Nitrite Therapy After Cardiac Arrest Reduces Reactive Oxygen Species Generation, Improves Cardiac and Neurological Function, and Enhances Survival via Reversible Inhibition of Mitochondrial Complex I

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Background—Three-fourths of cardiac arrest survivors die before hospital discharge or suffer significant neurological injury. Except for therapeutic hypothermia and revascularization, no novel therapies have been developed that improve survival or cardiac and neurological function after resuscitation. Nitrite (NO₂⁻) increases cellular resilience to focal ischemia/reperfusion injury in multiple organs. We hypothesized that nitrite therapy may improve outcomes after the unique global ischemia/reperfusion insult of cardiopulmonary arrest.

Methods and Results—We developed a mouse model of cardiac arrest characterized by 12 minutes of normothermic asystole and a high cardiopulmonary resuscitation rate. In this model, global ischemia and cardiopulmonary resuscitation were associated with blood and organ nitrite depletion, reversible myocardial dysfunction, impaired alveolar gas exchange, neurological injury, and an ≈50% mortality. A single low dose of intravenous nitrite (50 nmol/kg/1.85 μmol/kg=0.13 mg/kg) compared with blinded saline placebo given at cardiopulmonary resuscitation initiation with epinephrine improved cardiac function, survival, and neurological outcomes. From a mechanistic standpoint, nitrite treatment restored intracardiac nitrite and increased S-nitrosothiol levels, decreased pathological cardiac mitochondrial oxygen consumption resulting from reactive oxygen species formation, and prevented oxidative enzymatic injury via reversible specific inhibition of respiratory chain complex I.

Conclusion—Nitrite therapy after resuscitation from 12 minutes of asystole rapidly and reversibly modulated mitochondrial reactive oxygen species generation during early reperfusion, limiting acute cardiac dysfunction, death, and neurological impairment in survivors. (Circulation. 2009;120:897-905.)

Key Words: cardiopulmonary resuscitation ■ heart arrest ■ ischemia ■ nitric oxide ■ reperfusion

Nitrite (NO₂⁻), historically considered inert, functions as a reservoir for nitric oxide (NO).¹ During physiological hypoxia and pathological ischemia, nitrite is reduced to NO, regulating hypoxic vasodilation, cellular respiration, mitochondrial reactive oxygen species (ROS) generation, angiogenesis,² and cellular death programs.³ Nitrite in human plasma exists at concentrations of 100 to 300 nmol/L³⁻⁵ and may be reduced to NO by iron-containing enzymes,³⁻⁶ including hemoglobin, myoglobin, neuroglobin, xanthine oxidoreductase, endothelial NO synthase, mitochondrial electron transport chain proteins, and the hepatic cytochrome P450 system. The rate and extent of nitrite reduction are coupled to deoxygenation and proton generation. Thus, NO generation is coupled to oxygen and pH gradients³⁻⁷ and maximized in ischemic tissues.

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Nitrite therapy limits cellular injury and apoptosis after ischemia and reperfusion.⁶ Nitrite therapy is cytoprotective in numerous animal models of focal ischemia/reperfusion injury.⁶
including rodent heart, brain, liver, and kidney; canine heart; and primate brain. Systemic nitrite reduction by ceruloplasmin knockout or dietary nitrate/nitrite elimination increased infarction volume in the liver and heart after experimental ischemia. These studies indicate that physiological systemic nitrite levels modulate host resilience to ischemia. The established safety of human and animal nitrite dosing and its potent effects in limiting major organ injury suggest that nitrite represents an ideal therapy for cardiac arrest.

Cardiac arrest results in global multiorgan ischemic injury associated with significant morbidity and mortality. More than 70% of those resuscitated die before hospital discharge. Except for the selective application of hypothermia and revascularization, no novel postresuscitation therapies have been developed that improve survival or cardiac and neurological function. We explored nitrite therapy in a mouse model of cardiac arrest. Here, we provide evidence that nitrite therapy improves cardiac function, survival, and neurological function in survivors. Mechanistically, we show that nitrite specifically and reversibly inhibits cardiac complex I, limiting oxidative reperfusion injury. The ease of delivery, established human safety, and efficacy of nitrite in murine cardiac arrest suggest its promise as a novel therapy after cardiac arrest.

Table 1. Arterial Blood Gas Results Obtained From Sham Mice or 5 Minutes After CPR From Mice Randomized to Placebo or Nitrite

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sham</th>
<th>Placebo</th>
<th>Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.35±0.03</td>
<td>6.61±0.04*</td>
<td>6.73±0.05</td>
</tr>
<tr>
<td>PaCO₂, mm Hg</td>
<td>38.4±2.7</td>
<td>108.3±16.0*</td>
<td>66.2±16.2†</td>
</tr>
<tr>
<td>PaO₂, mm Hg</td>
<td>253.7±17.6</td>
<td>128.2±23.8*</td>
<td>210.7±25.7†</td>
</tr>
<tr>
<td>Bicarbonate, mg/dL</td>
<td>20.9±0.8</td>
<td>9.9±0.9*</td>
<td>8.4±0.9</td>
</tr>
<tr>
<td>Lactate, mg/dL</td>
<td>0.9±0.1</td>
<td>16.5±0.5*</td>
<td>15.6±1.2</td>
</tr>
</tbody>
</table>

Data are given as mean±SEM (n=5 per group) analyzed by ANOVA. *P<0.01, sham versus postarrest placebo; †P<0.05, postarrest placebo versus nitrite.

Methods

Mouse Cardiac Arrest Model

In initial model building, we adapted mouse cardiac arrest models from prior reports with the goal of nearly 100% resuscitation and 50% 24-hour mortality. To modulate mortality, we extended asystolic time from 8 to 12 minutes and added epinephrine to increase return of spontaneous circulation (ROSC). Seven- to 12-week-old (24 to 30 g) C57BL/6 male mice were used as approved by the National Institutes of Health Institutional Animal Care and Use Committee. Anesthesia consisting of intraperitoneal ketamine and xylazine (100 and 15 mg/kg, respectively) preceded surgical preparation, including rodent heart, brain, liver, and kidney; canine heart; and primate brain. Systemic nitrite reduction by ceruloplasmin knockout or dietary nitrate/nitrite elimination increased infarction volume in the liver and heart after experimental ischemia. These studies indicate that physiological systemic nitrite levels modulate host resilience to ischemia. The established safety of human and animal nitrite dosing and its potent effects in limiting major organ injury suggest that nitrite represents an ideal therapy for cardiac arrest.

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**Table 2. Baseline and CPR Characteristics of Randomized Animals**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo</th>
<th>Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at experiment, wk</td>
<td>10±1.8</td>
<td>10±1.8</td>
</tr>
<tr>
<td>Weight, g</td>
<td>27.0±2.3</td>
<td>26.5±2.3</td>
</tr>
<tr>
<td>Ischemic time, min</td>
<td>12.0±0.01</td>
<td>12.0±0.03</td>
</tr>
<tr>
<td>DBP 15 s before CPR, mm Hg</td>
<td>4.3±0.4</td>
<td>4.8±0.2</td>
</tr>
<tr>
<td>DBP during 15 s after CPR start, mm Hg</td>
<td>27.4±2.3</td>
<td>27.9±3.5</td>
</tr>
<tr>
<td>Successful resuscitation, n (%)</td>
<td>25/28 (89)</td>
<td>25/27 (93)</td>
</tr>
<tr>
<td>Time to ROSC, s</td>
<td>54.8±30.1</td>
<td>43.0±26.3*</td>
</tr>
</tbody>
</table>

DBP indicates diastolic blood pressure. n=21 per group. *P=0.034.

BLOOD GASES AND BLOOD/TISSUE NITRITE LEVELS

Carotid arterial blood was obtained in a heparinized syringe either 55 minutes after anesthesia (sham) or 5 minutes after CPR. Blood was used for blood gas analysis (Nova Biomedical, Waltham, Mass), mixed 4:1 with nitrite preservation solution, or centrifuged to isolate plasma for nitrite measurements. Animals were perfused with tissue nitrite preservation solution (1 mmol/L KCN, 0.2% NP-40, 0.8 mmol/L ferricyanide, 0.5 mol/L NEM, 100 μmol/L DTPA), and brains were homogenized for nitrite measurements. Snap-frozen hearts obtained 15 minutes after CPR were sectioned at −20°C, placed in ice-cold nitrite preservation solution, and homogenized for nitrite measurements. All nitrite measurements were determined by tri-iodide-based gas-phase reductive chemiluminescence with an NO analyzer (GE Analytic, Boulder, Colo) as described previously and tissue levels were normalized to protein content (BCA Protein Assay, Pierce, Rockford, Ill).

LEFT VENTRICULAR ECHOCARDIOGRAPHY

Animals had line removal, wound closure, and chest hair removal before transportation for echocardiogram 60 minutes after CPR or sham surgery. Animals were secured to the Vevo 770 (VisualSonics, Toronto, Ontario, Canada) platform, and temperature and ECG were monitored (temperature maintained at >35°C, heart rate at >300 bpm; supplemental oxygen delivered via nose cone). Parasternal long-axis 2-dimensional images of the left ventricle (LV) were obtained with the RMV707B scan head 75 to 90 minutes after CPR. M-mode images were used to measure end-systolic and end-diastolic ventricular size, and fractional shortening and ejection fraction (EF) were calculated with the manufacturer’s software (version 2.3.0).

CARDIAC MAGNETIC RESONANCE IMAGING

Anesthetized animals (1% to 1.5% isoflurane) underwent magnetic resonance imaging (MRI) 24 hours after CPR or sham surgery.
Magnetic resonance imaging experiments were carried out in a 7.0-T, 16-cm horizontal Bruker MR imaging system (Bruker, Billerica, Mass) with Bruker ParaVision 3.0.2 software. Magnevist (Bayer Healthcare, Montville, NJ) diluted 1:10 with sterile 0.9% saline was administered subcutaneously at a dose of 0.3 mmol/kg. Six short-axis slices were used to determine EF and ventricular volumes using CAAS-MRV-FARM software (Pie Medical Imaging, Maastricht, the Netherlands).

Histology
Mice were transcardiac perfused with saline followed by 10% buffered formalin. Brains were removed, further fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Seven serial high-powered (×400) fields of bilateral CA1 at Bregma −1.5 mm were examined, and live and dead cells were counted and normalized to hippocampal length as described elsewhere.

Mitochondria Isolation
Heart mitochondrial isolation was performed by differential centrifugation as described elsewhere, and protein concentration was determined. Fresh mitochondria were used for respirometry, ROS, and ATP generation assays, and aliquots were stored at −80°C for subsequent complex I activity assays as described previously.

Figure 1. Cardiac arrest experiment, end points, and cardiovascular effects. A, Experimental outline with sample blood pressure and ECG before arrest and during arrest, CPR, ROSC, and recovery phases with end points. B, Physiological data from 1 experiment depicting heart rate (HR), mean arterial blood pressure (MAP), and exhaled CO₂. The ischemic period is shaded. C through E, Postarrest placebo-treated mice vs nonarrested shams. C, Mean HR increases and MAP decreases 60 minutes after CPR vs 1 minute before arrest (n=21). D, Cardiac arrest results in significant reductions in LV fractional shortening (FS) and LVEF by echocardiography 75 to 90 minutes after CPR (n=6). E, Cardiac arrest results in diminished RVEF and increased dilation (n=5). Values denoted as mean±SEM analyzed by paired t test. RVEDV indicates RV end-diastolic volume. *P<0.01; †P=0.038.
Aconitase Activity
Hearts snap-frozen 15 minutes after CPR or sham surgery were homogenized in the manufacturer’s commercial buffer, and lysates were prepared by 3 freeze/thaw cycles. Aconitase activity was determined by spectrophotometric (340 nm) monitoring of NADPH formation with the Bioxytech Aconitase-340 kit (Oxis Research, Beverly Hills, Calif).

Statistical Analysis
Data are given as mean ± SEM; analyses were performed with GraphPad Prism 5 (La Jolla, Calif). Continuous data were compared between 3 groups with 1-way ANOVA with posthoc Bonferroni adjustment and between 2 groups with paired Student t test for variables that are normally distributed, Wilcoxon for variables that are not normally distributed, and repeated-measures ANOVA for variables measured at multiple time points from the same subjects. Mitochondrial experiments were performed as multiple paired experiments at discrete times and therefore analyzed at each time using a paired t test. Mortality was assessed by Kaplan–Meier survival analysis (log-rank test). A 2-tailed value of P < 0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Cardiac Arrest Physiological Effects
Cardiac arrest led to transient hyperemia soon after resuscitation (Figure 1B) as described by others. Cardiac arrest resulted in metabolic (lactic) and respiratory acidosis and oxygen depletion (Table 1). Ischemic times and resuscitation rates were similar between groups (Table 2).

Cardiac Arrest Depletes Systemic Nitrite, Which Is Reversed by Intravenous Therapy
Nitrite is reduced to NO and NO-modified proteins during focal ischemia in rodents. We tested the hypothesis that basal systemic levels of nitrite would be reduced by ischemic consumption during cardiac arrest and intravenous nitrite could restore levels (Figure 2A). In placebo-treated mice, ischemia depleted whole-blood nitrite levels (0.64 ± 0.05 μmol/L) and nitrite therapy restored these levels (1.01 ± 0.06 μmol/L) to near baseline (1.15 ± 0.10 μmol/L). These findings were mirrored in plasma (Figure 2B) and heart (Figure 2E), and a similar trend was noted in brain (Figure 2C). S-nitrosation of the cardiomyocyte L-type calcium channel is a protective posttranslational modification resulting from nitrite therapy or ischemic
preconditioning. We found that total S-nitrosothiol–modified protein concentration (Figure 2F) did not change with ischemia but nitrite therapy significantly increased these levels \((P < 0.05)\).

**Nitrite Repletion Improves Cardiac Function**

Increased systemic nitrite in treated mice was associated with improved cardiac function. Just before CPR, animals exhibited similar vascular loading based on identical asystolic pressures (Table 2). Nitrite did not decrease diastolic blood pressure, a coronary perfusion pressure surrogate, during CPR, and ROSC occurred sooner \((P = 0.034)\) than with placebo treatment. Consistent with improved cardiac function, nitrite-treated mice exhibited trends toward less tachycardia than placebo-treated controls \((68.7 \pm 12.1- \text{versus} \ 94.7 \pm 17.8-\text{bpm increase})\) and less hypotension \((9.1 \pm 3.2- \text{versus} \ 12.6 \pm 4.2-\text{mm Hg decrease})\).

Nitrite significantly improved postarrest LVEF \((54.4 \pm 2.4\%; \text{Figure 3A and 3C})\) compared with placebo-treated mice \((43.5 \pm 2.9\%; \text{P} = 0.007)\). Heart rate and blood pressure were similar between treatment groups throughout post-ROSC monitoring (data not shown; \(P > 0.2\)). Given similar baseline volume loading before CPR and similar mean blood pressures before imaging, the LVEF improvements likely indicate improved LV contractility. RVEF measured by magnetic resonance imaging (Figure 3B and 3D) 24 hours after CPR was significantly better in nitrite-treated mice \((54.7 \pm 1.3\%)\) than in placebo-treated mice \((42.8 \pm 1.7\%; \text{P} < 0.001)\). Consistent with improved pulmonary perfusion, gas exchange 5 minutes after CPR was improved with nitrite therapy compared with placebo (Table 1).

**Nitrite Repletion Improves Survival and Survivor Neurological Function**

Death after ROSC occurred 1 to 6 hours after CPR; after that point, all animals survived until the subsequent day (Figure 4). We observed bradycardia and hypotension progressing to asystole in mice dying early (while monitored), consistent with death from posts ischemic myocardial stunning and heart failure.
failure. Nitrite therapy significantly improved survival (19 of 25 [76%]) compared with placebo (12 of 25 [48%]; hazard ratio, 2.72; 95% confidence interval, 1.1 to 6.7; P=0.033).

Because a third of cardiac arrest survivors have neurological disability, we blindly assigned neurological scores to all mouse pairs surviving 1 day (n=11; Figure 5A). The median score for nitrite-treated mice was significantly better than for placebo-treated mice (9; P=0.020). In all pairs, placebo-treated mice exhibited impairment that was equal to or more severe than nitrite-treated mice. We measured rectal temperature 22 hours after resuscitation (Figure 5B) because rodent hypothermia correlates with severity of injury. Nitrite-treated mice had better thermoregulation (34.9±0.4°C) than placebo-treated mice (32.4±0.9°C; P=0.013). Sham surgery did not impair neurological scores (all 12) or thermoregulation (36.5±0.5°C). Hippocampal CA1, known to be selectively vulnerable to global ischemia, showed no histological injury at 24 hours, consistent with prior observations.

Seventy-two–hour survival experiments were therefore performed (n=3), demonstrating increased CA1 neuronal injury in placebo-treated compared with nitrite-treated mice (Figure 5C and 5D).

Nitrite Specifically and Reversibly Inhibits Complex I

On the basis of prior observations, we hypothesized that nitrite therapy in vivo would transiently S-nitrosate and inhibit mitochondrial complex I, resulting in a decrease in reperfusion ROS. Heart mitochondria from mice treated with nitrite isolated 5, 15, and 60 minutes after CPR exhibited reduced state 3 respiration using the complex I substrate pyruvate (Figure 6A) at 5 and 15 minutes but not 1 hour compared with placebo. Nitrite therapy did not reduce electron transport efficiency based on respiratory control ratio (5 minutes, 9.5±1.7; 60 minutes, 13.9±4.4), which was similar to the placebo-treated group (5 minutes, 7.5±1.0; 60 minutes, 10.4±4.6). Complex II (succinate)–dependent state 3 respiration was similar at all times (Figure 6B) and did not change with rotenone inhibition (data not shown), indicating the complex I specificity of nitrite. Complex I activity measured by NADH oxidation at 5 and 60 minutes confirmed the respirometry findings (Figure 6C). Using pyruvate as substrate, we consistently found increased oxygen consumption by placebo-treated postarrest mitochondria compared with prearrest despite reduced complex I (NADH consumption). This suggests pathological oxygen consumption to form ROS rather than to produce energy. Complex I inhibition was reversed 60 minutes after CPR as measured by respirometry (Figure 6A) and NADH oxidation (Figure 6C). ATP generation was similar between 60 minutes postarrest nitrite-treated mice (58.1±8.1 nmol · min⁻¹ · g⁻¹ protein) and placebo-treated mice (57.1±8.7 nmol · min⁻¹ · g⁻¹) and prearrest mice (65.1±4.3 nmol · min⁻¹ · g⁻¹), indicating no persistent functional complex I inhibition.

Nitrite Limits Reperfusion ROS Generation

Consistent with pathological oxygen conversion to ROS, peroxide generation by respiring mitochondria (pyruvate substrate) 5 and 15 minutes after CPR exceeded that of prearrest mice but normalized by 1 hour (Figure 7A). Cardiac mitochondria from nitrite-treated mice had significantly less peak (5 minutes after CPR) ROS production (14.0±3.2 pmol · min⁻¹ · mg⁻¹ protein) compared with placebo-treated mice (26.6±4.4 pmol · min⁻¹ · mg⁻¹; P<0.01). The abundant mitochondrial enzyme aconitase is susceptible to oxidative modification, which decreases its activity, and thus is useful as an indicator of oxidative damage. Cardiac arrest significantly decreased aconitase activity (Figure 7B). Compared with placebo (14.8±8.4 mU/mg protein), nitrite therapy attenuated this loss of aconitase function (61.6±11.4 mU/mg protein; P=0.05).

Discussion

We examined the effects of nitrite repletion on mitochondrial function, reperfusion ROS generation, organ function, and
Nitrite therapy effects on mitochondrial oxidative burst and injury. A, ROS generation was measured as mitochondrial peroxide production using the Amplex Red assay. Cardiac arrest increased ROS at 5 and 15 minutes, which resolved by 60 minutes. Compared with placebo, nitrite reduced ROS production 5 minutes (n=7) after CPR with a similar trend at 15 minutes (n=4). B, Cardiac arrest resulted in a significant loss of heart aconitase activity, which improves with nitrite therapy. Inset, Sample trace from each group. Values denoted as mean±SEM analyzed by paired t test (ROS) or ANOVA (aconitase). *P<0.01, †P=0.071, and ‡P<0.01, sham (n=11) vs placebo (n=8); #P=0.05, nitrite (n=8) vs placebo.

Nitrite therapy is complex I specific on the basis of the lack of effects with succinate. Complex I efficiency is unaffected; therefore, this is not due to complex I damage. Complex I inhibition is reversible on the basis of restored oxygen, NADH consumption, and ATP generation by 60 minutes. The increase in complex I oxygen consumption with placebo in the absence of increased NADH oxidation implies pathological oxygen consumption to form ROS rather than ATP, which is prevented with nitrite. On the basis of our ROS and aconitase data, nitrite is an antioxidant. This mechanism complements prior observations of reduced tissue nitrotyrosine staining, lipid peroxidation, and superoxide production with nitrite therapy.

The poor prognosis of cardiac arrest is driven primarily by brain and heart injury. Except for hypothermia, no beneficial postresuscitation therapies have emerged since the description of CPR 50 years ago. Present postresuscitation care is largely supportive. The role of nitrite as a novel therapeutic agent would be of great importance in this setting.

Human myocardial dysfunction (stunning) is common in cardiac arrest, is ultimately reversible, and is strongly associated with mortality. The molecular mechanisms of myocardial stunning after cardiac arrest remain unknown, but loss of excitation-contraction coupling is believed to result from ROS injury and calcium-mediated proteolysis. Nitrite, by reducing ROS, may mitigate stunning, similar to other antioxidants. The reduction in myocardial dysfunction likely explains the 50% relative survival advantage we noted. Further work is needed to characterize the effects of nitrite on brain injury, but our results are encouraging.
We designed a mouse model of cardiac arrest with prolonged asystole to study the effects of nitrite on heart and brain injury after resuscitation. Our model uses hyperkalemia to induce arrest, limiting its clinical relevance and potentially causing artifacts that may be organ protective (eg, cardioplegia) or injurious (endothelial damage perhaps causing RV dysfunction). In the context of these limitations, we demonstrate improvements in gas exchange, heart and brain function, and survival. We demonstrate that nitrite transiently inhibits complex I, resulting in an antioxidant effect. The ease in delivering intravenous nitrite, its established human safety, and the use of nitrite to detoxify hemoglobin-based oxygen carriers. Dr Gladwin is coinventor on National Institutes of Health/government patents for the use of nitrite salts for cardiovascular indications and the use of nitrite to detoxify hemoglobin-based oxygen carriers. The other authors report no conflicts.

Sources of Funding

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Disclosures

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References

Clinical Perspective

Cardiac arrest results in significant morbidity and mortality driven mainly by the cardiac and neurological injury resulting from global ischemia and reperfusion injury. Although resuscitation rates can exceed 65% for some rhythms, between 50% and 75% of these patients die before hospital discharge, and up to a third of survivors suffer significant brain injury. Only hypothermia has shown clinical benefit as a postresuscitation therapy in a subset of patients. Clearly, additional therapies are needed. The recent finding that nitrite acts as an ischemic reservoir for enzyme-independent nitric oxide generation has resulted in numerous animal studies in which it has been proven beneficial in reducing focal ischemic organ injury. On the basis of promising results in focal heart and brain ischemia, we have adapted a mouse model of cardiac arrest to model the high clinical mortality and myocardial and neurological dysfunction associated with cardiac arrest. In this model, nitrite therapy given at the start of resuscitation resulted in significant improvements in survival and myocardial and neurological function in survivors. We further investigate the potential mechanism for cardioprotection that involves the role of nitrite as a mitochondrial antioxidant early in resuscitation. The significant benefits attributed to nitrite, along with its ease of delivery and known primate and human safety data, make this a promising therapy for a condition with few current therapeutic options.
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