Inhaled Nitric Oxide Improves Outcomes After Successful Cardiopulmonary Resuscitation in Mice

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Background—Sudden cardiac arrest (CA) is a leading cause of death worldwide. Breathing nitric oxide (NO) reduces ischemia/reperfusion injury in animal models and in patients. The objective of this study was to learn whether inhaled NO improves outcomes after CA and cardiopulmonary resuscitation (CPR).

Methods and Results—Adult male mice were subjected to potassium-induced CA for 7.5 minutes whereupon CPR was performed with chest compression and mechanical ventilation. One hour after CPR, mice were extubated and breathed air alone or air supplemented with 40 ppm NO for 23 hours. Mice that were subjected to CA/CPR and breathed air exhibited a 10-day survival rate (4 of 13), depressed neurological and left ventricular function, and increased caspase-3 activation and inflammatory cytokine induction in the brain. Magnetic resonance imaging revealed brain regions with marked water diffusion abnormality 24 hours after CA/CPR in mice that breathed air. Breathing air supplemented with NO for 23 hours starting 1 hour after CPR attenuated neurological and left ventricular dysfunction 4 days after CA/CPR and markedly improved 10-day survival rate (11 of 13; \( P = 0.003 \) versus mice breathing air). The protective effects of inhaled NO on the outcome after CA/CPR were associated with reduced water diffusion abnormality, caspase-3 activation, and cytokine induction in the brain and increased serum nitrate/nitrite levels. Deficiency of the \( \alpha_1 \) subunit of soluble guanylate cyclase, a primary target of NO, abrogated the ability of inhaled NO to improve outcomes after CA/CPR.

Conclusions—These results suggest that NO inhalation after CA and successful CPR improves outcome via soluble guanylate cyclase–dependent mechanisms. (Circulation. 2011;124:00:00.)

Key Words: cardiopulmonary resuscitation ■ heart arrest ■ magnetic resonance imaging ■ nitric oxide synthase ■ physiology

Sudden cardiac arrest (CA) is a leading cause of death worldwide.1 Despite advances in cardiopulmonary resuscitation (CPR) methods, including the introduction of the automatic electric defibrillator and therapeutic hypothermia,2,3 <8% of adult out-of-hospital CA victims survive to hospital discharge,4 and up to 60% of survivors have moderate to severe cognitive deficits 3 months after resuscitation.5 The poor outcome after CA is due at least partly to the post-CA syndrome, which includes neurological and myocardial dysfunction and systemic inflammation. Although therapeutic hypothermia has proved effective in clinical studies,2,3 no pharmacological agent is available to improve outcomes of post-CA syndrome.

Clinical Perspective on p 000

Nitric oxide (NO) is produced from NO synthases (NOS1, NOS2, and NOS3). One of the primary targets of NO is soluble guanylate cyclase (sGC), which generates the second messenger cGMP on activation. sGC is a heme-containing heterodimeric enzyme composed of 1 \( \alpha \) and 1 \( \beta \) subunit. In most tissues, including heart, lung, and vascular smooth muscle cells, the sGC\( \alpha_1\beta_1 \) heterodimer is the predominant isoform. NO exerts a number of effects that would be expected to prevent ischemia/reperfusion (IR) injury, including inhibition of reactive oxygen species–producing enzymes and direct scavenging of reactive oxygen species. Nonethe-
less, the impact of endogenous and exogenous NO in the setting of CA/CPR, a whole-body IR injury complicated by systemic inflammation, is incompletely understood. In a previous study, we observed that deficiency of NOS3 or sGCα1 worsened outcomes of CA/CPR, whereas cardiomyocyte-specific overexpression of NOS3 rescued NOS3-deficient mice from myocardial and neurological dysfunction and death after CA.6,7 Along these lines, Dezfulian and colleagues5 recently reported that administration of nitrate at the initiation of CPR improved outcomes in a murine CA model, presumably by releasing NO. The protective effects of nitrate were associated with increased cardiac S-nitrosothiol levels and reversible inhibition of respiratory chain complex I in mitochondria. Although these results suggest that NO-dependent mechanisms have protective effects in CA/CPR, systemic administration of NO-donor compounds may induce systemic vasodilation and hypotension, frequently precluding its use in patients after CA in whom blood pressure may be low and unstable.

Although originally developed as a selective pulmonary vasodilator, inhaled NO has been shown to elicit systemic effects in a variety of preclinical and clinical studies without systemic vasodilation and hypotension, frequently precluding its use in patients after CA in whom blood pressure may be low and unstable.

Because of their sensitivity to prolonged CA,6,7 NOS3−/− mice were subjected to CA for only 6.5 minutes. Subsequent procedures, including CPR, in NOS3−/− mice were conducted as described above.

Assessment of Neurological Function
Neurological function was assessed at 24 and 96 hours after CA/CPR or sham surgery with a previously reported neurological function scoring system.8,9,10 Briefly, 5 parameters were assessed and scored: level of consciousness (no reaction to pinching of tail=0, poor response to tail pinch=1, normal response to tail pinch=2), corneal reflex (no blinking=0, sluggish blinking=1, normal blinking=2), respirations (irregular breathing pattern=0, decreased breathing frequency with normal pattern=1, normal breathing frequency and pattern=2), coordination (no movement=0, moderate ataxia=1, normal coordination=2), and movement/activity (no spontaneous movement=0, sluggish movement=1, normal movement=2). Total score was reported as the neurological function score (total possible score=10).

Assessment of Right Ventricular Systolic Pressure
In a group of WT mice, right ventricular (RV) systolic pressure was measured 1 hour after CPR (before initiation of NO inhalation) or sham surgery with a conductance pressure-volume catheter (SPR-839, Millar Instruments Inc, Houston, TX) inserted into the RV via the right jugular vein.

Effects of NO Inhalation on Myocardial Function
Left ventricular (LV) function was examined 4 days after CPR in WT mice that were subjected to CA/CPR and breathed air or air supplemented with NO or to sham surgery. Mice were anesthetized with fentanyl 250 μg/kg and ketamine 100 mg/kg IP, and LV function was measured with a conductance pressure-volume catheter, as previously described.6 Hemodynamic data were analyzed with a computer program (PVAN version 3.6, Millar Instruments).
Abnormality in the Brain 24 Hours After CA/CPR

Inhaled NO Improves Survival Rate at 10 Days After CA and CPR

Results

Inhaled NO Improves Survival Rate at 10 Days After CA and CPR

ROSIC was achieved in all 105 WT mice. Three WT mice died soon after extubation and therefore were excluded from further analysis. There was no difference between treatment groups in the CPR time to ROSC, total epinephrine dose, blood pressure, and heart rate 1 hour after CPR (Table I in the online-only Data Supplement). The partial pressure of oxygen (PaO2) and oxygen saturation (SaO2) of arterial blood samples obtained at 2 hours after CPR (1 hour after the initiation of air or NO breathing) did not differ between mice that breathed air and mice that breathed air supplemented with NO (data not shown). Body temperature was maintained at 37±0.5°C for the first hour after CPR. After mice were placed in the chambers at an ambient temperature of 27°C, body temperature fell to ~30°C within 3 hours but returned to baseline within 24 hours. There was no difference in core body temperature between mice that breathed air and those that breathed air supplemented with NO for the first 24 hours after CA/CPR (data not shown). Although only 4 of 13 mice that breathed air survived 10 days after CPR, 11 of 13 mice that breathed NO for 23 hours starting 1 hour after CPR survived for 10 days (P=0.003; Figure 1).

Inhaled NO Prevents Water Diffusion Abnormality in the Brain 24 Hours After CA and CPR

Magnetic resonance imaging acquired 24 hours after CA/CPR in mice that breathed air showed areas of hyperintense DWI in the brain (Figure 2A). Hyperintense DWI signal (or reduced ADC signal) is a measure of brain edema presumably caused by disruption of ion pump homeostasis and membrane failure.19,20 Breathing NO for 23 hours starting 1 hour after CPR largely prevented the development of hyperintense DWI. The degree of abnormal water diffusion was quantified by calculating the average ADC in several regions of interest, including the ventral-lateral hippocampus, caudoputamen, and lateral-frontal cortex (Figure 2B). Breathing NO prevented the reduction of ADC values in each region of interest and across the whole brain (Figure 2C). These results suggest that breathing NO reduced the development of the ischemia-induced edema in the brain 24 hours after CA/CPR.

Inhaled NO Prevents Neurological Dysfunction 4 Days After CA and CPR

Although neurological function did not differ between surviving mice that breathed air and those that breathed NO at 1 day after CA/CPR, the neurological function score at 4 days after CA/CPR was better in surviving mice that breathed air supplemented with NO than in mice that breathed air alone (P<0.01; Figure 3A). These results suggest that breathing NO prevented the development of neurological dysfunction 4 days after CA/CPR in mice.

Inhaled NO Prevents Neuronal Apoptosis After CA and CPR

Histological studies revealed that the number of neurons containing activated caspase 3 in the CA1 region of the hippocampus was markedly increased at 4 days after CA/CPR in mice that breathed air (Figure 3B and 3C). Breathing NO starting 1 hour after CPR prevented caspase 3 activation in the hippocampal neurons. These results suggest that NO inhalation starting 1 hour after CPR prevents neuronal apoptosis in the brain.

Inhaled NO Prevents Myocardial Dysfunction After CA and CPR

There was no difference in heart rate and mean arterial pressure among mice at ROSC or 1 hour after CPR that were subsequently randomized to breathe air or air supplemented with NO (Table I in the online-only Data Supplement). Furthermore, there was no difference in R V systolic pressure at 1 hour after CPR between mice subjected to CA and mice subjected to sham operation, suggesting the absence of pulmonary hypertension after CA (data not shown).

Four days after CA/CPR, indexes of LV systolic and diastolic function, LV end-systolic pressure, LV end-diastolic pressure, maximum rate of developed LV pressure (dP/dtmax), minimum rate of developed LV pressure (dP/dtmin), cardiac output, arterial elastance, end-systolic elastance, end-systolic elastance/arterial elastance, preload-recruitable stroke work, and the time constant of isovolumic relaxation (τ), were markedly impaired in mice that breathed air compared with sham-operated mice (the Table). Inhaled NO attenuated the impairment of heart rate, dP/dtmax, dP/dtmin, cardiac output, end-systolic elastance, end-systolic elastance/arterial elastance, and τ at 4 days after CA/CPR. These results show that inhalation of NO for 23 hours starting 1 hour after CPR...
ameliorates post-CA myocardial dysfunction at 4 days after CA/CPR in mice.

Inhaled NO Increased Serum Levels of Nitrite and Nitrate 24 Hours After CA/CPR
CA and CPR did not affect serum levels of nitrite and nitrate in mice that breathed air alone 24 hours after CA/CPR. Breathing air supplemented with NO for 23 hours markedly increased serum nitrite and nitrate levels compared with the levels in sham-operated mice (P<0.05 for nitrite and P<0.0001 for nitrate versus sham) and mice that breathed air alone after CPR (P<0.01 for nitrite and P<0.0001 for nitrate versus mice breathing air; Figure 4).

Deficiency of sGCα1, but Not NOS3, Abolishes the Salutary Effects of Inhaled NO on Survival Rate at 10 Days After CA/CPR
To elucidate the mechanisms responsible for the beneficial effects of NO inhalation on survival after CA/CPR, we examined whether inhaled NO improves outcomes of CA/CPR in sGCα1−/− mice.

Although ROSC was achieved in all 49 sGCα1−/− mice, 10 mice died soon after extubation and therefore were excluded from further analysis. The early mortality rate (in the first 2 hours after CPR) was higher in sGCα1−/− than in WT mice (3 of 105 WT mice died; P=0.0007 versus WT). In mice that survived long enough to be randomized to breathe air alone or air supplemented with NO for survival study (n=8 in each group), NO inhalation did not prevent neurological dysfunction on day 3 after CPR in sGCα1−/− mice (neurological function score 6±1 in mice that breathed air and 5±1 in mice that breathed NO; P=NS). Three of 8 sGCα1−/− mice that breathed air survived 10 days after CA/CPR. Inhalation of NO for 23 hours starting 1 hour after CPR did not improve the survival rate in sGCα1−/− mice (4 of 8 survived; Figure 5).

We considered the possibility that the reason for the failure of inhaled NO to improve the outcome in sGCα1−/− mice was that the injury induced by CA/CPR was too severe to be rescued by breathing NO. To test this hypothesis, we examined whether inhaled NO could improve outcomes in a strain of mice, NOS3−/− mice, that also manifest increased sensi-

Figure 2. A, Representative diffusion-weighted image (DWI) of mice 24 hours after cardiac arrest/cardiopulmonary resuscitation (CA/CPR) that breathed air (Air) or air supplemented with NO (iNO). White arrows indicate areas of hyperintense DWI. B, Representative magnetic resonance images showing 3 brain slices containing regions of interest (ROI). Slice positions are identified in millimeters (1.5, 0, or −3 mm) with respect to bregma in the coordinate space of the Allen mouse brain atlas.18 Colored outlines indicate portions of ROI (blue=caudoputamen [CPu], red=lateral cortex [Ctx]; green=ventral lateral hippocampus [Hipp]) that intersect with these slice planes (see Methods and Figure 1 in the online-only Data Supplement for further information). Average apparent diffusion coefficient (ADC) values of the slice plane for Air mice (n=6) and iNO mice (n=7). Color bar on the right side indicates the color code for ADC values (μm²/ms). C, Average ADC values of each 3-dimensional ROI across all planes in Air mice (n=6) or iNO mice (n=7) are shown in the bottom two rows after CA/CPR. *P<0.05 vs Air.
activity to CA/CPR.\(^6,13\) All NOS3\(^{-/-}\) mice that were subjected to 7.5 or 7 minutes CA died within 24 hours after CPR, confirming that NOS3\(^{-/-}\) mice were more sensitive to CA/CPR than WT and sGC\(^{1-/-}\) mice. In NOS3\(^{-/-}\) mice subjected to CA for 6.5 minutes, mean survival time was greater in those mice that breathed air supplemented with NO than in those that breathed air alone (3 ± 1 versus 1 ± 0 days, respectively; \(P=0.0064\) by log-rank test). These observations demonstrate that mice that are more sensitive to prolonged CA than sGC\(^{1-/-}\) mice can be rescued by NO inhalation after CA/CPR. Taken together, these results suggest that the protective effects of inhaled NO on neurological function and survival after CA/CPR are mediated at least in part via sGC-dependent mechanisms.

**Inhaled NO Prevents the Induction of Inflammatory Cytokines in WT but Not in sGC\(^{1-/-}\) Mice**

Expression of genes encoding tumor necrosis factor-\(\alpha\), interleukin-6, interleukin-\(1\beta\), and gp91phox (NOX2, a subunit of NADPH oxidase) was markedly greater in the brain cortex of WT mice that were subjected to CA/CPR and breathed air 24 hours after CA/CPR than in those of sham-operated mice (Figure 6). Breathing NO prevented the induction of tumor necrosis factor-\(\alpha\), interleukin-6, and NOX2 in the brain of WT mice subjected to CA/CPR.

**Table. Left Ventricular Function 4 Days After Cardiac Arrest and Cardiopulmonary Resuscitation**

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=4)</th>
<th>Air (n=5)</th>
<th>iNO (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>673 ± 7*</td>
<td>482 ± 26*</td>
<td>591 ± 28†</td>
</tr>
<tr>
<td>LVESP, mm Hg</td>
<td>100 ± 6</td>
<td>58 ± 4*</td>
<td>73 ± 5*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>2 ± 1</td>
<td>4 ± 1*</td>
<td>2 ± 0*</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}}), mm Hg/s</td>
<td>18.153 ± 1862</td>
<td>6159 ± 1007*</td>
<td>10 461 ± 803†</td>
</tr>
<tr>
<td>dP/dt(_{\text{min}}), mm Hg/s</td>
<td>-10 599 ± 1189</td>
<td>-4235 ± 432*</td>
<td>-7248 ± 640*</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>12.4 ± 0.5</td>
<td>7.6 ± 1.0*</td>
<td>11.4 ± 0.6†</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}})/IP, s(^{-1})</td>
<td>231 ± 9</td>
<td>198 ± 27</td>
<td>206 ± 18</td>
</tr>
<tr>
<td>Ea, mm Hg/(\mu)L</td>
<td>5 ± 0</td>
<td>4 ± 1*</td>
<td>4 ± 0*</td>
</tr>
<tr>
<td>Ees, mm Hg/(\mu)L</td>
<td>26 ± 5</td>
<td>6 ± 1*</td>
<td>15 ± 1†</td>
</tr>
<tr>
<td>Ees/Ea</td>
<td>4.7 ± 0.8</td>
<td>1.6 ± 0.3*</td>
<td>3.8 ± 0.1†</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>141 ± 25</td>
<td>74 ± 9*</td>
<td>102 ± 11</td>
</tr>
<tr>
<td>(r, ms)</td>
<td>4.9 ± 0.3</td>
<td>8.2 ± 0.6*</td>
<td>5.6 ± 0.4†</td>
</tr>
</tbody>
</table>

Sham indicates sham-operated mice; Air, mice that breathed air after cardiac arrest/cardio pulmonary resuscitation; iNO, mice that breathed air supplemented with NO starting 1 hour after cardiac arrest/cardio pulmonal resuscitation; HR, heart rate; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt\(_{\text{max}}\), maximum rate of developed left ventricular pressure; dP/dt\(_{\text{min}}\), minimum rate of developed left ventricular pressure; CO, cardiac output; dP/dt\(_{\text{max}}\)/IP, dP/dt\(_{\text{max}}\) divided by instantaneous pressure; Ea, arterial elastance; Ees, left ventricular end-systolic ventricular elastance; PRSW, preload-recruitable stroke work; and \(r\), time constant of isovolumic relaxation. Values are mean ± SEM.

\(\ast P<0.05\) versus Sham.

\(† P<0.05\) versus Air (by 1-way ANOVA with a Bonferroni post hoc test).

**Figure 3. Neuroprotective effects of inhaled nitric oxide (NO).** A, Neurological function score in surviving mice at 24 and 96 hours after cardiac arrest/cardiopulmonary resuscitation (CA/CPR). Dead mice (indicated by score=0) were excluded from the statistical analysis. B, Representative photomicrographs of brain sections of Air or iNO mice showing cleaved caspase 3-immunoreactive neurons (brown cells) at 4 days after CPR. Size bar=250 \(\mu\)m. C, Number of neurons per 1 mm\(^2\) containing cleaved caspase 3 in the CA-1 region of the hippocampus. n=4 for each group. \(\ast P<0.05\) vs Air.

**Figure 4. Serum nitrite and nitrate concentrations in mice 24 hours after sham surgery (Sham), after cardiac arrest/cardio pulmonary resuscitation (CA/CPR) and breathing air (Air), or after CA/CPR and breathing air supplemented with NO (iNO) for 23 hours starting 1 hour after CPR. n=6 to 9. \(\ast P<0.05\) vs Sham; \(\# P<0.05\) vs Air.**
Although CA/CPR induced tumor necrosis factor-\(\alpha\), interleukin-1\(\beta\), and NOX2 gene expression in the brains of sGC\(\alpha\)\textsuperscript{-/-} mice, the ability of NO inhalation to prevent the induction of these genes was abolished by sGC\(\alpha\) deficiency (Figure II in the online-only Data Supplement). Taken together, these observations suggest that NO breathing exerts antiinflammatory and antioxidant effects in the brain after CA/CPR via sGC-dependent mechanisms.

Discussion

The present study demonstrates that NO inhalation at 40 ppm for 23 hours starting at 1 hour after successful CPR markedly improves myocardial and neurological function and survival rate at 10 days after CA/CPR in mice. The neuroprotective effects of inhaled NO were associated with attenuation of the effects of inhaled NO on the outcome of CA/CPR was also associated with the inhibition of inflammatory cytokine induction in the brain and increased serum levels of nitrite and nitrate. Finally, deficiency of sGC\(\alpha\)1, but not NOS3, abrogated the protective effects of inhaled NO on the 10-day survival rate, neurological function, and inflammatory cytokine induction after CA/CPR. Taken together, these observations suggest that breathing NO after successful CPR confers organ protection and improves survival, at least in part, via sGC-dependent mechanisms.

It is increasingly recognized that post-CA care after ROSC can improve the likelihood of patient survival with good neurological function. Clinical trials showed that therapeutic hypothermia conferred neuroprotective effects when it was applied for 12 to 24 hours starting minutes to hours after successful CPR from CA caused by ventricular fibrillation.\(^2,3\) The apparent presence of a temporal therapeutic window after successful CPR is consistent with the observations that many of the mechanisms responsible for the post-CA brain injury are executed over hours to days after ROSC.\(^21-24\) These post-CA pathogenetic pathways include excitotoxicity, neuroinflammation, disrupted ion channel homeostasis, and membrane failure, as well as pathological activation of proteases and cell death signaling.\(^21,22\) The protective effects of breathing NO for 23 hours beginning 1 hour after successful CPR, observed in the present study, further support the notion that outcomes of sudden CA can be improved by implementing innovative therapies in the post-CA golden hours after successful CPR.

Conventional histopathological assessment of brain injury requires brain sections from individual animals euthanized at separate time points after injury. These methods not only diminish the statistical power, but may also introduce artifacts resulting from the postmortem tissue preparation. In the present study, mice that were successfully resuscitated from 7.5 minutes of CA and breathed air exhibited a marked abnormality in water diffusion in the hippocampus, caudoputamen, and cortex 24 hours after CPR. The presence of abnormal DWI signals in the vulnerable regions of the brain 1 day after CA/CPR correlated with worse neurological function and increased apoptosis of hippocampal neurons 4 days after CPR, as well as decreased rate of survival at 10 days. In contrast, NO breathing markedly attenuated the development of abnormality in water diffusion in the brain and improved neurological outcomes and survival rate. These observations are consistent with a recent clinical study that showed that diffuse cortical abnormalities in DWI are associated with poor outcomes in patients resuscitated from CA.\(^25\) Hyperintense DWI signals indicate the presence of brain edema presumably resulting from disruption of ion pump function and membrane failure. The present observations therefore suggest that NO inhalation after successful CPR can preserve ion pump homeostasis and membrane integrity early after CA/CPR.

Although the greatest proportion of the post-CA mortality and morbidity is caused by global ischemic brain damage, the severity of myocardial dysfunction correlates with poor neurological outcome.\(^26\) We found that the degree of LV

Figure 5. Survival rate of sGC\(\alpha\)\textsuperscript{-/-} mice during the first 10 days after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Air sGC\(\alpha\)\textsuperscript{-/-} indicates sGC\(\alpha\)\textsuperscript{-/-} mice subjected to CA/CPR that breathed air; iNO sGC\(\alpha\)\textsuperscript{-/-} mice subjected to CA/CPR that breathed air supplemented with nitric oxide for 23 hours starting 1 hour after CPR. \(n=8\) in each group. There was no difference in survival rates between the 2 groups.

Figure 6. Expression of genes encoding tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-6 (IL-6), IL-1\(\beta\), and gp91phox (NOX2) in control (Sham), after CA/CPR and breathing air (Air), or after CA/CPR and breathing air supplemented with NO (iNO) for 23 hours starting 1 hour after CPR. \(n=4\) to 8. *\(P<0.05\) vs Sham; #\(P<0.05\) vs Air.
RV dysfunction may also contribute to the circulatory failure after CA/CPR. Given the ability of inhaled NO to selectively reduce pulmonary artery pressure, it is conceivable that breathing NO improved outcomes of CA/CPR by reducing RV afterload. However, we did not find evidence of pulmonary hypertension in WT mice 1 hour after CA/CPR (before initiation of NO inhalation). Because inhaled NO reduces pulmonary artery pressure only in the presence of pulmonary hypertension, it is unlikely that inhaled NO improved outcomes after CA/CPR by reducing RV afterload in our model.

Neuroinflammation triggered by the whole-body IR injury associated with CA/CPR hinders the neurological recovery from prolonged CA. We observed that CA/CPR markedly upregulated the expression of genes encoding inflammatory cytokines and NADPH oxidase in the brain of WT mice that breathed air but not in WT mice that breathed air supplemented with NO. These observations suggest that NO inhalation prevents neuroinflammation after CA/CPR. Furthermore, these results demonstrate a correlation between neuroinflammation, neurological dysfunction, and mortality after CA/CPR.

NO elicits biological effects via sGC-dependent and/or-independent mechanisms. To determine the role of sGC in the protective effects of inhaled NO on the outcome of CA/CPR, we studied sGCα1−/− mice. We observed that sGCα1 deficiency increased the early mortality rate (in the first 2 hours after CPR) compared with WT mice after CA/CPR, consistent with our previous report. Although the cause of these early deaths is unknown, we previously reported that sGCα1 deficiency markedly exacerbated LV dysfunction early after CA/CPR. After the exclusion of mice that died early after CPR, sGCα1−/− mice that breathed air had a 10-day survival rate comparable to that in WT mice that breathed air after CA/CPR. These observations suggest that NO activity is critically important for initial recovery after CA/CPR but may not be necessary for long-term survival after CA/CPR. In contrast, sGCα1 deficiency abolished the ability of NO inhalation to inhibit the induction of inflammatory cytokines in the brain and to improve neurological function and 10-day survival rate after CA. These observations suggest that protective effects of inhaled NO on the outcome of CA/CPR are mediated largely via sGC-dependent mechanisms.

Inhaled NO may exert systemic effects via interaction with circulating bone marrow–derived cells (eg, leukocytes) as they transit lungs. Alternatively, some NO, once inhaled, may escape scavenging by hemoglobin and be converted to relatively stable NO metabolites (eg, nitrite, S-nitrosothiols) that can regenerate NO in the periphery and directly protect neurons. In fact, in the present study, we found that breathing NO increased levels of nitrite and nitrate 24 hours after CA/CPR. We previously reported that neutrophils are required for inhaled NO to reduce myocardial infarction size in WT mice subjected to transient left coronary artery occlusion. Along these lines, we recently observed that breathing NO markedly decreased myocardial infarction size in WT but not in sGCα1−/− mice. Furthermore, breathing NO decreased myocardial infarction size in chimeric sGCα1−/− mice carrying WT bone marrow generated by bone marrow transplantation. These results raise the possibility that the neuroprotective effects of inhaled NO after CA/CPR may be mediated by bone marrow–derived cells in an sGC-dependent manner.

Our data do not exclude the possibility that sGC-independent mechanisms could contribute to the protective effects of inhaled NO on peripheral organs after CA/CPR. It is conceivable that NO modifies functions of enzymes and ion channels in an sGC-independent manner. For example, ischemic preconditioning has been shown to protect cardiomyocytes from subsequent IR injury by preventing Ca2+ overload via S-nitrosoylation–mediated inhibition of L-type Ca2+ channel α1 subunit. Further studies are warranted to elucidate the mechanisms responsible for the protective effects of inhaled NO on outcome after CA/CPR.

From the viewpoint of translating the present results into clinical benefit, it is of particular importance that NO inhalation started 1 hour after successful CPR and continued for 23 hours markedly improves neurological and myocardial function and survival rate 10 days after CA/CPR. For example, NO inhalation can be started after patients are transferred to hospital and informed consent is obtained. To date, therapeutic hypothermia is the only therapeutic approach proven to improve outcomes after CA/CPR when applied hours after successful CPR. Because the body temperature of mice was allowed to decrease to 30°C during NO inhalation in the first 24 hours after CA/CPR in the present study, our data suggest that NO breathing may confer protection in the setting of mild hypothermia. Nonetheless, the effects of the combination of inhaled NO and therapeutic hypothermia, compared with either alone, on outcomes after CA/CPR remain to be formally determined in future studies.

This study has several limitations. The induction of CA by bolus administration of potassium chloride may have limited clinical relevance. However, we believe this model provides a valuable platform for elucidating the molecular mechanisms of organ dysfunction associated with CA/CPR and the impact of inhaled NO on the post-CA syndrome. All mice were anesthetized when subjected to CA/CPR. It is possible that drugs used to induce anesthesia may affect the outcomes of CA/CPR.

Conclusions

The present study revealed robust protective effects of NO inhalation on the outcome of CA/CPR in mice. Breathing NO at 40 ppm for 23 hours starting 1 hour after successful CPR markedly improved myocardial and neurological function and survival rate 10 days after CA/CPR, at least in part, via sGC-dependent mechanisms. The ability of delayed NO breathing to prevent post-CA brain injury and to promote survival in mice, if extrapolated to human beings, is highly clinically relevant and may serve as the experimental basis for future clinical trials in which the effects of inhaled NO on outcome after CA/CPR are examined. We anticipate that the
established safety profile of NO inhalation\(^3\) will enable the rapid translation of findings in animal models to patients suffering from the post-CA syndrome.

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**Disclosures**


**References**


**CLINICAL PERSPECTIVE**

Sudden cardiac arrest is one of the leading causes of death worldwide. Despite advances in resuscitation techniques, <8% of the 300 000 adults who experience cardiac arrest in the United States each year survive to hospital discharge, and up to 60% of survivors have long-lasting neurological deficits. Although therapeutic hypothermia has proven effective in clinical studies, no pharmacological agent is available to improve outcome from cardiac arrest. Although originally developed as a selective pulmonary vasodilator, inhaled nitric oxide (NO) has been shown to have systemic effects in a variety of preclinical and clinical studies without causing systemic vasodilation. In the present study, we found that breathing a low concentration of NO starting 1 hour after successful cardiopulmonary resuscitation for 23 hours markedly improves long-term neurological and cardiac outcomes and survival in mice subjected to cardiac arrest and cardiopulmonary resuscitation. The ability of NO breathing to improve outcomes after cardiac arrest when begun after cardiopulmonary resuscitation, if extrapolated to human beings, makes inhaled NO a practical therapeutic approach that can be initiated after patients are transferred to a hospital. Furthermore, because inhaled NO does not cause systemic hypotension, in contrast to systemic NO donors, it is uniquely suited for the treatment of post–cardiac arrest patients in whom blood pressure is often unstable. We anticipate that the established safety profile of NO inhalation will enable the rapid translation of findings in animal models to patients suffering from the post–cardiac arrest syndrome.
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SUPPLEMENTAL MATERIAL for “Inhaled Nitric Oxide Improves Outcome After Successful Cardiopulmonary Resuscitation in Mice.”

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Supplemental Methods

MRI Acquisition—Magnetic resonance imaging data were obtained using a 9.4 Tesla horizontal-bore magnet (Bruker Biospin Corp., Billerica MA) equipped with a custom surface coil for transmission and reception of radio frequencies for MRI of mouse brain. During imaging, mice were anesthetized by 2% isoflurane administered through a nose cone. Following image localization, multi-slice diffusion-weighted images (DWI) were acquired using a conventional spin-echo pulse sequence with an echo time of 26 ms, a
repetition time of one second, sixteen contiguous slices of 750 microns, and an isotropic resolution of 260 microns in the image plane. Two coronal diffusion weightings ("b values" of 154 and 1294 sec/mm²) were acquired every three minutes, and six pairs of diffusion values were acquired for each animal in order to reduce motion artifacts (e.g., due to respiration) by averaging.

**MRI Analysis**—For each animal, volumetric MRI data were aligned to the coordinate space of the Allen Mouse Brain atlas¹ using publicly available software developed by one of the authors (JBM: [www.nitrc.org/projects/jip](http://www.nitrc.org/projects/jip)). Briefly, digitized slides of the Allen Mouse Brain “Reference Atlas” were segmented manually into gray matter, white matter, and cerebrospinal fluid in order to form a template for automated alignment of T2-weighted MR brain images from a cohort of mice. Subsequently, data from each animal were aligned to this cross-subject MRI template using automated adjustment of linear and non-linear transformations. Linear “affine” alignment (6-parameter rigid-body transformation plus 3 uniform inflations and 3 uniform skews) was followed by adjustment of three-dimensional distortion fields to reduce residual alignment errors due to MRI artifact or anatomical variance. All MRI data were registered into brain volumes with a slice thickness of 500 microns and an in-plane resolution of 250 microns.

Because DWI is sensitive to motion, respiration produced subtle artifacts in images with heavy diffusion weighting. To minimize these artifacts, each series of six b-values were averaged using a weighting function equal to the inverse of the global residue with respect to mean in order to produce a single time-averaged brain volume for each b-value. Subsequently, the apparent diffusion coefficient (ADC) for each brain
voxel was computed using the standard formulation for the MRI signal: \( S = S_0 \exp(-b \times \text{ADC}) \). Brains maps of average ADC were computed for each subject group, and the average ADC value within each region of interest was computed for each animal for entry into histograms and statistical tables.

**MRI Regions of Interest**—Anatomical regions of interest were determined by the Allen Brain Atlas and cross-subject maps of average ADC value in the two subject groups. These regions included (1) whole brain, (2) whole caudoputamen (blue outline in Supplemental Figure 1), and (3) whole hippocampus, as defined from the Allen Brain Atlas. Based on our pilot studies, two additional bilaterally symmetric regions of interest were defined: (1) lateral cortex (red outlines in Supplemental Figure 1) and (2) ventral lateral hippocampus (green outlines in Supplemental Figure 1).

**Histological studies**—Four days after CA/CPR, mice were sacrificed, and brains were perfusion-fixed in 4% formalin in PBS and embedded in paraffin. Brains were cut with a microtome in coronal planes including the hippocampus (6 µm thickness). Activation of caspase 3 was assessed by immunohistochemistry in paraffin-embedded brain sections obtained 96h after CA/CPR using a rabbit monoclonal antibody against cleaved caspase 3 (1:80, Cell Signaling) according to the protocol recommended by the manufacturer. Cleaved caspase 3-positive neurons in the CA1 sector of the hippocampus were manually counted by an investigator blinded to the treatment group, and the number of these neurons per square millimeter of examined area was reported.
Measurements of gene expression—Total RNA was extracted from cortex of mice 24h after CA/CPR or sham surgery using the illustra RNA spin Mini kit (GE Healthcare, Waukesha, WI), and cDNA was synthesized using MMLV-RT (Promega, Madison, WI). Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), gp91phox (NOX2), and 18S ribosomal RNA transcript levels were measured by real-time PCR using a Realplex 2 system (Eppendorf, Westbury, NY). The following primer sets were used: TNF-α (5’-CAGCCTCTTCTCATTCTGTC-3’, 5’-GGTCTGGGCCCATAGAACTGA-3’), IL-1β (TaqMan, Applied Biosystems), IL-6 (5’-CCGGAGAGGAGACTTCACAGA-3’, 5’-CAGAATTGCCATTGCACAAC-3’), NOX2 (5’-CTGCTCTCTTTTCTCAGGGGT-3’, 5’-GTGTGCAGTGCTATCATCCAA-3’), and 18S rRNA (5’-CGGCTACCACATCCAGGA-3’, 5’-GCTGGAATTACCGCGGCT-3’). Changes in the relative gene expression normalized to levels of 18S rRNA were determined using the relative C_T method. The mean value of samples from control mice was set as 1.
Supplemental Table 1. Group characteristics before cardiac arrest and in the first hour after cardiac arrest and CPR

<table>
<thead>
<tr>
<th></th>
<th>Air (n=13)</th>
<th>Inhaled NO (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>24.6±0.5</td>
<td>24.3±0.4</td>
</tr>
<tr>
<td>HR before CA, bpm</td>
<td>594±18</td>
<td>587±11</td>
</tr>
<tr>
<td>MAP before CA, mmHg</td>
<td>123±3</td>
<td>123±3</td>
</tr>
<tr>
<td>Total dose of Epinephrine, µg</td>
<td>0.9±0.1</td>
<td>0.8±0.0</td>
</tr>
<tr>
<td>CPR time to ROSC, s</td>
<td>262±16</td>
<td>255±14</td>
</tr>
<tr>
<td>HR at ROSC, bpm</td>
<td>523±5</td>
<td>512±3</td>
</tr>
<tr>
<td>MAP at ROSC, mmHg</td>
<td>106±7</td>
<td>111±8</td>
</tr>
<tr>
<td>HR at 60 min after CPR, bpm</td>
<td>323±18</td>
<td>294±12</td>
</tr>
<tr>
<td>MAP at 60 min after CPR, mmHg</td>
<td>49±3</td>
<td>48±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Air, mice subjected to cardiac arrest and CPR that breathed air; Inhaled NO, mice subjected to cardiac arrest and CPR that breathed NO; CA, cardiac arrest; HR, heart rate; MAP, mean arterial pressure; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation. No differences were found statistically significant.
Supplemental Figures and Figure legends

Supplemental Figure 1. Representative fast spin-echo images derived from the mouse showing the definition of the regions employed for analysis. Slices are labeled with respect to bregma in the coordinate space of the Allen Mouse Brain Atlas. Using the nomenclature employed in the manuscript, colored outlines identify caudoputamen (blue), lateral cortex (red), and ventral lateral hippocampus (green).
Supplemental Figure 2. Expression of genes encoding tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β, and gp91phox (NOX2, a subunit of NADPH oxidase) in the brain cortex of sGCα1−/− mice 24h after sham surgery (Sham), after CA/CPR and breathing air (Air), or after CA/CPR and breathing NO (iNO) starting 1h after CPR for 23h. N=4-8. *P<0.05 vs Sham.
Supplemental References