 were considered statistically significant.

**Results**

**Permissive Spontaneous Hypothermia Improves Survival Rate after CA/CPR in WT, but Not in NOS3\(^{-/-}\), Mice**
To examine the impact of NOS3 on the beneficial effects of hypothermia after CA/CPR, WT and NOS3\(^{-/-}\) mice were subjected to CA/CPR followed by permissive spontaneous hypothermia. When mice were kept in a warmed environment to maintain normothermia for 24 h, all mice of both genotypes died within 24 h after CA/CPR. When mice were maintained at room temperature after CA/CPR, their body temperature declined spontaneously (permissive spontaneous hypothermia [approximately 33°C]). Spontaneous hypothermia markedly improved the survival rate in WT mice after 7.5 min of CA and subsequent CPR (fig. 1A). In contrast, spontaneous hypothermia failed to improve the survival rate in NOS3\(^{-/-}\) mice subjected to 6.5 min of CA and CPR (fig. 1B). These results suggest that NOS3 is required for the ability of hypothermia to improve survival rate after CA/CPR.

**Inhaled Nitric Oxide Rescues NOS3\(^{-/-}\) Mice Treated with Permissive Spontaneous Hypothermia after CA/CPR**
To examine whether supplementing nitric oxide \textit{via} inhalation improves the survival rate in NOS3\(^{-/-}\) mice, we studied the impact of breathing nitric oxide at 40 ppm on survival after CA/CPR in NOS3\(^{-/-}\) mice that were treated with spontaneous hypothermia. Nitric oxide inhalation at 40 ppm starting 1 h after ROSC and continuing for 24 h, which markedly improved survival rates in WT mice in a previous study,14 only modestly prolonged the survival time in NOS3\(^{-/-}\) mice. In contrast, breathing nitric oxide for 48 h markedly improved the 10-day survival rate in NOS3\(^{-/-}\) mice treated with spontaneous hypothermia (fig. 1B). These results suggest that nitric oxide inhalation improves outcomes after CA/CPR even when hypothermia is ineffective due to deficiency of NOS3.

**TH Up-regulates NOS3-dependent Signaling in the Brain after CA/CPR**
To elucidate the role of NOS3 in the beneficial effects of TH, we examined expression levels of total and phosphorylated NOS3 and VASP, a downstream target of protein kinase G, in the brain tissue of mice. Although CA/CPR did not affect the total NOS3 expression, TH markedly increased the expression of phosphorylated NOS3 at Ser\(^{1177}\) in the brain compared with sham-operated control mice and mice subjected to CA/CPR and maintained at normothermia (fig. 2A). However, TH decreased the expression of phosphorylated NOS3 at Thr\(^{495}\) compared with sham control (fig. 2B). Because phosphorylation at Ser\(^{1177}\) activates whereas phosphorylation at Thr\(^{495}\) inhibits NOS3, these observations suggest that TH activates NOS3 in the brain after CA/CPR. We also found that TH increased phosphorylated VASP at Ser\(^{239}\) compared with sham control and mice maintained at normothermia after CA/CPR corroborating the augmented...
To characterize the mechanisms responsible for the neuroprotective effects of inhaled nitric oxide after CA/CPR, we examined the effects of nitric oxide inhalation at 10, 20, 40, 60, and 80 ppm in WT mice treated with TH. To mimic the clinical care of patients with CA more accurately, we developed a new protocol that includes a mechanical chest compression device and targeted temperature control (fig. 4, see Protocol 2 in Materials and Methods). When maintained at normothermia (37°C) for the first 24 h after 8 min of CA and CPR, 100% of WT mice died within 24 h. TH initiated at 30 min after ROSC markedly improved survival rate of WT mice (fig. 5). Blood pressure after ROSC did not differ between surviving mice maintained at normothermia or treated with TH (fig. 6, A and B). Inhalation of nitric oxide at 40 or 60 (data not shown) ppm starting at 30 min after ROSC and continued for 24 h further improved the survival rate after CA/CPR in mice treated with TH (fig. 5). Although breathing nitric oxide at 10, 20, and 80 ppm tended to improve the survival rate in TH-treated mice, inhaled nitric oxide failed to provide statistically significant improvement in survival rate compared with TH alone at these doses (data not shown).

**Inhaled Nitric Oxide Remains Beneficial during TH When Initiated up to 2h after CA/CPR**

To determine the window of opportunity during which inhaled nitric oxide is able to improve outcomes after CA/CPR in TH-treated mice, we examined the effects of nitric oxide inhalation starting 2 or 6 h after ROSC on the survival rate after CA/CPR. Nitric oxide inhalation at 40 ppm starting up to 2 h after ROSC improved the survival rate after CA/CPR (fig. 5). However, initiation of nitric oxide breathing 6 h after ROSC failed to improve the survival rate in TH-treated mice.

**Inhaled Nitric Oxide Prevents the Brain Injury after CA/CPR in TH-treated WT Mice**

Therapeutic hypothermia confers neuroprotective effects after CA/CPR. To determine whether or not nitric oxide inhalation confers additional brain protection in mice treated with TH, we performed brain magnetic resonance imaging 24 h after CA/CPR in WT mice that were treated with TH alone or TH combined with inhaled nitric oxide at 40 ppm. T2-weighted images revealed that the brains of mice treated with TH alone exhibited hyperintense signal areas suggesting the development of vasogenic edema (fig. 7A). Breathing nitric oxide decreased the T2 intensity in the brain stem and ventral hippocampus of TH-treated WT mice (fig. 7, A and B). These results suggest that inhaled nitric oxide exerts brain protection above and beyond what is afforded by TH alone after CA/CPR.

**Inhaled Nitric Oxide Does Not Increase the CBF after CA/CPR**

To characterize the mechanisms responsible for the neuroprotective effects of inhaled nitric oxide after CA/CPR, we
Fig. 2. Protein expression in brain cortex 3 h after sham operation or cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Sham = mice that were subjected to sham operation without CA/CPR. NT = mice that were maintained normothermia for 3 h after CA/CPR. TH = mice that were treated with therapeutic hypothermia (TH) for 3 h after CA/CPR. (A) Relative phosphorylated nitric oxide synthase 3 (NOS3) at Ser\textsuperscript{1177} levels were quantified by dividing the phosphorylated at NOS3 at Ser\textsuperscript{1177} immunoreactivity by NOS3 immunoreactivity and normalized to values of sham-operated mice. (B) Relative phosphorylated NOS3 at Thr\textsuperscript{495} levels were quantified by dividing the phosphorylated NOS3 at Thr\textsuperscript{495} immunoreactivity by NOS3 immunoreactivity and normalized to values of sham-operated mice. (C) Relative phosphorylated vasodilator-stimulated phosphoprotein (VASP) at Ser\textsuperscript{239} levels were quantified by dividing the phosphorylated VASP at Ser\textsuperscript{239} immunoreactivity by VASP immunoreactivity and normalized to values of sham-operated mice. (D) Relative phosphorylated Akt at Ser\textsuperscript{473} levels were quantified by dividing the phosphorylated Akt at Ser\textsuperscript{473} immunoreactivity by Akt immunoreactivity and normalized to values of sham-operated mice. (E) Relative NOS1 levels were quantified by dividing the NOS1 immunoreactivity by \( \beta \)-tubulin immunoreactivity and normalized to values of sham-operated mice. (F) Relative NOS2 levels were quantified by dividing the NOS2 immunoreactivity by \( \beta \)-tubulin immunoreactivity and normalized to values of sham-operated mice. (G) Relative phosphorylated Akt at Thr\textsuperscript{308} levels were quantified by dividing the phosphorylated Akt at Thr\textsuperscript{308} immunoreactivity by Akt immunoreactivity and normalized to values of sham-operated mice. (H) Relative phosphorylated AMP-activated protein kinase \( \alpha \) (AMPK\( \alpha \)) at Thr\textsuperscript{172} levels were quantified by dividing the phosphorylated AMPK\( \alpha \) at Thr\textsuperscript{172} immunoreactivity by AMPK\( \alpha \) immunoreactivity and normalized to values of sham-operated mice. (I) Relative heat shock protein 90 levels were quantified by dividing the heat shock protein 90 immunoreactivity by \( \beta \)-tubulin immunoreactivity and normalized to values of sham-operated mice. Representative blots are shown of six blots in each group. N = 6–7. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \). Data were analyzed using one-way ANOVA with a Bonferroni post hoc test.
measured CBF by continuous ASL technique with magnetic resonance imaging in WT mice. ASL demonstrated that breathing nitric oxide gas at 40 ppm mixed with air did not increase CBF after CA/CPR in any regions in the brain during hypothermia (approximately 33°C). In contrast, inhalation of 7% CO₂ with 21% oxygen markedly increased CBF after CA/CPR and treated with TH combined with iNO at 40 ppm starting 2h after ROSC. TH + iNO 6h after ROSC = WT mice that were subjected to CA/CPR and treated with TH combined with iNO at 40 ppm starting 6h after ROSC. N = 9, 9, 7, 51, and 5 respectively. *P < 0.05 versus TH alone. #P < 0.05 versus NT. Differences in survival rates were analyzed with log-rank test.

Discussion

Potential role of nitric oxide–dependent signaling in the beneficial effects of hypothermia has been suggested previously.²⁰ Along these lines, we previously reported that short-term (24 h) survival rate of mice resuscitated from CA with intra-arrest cooling was markedly worsened by deficiency of NOS3 or α₁ subunit of soluble guanylate cyclase in mice.¹⁰ By directly comparing the survival rates of WT and NOS3⁻/⁻ mice maintained at normothermia or treated with hypothermia after resuscitation from CA, the current results extended these findings and revealed that NOS3 is required for the protective effect of hypothermia in a mouse model of CA/CPR. The critical role of NOS3 in the salutary effects of TH is further supported by the observation that
Inhaled NO with Hypothermia Improves Outcomes after CPR

Fig. 7. (A) Representative brain T2 images of magnetic resonance imaging in live mice at 24 h after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Yellow circles indicate the region of interest containing the ventral hippocampus and brain stem. Yellow arrows indicate hyperintense signal areas. TH alone = mice that were treated with therapeutic hypothermia (TH) after CA/CPR. TH + iNO = mice that were treated with TH combined with inhaled nitric oxide (NO) at 40 ppm starting at 30 min after return of spontaneous circulation and continued for 24 h. (B) Averaged T2 value in ventral hippocampus and brain stem. N = 7 in each group. *P < 0.05. Data were analyzed using unpaired t test.

beneficial effects of TH in WT mice resuscitated from CA were associated with activation of NOS3 and phosphorylation of VASP in the brain. Taken together, our observations suggest that beneficial effects of TH are mediated via the NOS3-cGMP-dependent signaling mechanisms after CA and CPR.

Because hypothermia failed to improve the survival rate in NOS3−/− mice, we examined whether or not replacement of nitric oxide by nitric oxide inhalation could improve outcomes after CA/CPR in hypothermia-treated NOS3−/− mice. We observed that breathing nitric oxide at 40 ppm for 48 h, but not for 24 h, markedly improved the survival rate in NOS3−/− mice compared with NOS3−/− mice treated with or without hypothermia. These results suggest that nitric oxide inhalation improves outcomes after CA/CPR in NOS3−/− mice in which hypothermia alone is insufficient to improve survival rates. If these observations are extrapolated to human beings, nitric oxide inhalation combined with hypothermia may be able to improve outcomes after CA/CPR in patients in whom TH alone is ineffective due to the impaired ability to produce nitric oxide (e.g., endothelial dysfunction). Further studies are warranted to examine effects of nitric oxide inhalation on outcomes after CA/CPR in mouse models of endothelial dysfunction (e.g., mice with diabetes mellitus or obesity).

To determine the effects of nitric oxide inhalation in WT mice resuscitated from CA and treated with targeted hypothermia of 33°C, we modified our experimental protocol. Because TH alone markedly improved survival rate of WT mice subjected to 7.5 min of CA (e.g., >90% at 10 days after CA), we prolonged the arrest time to 8 min in the new protocol (see Protocol 2 in the Materials and Methods). We also developed and used a novel mechanical mouse CPR device in the new protocol to increase the consistency of chest compression across multiple experimental groups. All normothermic control mice maintained at 37°C died within 24 h after subjected to 8 min of CA. TH at 33°C markedly improved the survival rate of WT mice to approximately 40% at 10 days after CA. By using this robust protocol, we observed that nitric oxide breathing at 40 or 60 ppm markedly improved outcomes after CA/CPR in TH-treated mice above and beyond TH alone.

Although nitric oxide inhalation at 10, 20, and 80 ppm failed to improve the survival rate after CA/CPR in mice treated with TH, inhaled nitric oxide did not impair the beneficial effects of TH at any concentrations. The reason why some concentrations of inhaled nitric oxide exerted additional beneficial effects above and beyond TH, whereas higher and lower concentrations did not, is unclear. Absence or attenuation of the beneficial effects of inhaled nitric oxide at 80 ppm on ischemic brain injury seems to be consistent with previous studies in stroke models. For example, Li et al.21 reported that inhaled nitric oxide at 10, 20, 40, and 60 ppm, but not at 80 ppm, reduced the infarct volume in adult mice subjected to middle cerebral artery occlusion and reperfusion. Charriaut-Marlangue et al.22 reported that breathing nitric oxide at 20 ppm during ischemia, but not at 5 or 80 ppm, reduced the infarct volume in a model of neonatal rat brain ischemia. It is of note that breathing 80 ppm nitric oxide during ischemia increases nitrotyrosine-positive neurons in cortex of newborn rats compared with rats that breathed air in the latter study. It is possible that breathing high concentration of nitric oxide may increase production of injurious peroxynitrite during I/R when the production of reactive oxygen species is also increased.

We observed that initiating nitric oxide inhalation 30 min or 2 h, but not 6 h, after ROSC markedly improved the survival rates in mice that were treated with TH. There was no difference between the survival rates of mice that breathed nitric oxide starting 30 min and 2 h after ROSC. These results suggest that there is a therapeutic window after which the benefit of inhaled nitric oxide on survival after CA/CPR is lost. We found this therapeutic window for inhaled nitric oxide to close between 2 and 6 h after ROSC in our mouse model. Although the therapeutic window in humans remains to be determined, our results suggest that...
Nitric oxide inhalation can improve neurological outcomes after CA/CPR, even if it is initiated several hours after ROSC. The implication of these observations for clinical implementation of inhaled nitric oxide after CA/CPR is significant because our results suggest that inhaled nitric oxide can be started after patients are transported to the hospital, and informed consent is obtained.

In previous studies, inhaled nitric oxide has been shown to cause cerebral vasodilation during brain ischemia in rodents and sheep. To determine whether or not the beneficial effects of nitric oxide inhalation after CA/CPR are associated with augmentation of cerebral perfusion, we measured whole-brain CBF by ASL technique. Surprisingly, nitric oxide inhalation starting 1 h after CPR during hypothermia failed to alter CBF in mice. In contrast, inhalation of 7% CO₂ robustly increased CBF in WT mice 1 h after CA/CPR. These observations suggest that vasoreactivity of cerebral vessels are intact after CA/CPR and that the ASL technique

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**Fig. 8.** (A) Time series of averaged percentage cerebral blood flow (CBF) change relative to the baseline after cardiac arrest and cardiopulmonary resuscitation. CBF was measured by continuous arterial spin labeling in cortex, caudoputamen, hippocampus, and whole brain. Gray shaded areas indicate inhalation of either 40 ppm nitric oxide (NO) mixed with air or 7% carbon dioxide (CO₂) with 21% oxygen. N = 6. (B) Averaged percentage CBF change relative to the baseline after cardiac arrest and cardiopulmonary resuscitation in response to the inhalation of NO and CO₂. N = 6. ***P < 0.001 versus NO and baseline. Data were analyzed using one-way ANOVA with a Bonferroni post hoc test.
used in our study has sufficient sensitivity to detect changes in CBF in mice. The reasons why our findings differ from those of Terpolilli et al. are likely multifactorial and include differences in the models studied and methods used to assess CBF. Nonetheless, our results do not support the hypothesis that nitric oxide inhalation improves outcomes after CA/CPR by increasing cerebral perfusion.

Although we observed that serum nitrite levels were markedly increased at 3 h after CPR, TH did not further increase serum nitrite levels compared with normothermia. In addition, brain nitrite levels were not affected by CA/CPR or TH. Although these results seem to conflict with the apparent activation of NOS3 in the brain, serum and brain nitrite levels are regulated not only by NOS activity but also by multiple factors including enzymatic and nonenzymatic reduction of nitrite/nitrate to nitric oxide, and nitrite and nitrate levels in diet.24 Furthermore, NOS3 is more concentrated in CA1 hippocampal pyramidal neurons than in any other brain areas.25 It is conceivable that TH may augment nitric oxide levels in selected regions of the brain without affecting global levels of nitrite.

A previous study suggested the potential role of Akt-dependent signaling in the beneficial effects of TH after CA.26 Because Akt phosphorylates NOS3, we examined expression of phosphorylated Akt in the brain of WT mice resuscitated from CA/CPR. Although CA/CPR markedly increased Akt phosphorylation at Ser473, no difference was detected between brains treated with normothermia or hypothermia. Levels of phosphorylated Akt at Thr308, phosphorylated AMP-activated protein kinase α at Thr172, and heat shock protein 90 were not affected by CA/CPR or TH, suggesting that phosphorylation of NOS3 was augmented via other mechanisms during TH. Mechanisms responsible for the TH-induced NOS3 phosphorylation remains to be determined in the future studies.

In summary, the current study revealed that nitric oxide breathing remains beneficial in TH-treated mice. Our observations also suggest that NOS3-derived nitric oxide is required for the beneficial effects of TH to improve outcomes after CA/CPR. Because endothelial dysfunction is associated with reduced vascular nitric oxide bioavailability, our findings in mice that are genetically deficient in endothelial nitric oxide synthase (NOS3−/− mice) raises the possibility that patient with endothelial dysfunction (due a broad spectrum of cardiovascular disorders) may be less likely to benefit from TH after CA/CPR. Finally, our current results suggest an exciting possibility that nitric oxide breathing may improve outcomes after CA/CPR in patients in whom TH alone is ineffective due to impaired endogenous nitric oxide production.

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Competing Interests

The authors declare no competing interests.

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