Preconditioning Results in S-Nitrosylation of Proteins Involved in Regulation of Mitochondrial Energetics and Calcium Transport

Junhui Sun, Meghan Morgan, Rong-Fong Shen, Charles Steenbergen, Elizabeth Murphy

Abstract—Nitric oxide has been shown to be an important signaling messenger in ischemic preconditioning (IPC). Accordingly, we investigated whether protein S-nitrosylation occurs in IPC hearts and whether S-nitrosoglutathione (GSNO) elicits similar effects on S-nitrosylation and cardioprotection. Preceding 20 minutes of no-flow ischemia and reperfusion, hearts from C57BL/6J mice were perfused in the Langendorff mode and subjected to the following conditions: (1) control perfusion; (2) IPC; or (3) 0.1 mmol/L GSNO treatment. Compared with control, IPC and GSNO significantly improved postischemic recovery of left ventricular developed pressure and reduced infarct size. IPC and GSNO both significantly increased S-nitrosothiol contents and S-nitrosylation levels of the L-type Ca\(^{2+}\) channel α1 subunit in heart membrane fractions. We identified several candidate S-nitrosylated proteins by proteomic analysis following the biotin switch method, including the cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, α-ketoglutarate dehydrogenase, and the mitochondrial F1-ATPase α1 subunit. The activities of these enzymes were altered in a concentration-dependent manner by GSNO treatment. We further developed a 2D DyLight fluorescence difference gel electrophoresis proteomic method that used DyLight fluoros and a modified biotin switch method to identify S-nitrosylated proteins. IPC and GSNO produced a similar pattern of S-nitrosylation modification and cardiac protection against ischemia/reperfusion injury, suggesting that protein S-nitrosylation may play an important cardioprotective role in heart. (Circ Res. 2007;101:1155-1163.)

Key Words: preconditioning • S-nitrosylation • cardioprotection

Ischemic preconditioning (IPC) is a cellular adaptive phenomenon whereby brief episodes of myocardial ischemia and reperfusion (I/R) render the heart resistant to subsequent prolonged ischemic injury.\(^1\) Through activation of a complex cascade of signaling events, IPC has been shown to reduce arrhythmias, infarct size, and posts ischemic contractile dysfunction.\(^2\)–\(^5\)

Nitric oxide (NO) has been shown to be an important signal in cardioprotection.\(^6\)–\(^8\) In acute IPC, NO has been shown to mediate protection at least in part by activation of guanylyl cyclase, resulting in the production of cyclic guanosine monophosphate (cGMP) and the activation of protein kinase G, which in turn leads to the opening of the mitochondrial K\(_{\text{ATP}}\) channel.\(^6\) Recently, it has also been shown that protein kinase G results in activation of an endogenous mitochondrial protein kinase Ce that is involved in activation of the mitochondrial K\(_{\text{ATP}}\) channel.\(^9\)–\(^10\) The opening of the mitochondrial K\(_{\text{ATP}}\) channel is reported to reduce mitochondrial Ca\(^{2+}\) loading\(^11\),\(^12\) and also to lead to generation of reactive oxygen species, which activate signaling cascades in a feed-forward manner to elicit cardioprotection.\(^13\),\(^14\)

In addition to activating cGMP/protein kinase G–dependent signaling pathways, NO can directly modify sulfhydryl residues of proteins through S-nitrosylation, which has emerged as an important posttranslational protein modification.\(^15\)–\(^17\) Furthermore, S-nitrosylation of critical protein thiols has been shown to protect them from further oxidative modification by reactive oxygen species.\(^15\)–\(^18\),\(^19\) S-Nitrosylation has recently been suggested to be important in cardioprotection.\(^20\),\(^21\) We have recently shown that cardioprotection in female hearts involves inhibition of I\(_{\text{Ca,L}}\) by S-nitrosylation.\(^20\) A recent study has shown that S-nitrosothiols (SNOs) were detected in mitochondria isolated from IPC hearts, suggesting that protein S-nitrosylation may play an important role in IPC cardioprotection.\(^22\)

The goal of this study was to examine the role of S-nitrosylation in cardioprotection and to identify S-nitrosylation–modified proteins and the functional effects of S-nitrosylation. We found that IPC results in S-nitrosylation
of a number of proteins that have important roles in cardio-
protection. We report the novel finding that cardioprotection
results in \( S \)-nitrosylation and decreased activity of the mito-
chondrial F1-ATPase. A decrease in F1-ATPase activity
would provide a mechanism for the reduced rate of decline in
ATP observed in IPC hearts.1

Materials and Methods

Animals

C57BL/6J mice were obtained from The Jackson Laboratory (Bar
Harbor, Me). All animals were adults, reproductively viable, and
between 12 and 15 weeks of age at the time of experimentation.
All animals were treated in accordance with NIH guidelines and
the Guiding Principles for Research Involving Animals and
Human Beings.

Treatment Protocol, Hemodynamic, and Infarct
Size Measurements

Hearts were Langendorff perfused in the dark and randomly assigned
to 1 of 6 groups (Figure 1, left). For details regarding the protocol,
hemodynamic, and infarct size measurements, see the online data
supplement at http://circres.ahajournals.org.

Preparation of Crude Homogenate and Membrane
Fractions From Mouse Hearts

All preparative procedures were performed in the dark to prevent
light-induced cleavage of SNOs. The crude heart homogenate and
the membrane fraction was prepared as described previously20 (see
the online data supplement for details).

SNO Content and Biotin Switch

\( S \)-Nitrosylation Detection

SNO in heart membrane fractions was detected by the 2,3-
diaminonaphthalene (Sigma) fluorometric method.20,22 \( S \)-Nitrosylated proteins were detected using a modified biotin switch method,23 as
described in our previous study20 (also see the online supplement).
To identify additional \( S \)-nitrosylated proteins in IPC or GSNO-
treated hearts, we tried several approaches. For samples from IPC
hearts, after the biotin switch method the respective protein bands
with the same migration as positive anti-biotin detection were
excised from the EZ-blue–stained 4% to 20% SDS-PAGE gel and
subjected to in-gel tryptic digestion. For the GSNO-treated samples,
after the biotin switch method, the biotinylated proteins were
purified on streptavidin–agarose beads (Sigma) for 1 hour at 25°C,
and in-bead tryptic digestion was performed for the MALDI-TOF
(matrix-assisted laser desorption ionization time-of-flight) peptide
mass fingerprinting.

S-Nitrosylation Identification by Two-Dimensional
DyLight Fluorescence Difference Gel
Electrophoresis Proteomic Analysis

The newly developed DyLight maleimide sulhydryl-reactive fluors
(Pierce, Rockford, Ill) were used to replace the biotin-HPDP\{N-[6-
(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide\} in the bi-
otin switch method at pH 7.0. The SNO-derived cysteine residues in
each sample taken before sustained ischemia were individually labeled by DyLight 488 (for perfusion control), DyLight 549 (for
IPC heart), and DyLight 649 (for GSNO-treated heart). After labeling, equal amounts of each sample were mixed together and subjected to 2D DyLight fluorescence difference gel electrophoresis (DyLight Fluor DIGE), ie, running on the same isoelectric focusing (pI 3 to 10; Amersham Biosciences, Piscataway, NJ) and 10% to 15% gradient SDS-PAGE (NextGen, Ann Arbor, Mich). Gels were scanned on a Typhoon 9400 variable mode imager (Amersham Biosciences) at a resolution of 100 μm. Each of the individual samples was visualized independently by selecting the individual excitation and emission wavelength with fluorescence scanning. All of the images scanned from the same gel were aligned by 2 internal fluorescence anchor spots in the gel and the image analysis was performed using single-stain analysis with intelligent noise correction algorithm processing by Progenesis Discovery software (Nonlinear Dynamics, Newcastle on Tyne, UK). DyLight-labeled protein will cause a positive/acidic shift (on isoelectric focusing) and thereby a left shift (on 10% to 15% SDS-PAGE) because each DyLight fluor molecule contains 3 to 4 negative charges. Meanwhile, the ~1-KDa mass from each DyLight fluor molecule will also cause a minor shift upwards. To sample the protein spots from a gel with a shifted DyLight pattern, the gel was poststained with SYPRO Ruby (Sigma) to ensure that a protein from a spot will provide sufficient sample for mass spectrometric (MS) identification. In addition, some spots with strong DyLight signal were directly picked from the gel for further clarification. The Ettan Spot Handling Workstation (Amersham Biosciences) performed automated extraction and in-gel trypsin digestion of selected protein spots according to the instruction. Peptides were analyzed using a MALDI-TOF mass spectrometer (4700 Proteomics Discovery System; Applied Biosystems, Foster City, Calif) for peptide mass fingerprinting and tandem mass spectrometry. Proteins were identified from the acquired spectra using the MASCOT database search function.

Enzyme Assays

All enzyme assays were conducted at 24°C in the dark in the presence of 0 to 1.0 mmol/L GSNO. Sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) activity in membrane fractions was assayed by the Pi-sensitive malachite green ATPase method. The reaction was started by adding Mg2+-ATP to a medium containing membrane fractions in the absence and presence of 1 μmol/L ionomycin (Ca2+ ionophore, Sigma). The reaction was stopped by trichloroacetic acid precipitation, and SERCA activity was assayed by the Pi-sensitive malachite green ATPase method.24

Figure 2. S-Nitrosylation of the L-type Ca2+ channel α1 subunit in IPC and GSNO-treated hearts. Following the biotin switch method, the biotinylated/S-nitrosylated proteins in heart membrane fraction from IPC (A) and GSNO-treated (B) hearts were immunoprecipitated (IP) with anti-L-type Ca2+ channel α1 antibody, and the precipitants were detected by anti-L-type Ca2+ channel α1 and anti-biotin immunoblot (IB) under nonreducing conditions. Mean densitometry data from 3 individual experiments. *P<0.05, compared with perfusion (Perf) control.

Results

IPC Reduces I/R Injury and Increases SNO Content

We were interested in examining the role of S-nitrosylation in cardioprotection. As shown in Figure 1A (right), IPC significantly increased the postischemic recovery of left ventricular developed pressure (LVDP). The LVDP at 40 minutes of reperfusion and 20 minutes of ischemia was 36.8±4.8% (n=8) of preischemic LVDP, whereas it was 62.1±6.7% (n=10) of preischemic LVDP, in IPC hearts. Consistent with the improved functional recovery, infarct size (Figure 1B) was also significantly less in IPC hearts (6.4±1.1%) compared with control hearts without IPC (28.2±5.6%). The SNO contents (Figure 1C) in membrane fractions of perfusion control heart was 3.4±0.6 pmol/mg protein, whereas there was almost a 3-fold increase of SNO content in IPC hearts (9.9±0.4 pmol/mg protein). After 20 minutes of ischemia and 40 minutes of reperfusion, the SNO content in IPC hearts was decreased to 5.6±0.4 pmol/mg protein, a value still significantly higher than perfusion control. Thus, IPC leads to an increased formation of SNO, consistent with a role for protein S-nitrosylation in IPC.
IPC Increases S-Nitrosylation Level of the L-type Ca\(^{2+}\) Channel \(\alpha 1\) Subunit

We have previously reported that the L-type Ca\(^{2+}\) channel \(\alpha 1\) subunit is among the predominant S-nitrosylated proteins in the membrane fraction of cardiac muscle and that S-nitrosylation of the L-type Ca\(^{2+}\) channel \(\alpha 1\) subunit reduces Ca\(^{2+}\) entry via the channel, which reduces Ca\(^{2+}\) overload and contributes to cardioprotection in female hearts.\(^{20}\) Therefore, we examined whether IPC results in any change in the level of S-nitrosylation of the L-type Ca\(^{2+}\) channel. Following the biotin switch method, we immunoprecipitated the membrane fraction with anti-L-type Ca\(^{2+}\) channel \(\alpha 1\) antibody and then probed the immunoprecipitate with anti-biotin antibody. The anti-biotin signal of the L-type Ca\(^{2+}\) channel \(\alpha 1\) (Figure 2A) was significantly higher in IPC hearts. After I/R, the level of S-nitrosylation of the L-type Ca\(^{2+}\) channel \(\alpha 1\) was decreased but still higher than perfusion control.

IPC Increases S-Nitrosylation Level of Other Proteins

To investigate other S-nitrosylated proteins in IPC hearts, in addition to the membrane fractions used in Figure 2, whole cell homogenates were also subjected to the biotin switch method. The EZ-blue–stained protein bands that comigrated with positive anti-biotin blots were excised and subjected to in-gel trypsin digestion and MS analysis. Several S-nitrosylated candidate proteins in IPC hearts were identified, including (Figure 3) the cardiac isoform SERCA (SERCA2a), glycogen phosphorylase, \(\alpha\)-KGDH, myomesin, and cardiac \(\alpha\)-myosin heavy chain.

GSNO Preconditioning Increases LVDP Recovery and SNO Content

To determine whether an increase of NO/SNO elicits similar effects on S-nitrosylation and protection, we examined the effect of treatment with GSNO. Pharmacological preconditioning using GSNO has been reported in other studies.\(^{27,28}\) However, its molecular mechanism is still unclear. Perfusion of hearts with 0.1 mmol/L GSNO for 30 minutes did not significantly change hemodynamics (see the online data supplement). As shown in Figure 1, GSNO significantly improved posts ischemic recovery of LVDP (54.8 ± 7.9% versus 36.8 ± 4.8% in control) and also reduced infarct size (8.2 ± 2.4% versus 28.2 ± 5.6% in control). GSNO also increased the SNO content in the heart membrane fraction to a level comparable to IPC.

**S-Nitrosylated Protein Induced by GSNO Preconditioning**

We also examined whether GSNO-mediated protection would result in S-nitrosylation of the L-type Ca\(^{2+}\) channel \(\alpha 1\) subunit, which was S-nitrosylated in IPC hearts (Figure 2A). As shown in Figure 2B, the S-nitrosylation level of the L-type Ca\(^{2+}\) channel \(\alpha 1\) subunit was also found to be significantly higher in GSNO-treated hearts compared with perfusion control. Thus, IPC and GSNO both significantly increase S-nitrosylation of the L-type Ca\(^{2+}\) channel.

To identify additional S-nitrosylated proteins occurring with GSNO treatment, after the biotin switch procedure to label the S-nitrosylated proteins, the samples were subjected to streptavidin–agarose chromatography. The MS analysis after in-bead tryptic digestion identified several S-nitrosylated proteins, including the mitochondrial F1-ATPase \(\alpha 1\) subunit, SERCA2a, cardiac \(\alpha\)-myosin heavy chain, and myosin light chain 1. Interestingly, SERCA2a was also identified as an S-nitrosylated protein in IPC hearts (Figure 3).

**Identification of S-Nitrosylated Proteins by DyLight Fluor DIGE Proteomic Analysis**

We were interested in obtaining a direct comparison of S-nitrosylated proteins in IPC and GSNO-treated hearts. In addition, it would be useful to have a 2D method for S-nitrosylated proteins to provide better separation of proteins for MS identification. The biotin labeling of proteins in the biotin switch method is somewhat labile, and the biotin would be lost if gels were run under reducing conditions, as is common for most 2D methods. As described in Materials and Methods, we therefore developed a DyLight Fluor DIGE proteomic method that would allow labeling of S-nitrosylated proteins in IPC hearts. Total crude homogenates were prepared from the following groups: perfusion (lane 1), IPC (lane 2), IPC-I/R (lane 3), and Perf-I/R (lane 4). After the biotin switch method, the biotinylated/S-nitrosylated proteins were subjected to 4% to 20% nonreducing gradient SDS-PAGE. The protein bands in EZ-blue–stained gel corresponding to the anti-biotin signal (as shown in dashed box) were trypsin digested for MS peptide identification. One representative anti-biotin blot and EZ-blue gel staining, from 3 separate experiments, is shown, and the proteins identified are listed in the inset table.
proteins by DyLight–maleimide fluors and separation by a 2D fluorescence difference gel electrophoresis. Perfusion control samples were labeled with DyLight 488. As shown in Figure 4A, the very faint DyLight 488 signal suggests that a small level of endogenous $S$-nitrosylation was present under control conditions. IPC and GSNO significantly increased the protein $S$-nitrosylation, as shown by the increased fluorescence signal of DyLight 549 (labeling IPC sample) and DyLight 649 (labeling GSNO-treated sample). By overlaying the DyLight images to the Ruby images (Figure 4B), the Ruby protein spots with the same pattern as DyLight positive signal were chosen for MS identification, taking into account the shift attributable to DyLight modification of the proteins.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Protein ID</th>
<th>Mass (KDa)</th>
<th>pI</th>
<th>SNO Level (Arbitrary Ratio of DyLight Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitase</td>
<td>Q99K10</td>
<td>86</td>
<td>8.08</td>
<td>IPC/Control 5.8±0.6, GSNO/Control 23.5±2.6, GSNO/IPC 4.0±0.2</td>
</tr>
<tr>
<td>Mitochondrial complex I-75 KDa</td>
<td>Q91VD9</td>
<td>80</td>
<td>5.51</td>
<td>...</td>
</tr>
<tr>
<td>Serum albumin precursor</td>
<td>P07724</td>
<td>70</td>
<td>5.75</td>
<td>...</td>
</tr>
<tr>
<td>Heat shock protein 60</td>
<td>P63038</td>
<td>61</td>
<td>5.91</td>
<td>...</td>
</tr>
<tr>
<td>Mitochondrial F1-ATPase α1 subunit</td>
<td>Q03265</td>
<td>59</td>
<td>9.22</td>
<td>...</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>Q6P8J7</td>
<td>47</td>
<td>8.64</td>
<td>...</td>
</tr>
<tr>
<td>Acyl-CoA dehydrogenase</td>
<td>P45952</td>
<td>46</td>
<td>8.60</td>
<td>...</td>
</tr>
<tr>
<td>Creatine kinase M chain</td>
<td>P07310</td>
<td>43</td>
<td>6.58</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>α-Cardiac muscle actin</td>
<td>P68033</td>
<td>42</td>
<td>5.23</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>P14152</td>
<td>36</td>
<td>6.16</td>
<td>...</td>
</tr>
<tr>
<td>Electron transfer flavoprotein α</td>
<td>Q99LCS</td>
<td>35</td>
<td>8.62</td>
<td>...</td>
</tr>
<tr>
<td>Myosin light chain 1</td>
<td>P09542</td>
<td>22</td>
<td>5.03</td>
<td>3.6±0.6</td>
</tr>
</tbody>
</table>

Each protein was reproducibly identified from three individual DyLight DIGE proteomic analyses, which was identified by at least 2 peptides with the scoring criteria of the best ion score confidence interval (≥95%). — indicates not detectable in control DyLight 488 fluorescence.
(arrowheads in Figure 4B). The S-nitrosylated proteins identified by DyLight Fluor DIGE proteomics are listed in Table.

**Functional Relevance of S-Nitrosylation to Cardioprotection**

We identified several proteins that could be important in cardioprotection that were S-nitrosylated by either IPC or treatment with GSNO. We next examined whether S-nitrosylation altered the activities of these proteins. We examined the effect of GSNO treatment (1) on the activity of SERCA2a in the membrane fractions and (2) on the activity of α-KGDH and F1-ATPase in the mitochondrial fractions. As shown in Figure 5A, GSNO concentration-dependently increased SERCA activity. GSNO also concentration-dependently increased the activity of α-KGDH, either in a purified enzyme from porcine heart (Sigma, data not shown) or in our isolated mitochondrial fractions (Figure 5B). We next examined the effect of GSNO treatment on the activity of the mitochondrial F1-ATPase in submitochondrial particles prepared from sonicated mitochondrial fractions. As shown in Figure 5C, there was a decrease in the mitochondrial F1-ATPase activity with increasing concentrations of GSNO. This would be consistent with the decrease in ATP consumption observed previously in IPC hearts.1

**Effect of GSNO on Ca\(^{2+}\) Transient and Ca\(^{2+}\) Release by Caffeine in Cardiomyocytes**

As shown in Figure 6A and B, GSNO (0.1 mmol/L) significantly decreased the Ca\(^{2+}\) transient in field-stimulated cardiomyocytes, consistent with reduced Ca\(^{2+}\) entry caused by inhibition of the L-type Ca\(^{2+}\) channel by S-nitrosylation and the resultant reduction of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release.20 Furthermore, the half-decay time of the Ca\(^{2+}\) transient (Figure 6C) was significantly shortened in GSNO-treated cardiomyocytes, consistent with increased SR Ca\(^{2+}\) uptake attributable to activation of SERCA2a by S-nitrosylation (Figure 5A). However, GSNO did not alter SR Ca\(^{2+}\), because Ca\(^{2+}\) release by caffeine in the presence of GSNO was comparable to the control (Figure 6B). These results show that GSNO could significantly decrease the cytosolic Ca\(^{2+}\) transient, which would reduce Ca\(^{2+}\) overload after I/R.

**Discussion**

**S-Nitrosylated Proteins and Cardioprotection**

The consistent relationship between the increase of protein S-nitrosylation and reduced infarct size suggests that S-nitrosylation might play a cardioprotective role. We found that cardioprotection was associated with increased S-nitrosylation of a number of proteins and we further
showed that SNO altered enzymatic activities of these proteins. Of particular interest, we observed that GSNO treatment resulted in increased S-nitrosylation of the mitochondrial F1-ATPase, which resulted in decreased activity. It has been reported that ~50% of the ATP generated during ischemia by glycolysis is consumed by reverse mode of the mitochondrial F1-ATPase. Therefore inhibition of the F1-ATPase during ischemia would conserve ATP. Indeed, inhibition of the F1-ATPase was an early hypothesis to explain the reduced rate of decline in ATP observed during ischemia in IPC hearts. Furthermore, the ATP consumed by the reverse mode of the F1-ATPase is used to maintain the mitochondrial $\Delta\psi$ and transport $Ca^{2+}$ into the mitochondria. Thus inhibition of the F1-ATPase could be beneficial by conserving cytosolic ATP and by reducing $Ca^{2+}$ uptake into the mitochondria. However, when the rate of the F1-ATPase was measured in submitochondrial particles, no differences in activity were observed between IPC and non-IPC hearts. Because S-nitrosylation is easily reversed, particularly if the samples are exposed to light and metal ions, it is likely that the IPC mediated S-nitrosylation of the F1-ATPase would be lost during isolation of the submitochondrial particles in those studies.

It has recently been reported that IPC prevents the I/R-induced loss of activity of $\alpha$-KGDH. Interestingly, we found that IPC resulted in increased S-nitrosylation of $\alpha$-KGDH. We further found that GSNO treatment increased activity of $\alpha$-KGDH, perhaps contributing to the IPC-mediated maintenance of $\alpha$-KGDH activity. It is tempting to speculate that the S-nitrosylation of $\alpha$-KGDH protects it from oxidative damage during I/R.

The original report describing IPC indicated that IPC reduced the accumulation of lactate during sustained ischemia. IPC has also been reported to reduce ischemic acidosis, and Weiss et al demonstrated that IPC results in the attenuation of glycolysis. It is therefore of interest that IPC increased S-nitrosylation of glycogen phosphorylase, the enzyme responsible for catabolism of glycogen. Furthermore, by DyLight Fluor DIGE proteomic analysis, several other important mitochondrial proteins, such as aconitase, creatine kinase, malate dehydrogenase, acyl-CoA dehydrogenase, complex I-75 KDa, heat shock protein 60, and electron transfer flavoprotein $\alpha$, were also found to be S-nitrosylated in IPC and GSNO-treated hearts (Table). Future studies will be directed at determining whether S-nitrosylation alters the activities of these metabolic enzymes.

In this study, we found that IPC resulted in an increase in S-nitrosylation of the L-type $Ca^{2+}$ channel $\alpha1$ subunit. This is consistent with our previous study of cardioprotection in hypercontractile female hearts, in which we observed increased S-nitrosylation that resulted in decreased $Ca^{2+}$ entry via the L-type $Ca^{2+}$ channel. The increase in S-nitrosylation of the L-type $Ca^{2+}$ channel and reduced $Ca^{2+}$ entry resulted in reduced I/R injury. Consistent with this hypothesis, we have previously reported that preconditioning reduces cytosolic $Ca^{2+}$ levels during ischemia, and in this study, we found that GSNO treatment also led to reduction of the cytosolic $Ca^{2+}$ transient (Figure 6). NO has been reported to mediate the activation of SERCA2a. Protein modification mediated by NO carriers could result from S-nitrosylation or from other secondary oxidative modifications such as S-glutathiolation. We found that both IPC and GSNO result in S-nitrosylation of SERCA2a, and we further showed that GSNO treatment causes an increase in SERCA2a activity (Figures 5A and 6). An increase in SERCA2a activity during ischemia and early reperfusion would provide for improved $Ca^{2+}$ uptake into the SR, which could reduce cytosolic $Ca^{2+}$ and reduce diastolic $Ca^{2+}$ during I/R, making SERCA2a a plausible target for cardioprotection. Indeed, adenoviral-mediated overexpression of SERCA2a has been reported to reduce infarct size and improve function following ischemia. In addition, the SR has been suggested to be a primary target of reperfusion protection. $Ca^{2+}$-induced $Ca^{2+}$ release is the well-known molecular mechanism of excitation–contraction coupling in cardiac muscle. The overall effect of S-nitrosylation on intracellular $Ca^{2+}$ handling, ie, decreased $Ca^{2+}$ entry by the inhibition of the L-type $Ca^{2+}$ channel and increased SR $Ca^{2+}$ uptake by the activation of SERCA2a, will lead to the attenuation of the rise in cytosolic $Ca^{2+}$ during ischemia and $Ca^{2+}$ overload during reperfusion.

**Relationship Between SNO and Other PC-Signaling Mechanisms**

It has been well established that NO is an essential component of IPC (see Figure 7). However, other signaling molecules have also been shown to be essential. How do we reconcile the observation that inhibition of any of these pathways blocks IPC? Two explanations are generally proposed. It has been suggested that IPC is a linear pathway, and therefore inhibition at any part of the pathway blocks IPC. This model would suggest that there is a single final effector. An alternative proposal is that IPC involves multiple signaling pathways and to achieve protection requires integration of these multiple pathways. In this latter case, inhibition of one arm of the protective signaling cascade may or may not block cardioprotection depending on the strength of the cardioprotective initiator (eg, 1 versus 4 cycles of IPC) and the length of the sustained ischemic period. These factors may account for some of the variability of the data in the literature. Recent data have suggested that inhibition of the mitochondrial permeability transition (MPT) pore is an important (if not final effector) component of cardioprotection. Many of the cardioprotective signals appear to converge on inhibition of MPT. MPT is regulated by reactive oxygen species and calcium, and there may be multiple mechanisms that can inhibit MPT. Our data suggest that NO has additional actions that can also reduce ischemic injury and reduce MPT opening. As discussed, S-nitrosylation and inhibition of the L-type $Ca^{2+}$ channel would reduce $Ca^{2+}$ loading of the myocytes, which would reduce $Ca^{2+}$ available to activate the MPT. S-Nitrosylation of the SERCA2a, which has been previously reported to reduce infarct size and improve function following myocardial ischemia, would reduce cytosolic and mitochondrial $Ca^{2+}$, which would reduce MPT. We also found a number of mitochondrial targets, such as the F1-ATPase, that were S-nitrosylated, and many of these targets might also result in inhibition of MPT. We found that the addition of GSNO resulted in inhibition of the F1-ATPase. Inhibition
of the F1-ATPase during ischemia would reduce breakdown of glycolytic ATP and accelerate the fall in the mitochondrial membrane potential. The reduction in mitochondrial membrane potential would reduce reactive oxygen species generation and would reduce the driving force for Ca\(^{2+}\) entry into the mitochondria; these would both result in less activation of MPT. Our working hypothesis is that IPC results in activation of several interacting pathways that all seem to converge to block activation of the MPT, but the mechanisms may be different.

**Summary**

IPC has been reported to increase the formation of NO.\(^{41,42}\) Our study suggests that the increase in NO occurring in IPC hearts results in protein modifications such as S-nitrosylation, which is also likely to be involved in cardioprotection. An increase in SNO content in the mitochondrial fraction has been reported recently in IPC rat hearts.\(^{21}\) Recent studies have shown that protein S-nitrosylation could affect cell death or survival by reversibly regulating mitochondrial respiration\(^{21}\) and redox status.\(^{41}\) Because S-nitrosylation is a reversible modification, it can modify cysteine residues and thereby protect them from irreversible oxidation during sustained ischemia.\(^{15,18,44}\) In addition, S-nitrosylation can alter enzymatic activity, and this altered activity may play a role in cardioprotection.\(^{15,44}\) IPC might provide an environment that favors SNO, for example, favorable ion content, pH, and redox equilibrium. The increase of SNO contents and S-nitrosylated proteins in IPC hearts suggests that protein S-nitrosylation, similar to phosphorylation, may play an important role in IPC. The data suggest that protein S-nitrosylation might elicit cardioprotective effects by regulating intracellular Ca\(^{2+}\) handling, mitochondrial energetics, and sarcomeric ultrastructure. Further investigation correlating protein S-nitrosylation modification and functional regulation will provide a better understanding of the molecular mechanisms of its cardioprotective effect and provide new therapeutic opportunities and targets for intervention in ischemic injury.

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**Disclosures**

None.

**References**

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Treatment protocol

Hearts were Langendorff perfused in the dark and randomly assigned to one of six groups (as shown in left panel of Figure 1): (1) hearts in the perfusion control group were perfused for 60 min; (2) hearts in the ischemic preconditioning (IPC) group were perfused for 20 min, followed by four cycles of 5 min of ischemia and 5 min of reperfusion; (3) hearts in S-nitrosoglutathione (GSNO) treated group were perfused with control buffer for 30 min followed by 30 min perfusion with 0.1 mmol/L GSNO; (4) hearts in the perfusion-ischemia reperfusion (I/R) group were subjected to 60 min of perfusion, 20 min of no-flow ischemia, and 40 min of reperfusion; (5) hearts in the IPC-I/R group were subjected to IPC before I/R; (6) hearts in the GSNO-I/R group were subjected to GSNO treatment before I/R. An NO electrode (Apollo Free Radical Analyzer 4000, World Precision Instruments, Sarasota, FL) was used to characterize the peak concentrations and the duration of NO released by S-nitrosoglutathione (GSNO, Sigma, St. Louis, MO) in heart perfusion buffer. GSNO (0.1 mmol/L) attained peak concentrations of NO at $1.1 \pm 0.2 \mu$mol/L ($n=3$) with half lifetimes of $\sim 0.5$ hour, which is within the physiological range. Perfusion of hearts with 0.1 mmol/L GSNO for 30 minutes did not significantly change hemodynamics. The recording of left ventricular pressure (LVP) before and after infusion with 0.1 mmol/L GSNO are shown in the Supplemental Figure and the hemodynamic values are listed in Supplemental Table.
Hemodynamic and infarct size measurements

To monitor left ventricular developed pressure (LVDP), a latex balloon connected to a pressure transducer was inserted into the left ventricle of Langendorff perfused hearts. Pressure was recorded and digitized using a PowerLab system (ADInstruments, Colorado Springs, CO). The recovery of LVDP after 20 min of no-flow ischemia was expressed as a percentage of pre-ischemic LVDP before IPC or GSNO administration. For infarct size, the protocol was the same except that reperfusion was extended to 2 hrs. After that, the hearts were perfused with 1% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated in TTC at 37°C for 15 min, followed by fixation in 10% formaldehyde. Infarct size was expressed as the percentage of total area of cross-sectional slice through the ventricles.

Preparation of crude homogenate and membrane fractions from mouse hearts

All preparative procedures were performed in the dark to prevent light-induced cleavage of S-nitrosothiols. The crude heart homogenate was obtained by homogenizing the heart (3×5 s of Ultra Turrax T25 set at 22,000 rpm) on ice in 1.5 ml buffer containing (in mmol/L) sucrose 300, imidazole-HCl 20 (pH7.0), EDTA 1, neocuproine 0.1. An EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation, Indianapolis, IN) was introduced just before use. To obtain the membrane fraction, the crude homogenates were centrifuged for 15 min at 3,800×g at 4°C, the supernatant was filtered through cheesecloth and ultracentrifuged for 2 hours at 100,000×g at 4°C. The pellet was resuspended on ice in 1.5 ml buffer containing (in mmol/L) KCl 600, sucrose 300, imidazole-HCl 20 (pH7.0), EDTA 1, neocuproine 0.1, leupeptin 0.025, and pefabloc 0.25 using a dounce glass homogenization. The resuspended pellets were incubated on ice for 1 hour, followed by a 100,000×g ultracentrifugation at 4°C. The membrane fraction was obtained by resuspension of the pellet in 0.5 ml of homogenization buffer.
Detection of S-nitrosylated proteins in IPC or GSNO treated hearts

Following the biotin switch method, the biotinylated membrane fractions were immunoprecipitated with anti-L-type Ca^{2+} channel α1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and the precipitates were probed with anti-biotin (Sigma) and anti-L-type Ca^{2+} channel α1 antibodies, respectively.

Intracellular Ca^{2+} recording in cardiomyocytes

Cardiomyocytes loaded with Fluo-4/AM (Invitrogen, Carlsbad, CA) on coverslips were placed on the stage of a Nikon microscope connected to a spectrofluorometer EasyRatioPro (Photon Technology International, Birmingham, NJ) and superfused with 1.2 mmol/L CaCl_2 Tyrode’s solution at room temperature. After measurement of basal Ca^{2+} concentration, myocytes were field stimulated (25 V) at 0.5 Hz for 0.5 min. Ca^{2+} release by caffeine was introduced by bolus adding 10 mmol/L of caffeine after 0.5 min of perfusion without field stimulation. Cardiomyocytes were perfused with 0.1 mmol/L GSNO for one minute, then subjected to 0.5 min of field stimulation, and GSNO was also present in the following caffeine treatment.
Supplemental Figure Legend

To monitor left ventricular pressure (LVP), a latex balloon connected to a pressure transducer was inserted into the left ventricle of Langendorff perfused hearts. Pressure was recorded and digitized using a PowerLab system. A real trace of 5 seconds of LVP was representatively shown before (pre-GSNO) and after (post-GSNO) infusion with 0.1 mmol/L GSNO.
Supplemental Figure
Supplemental Table

<table>
<thead>
<tr>
<th>Hemodynamic values</th>
<th>Pre-GSNO</th>
<th>Post-GSNO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beat per min)</td>
<td>363 ± 12</td>
<td>336 ± 19</td>
</tr>
<tr>
<td>CFR (ml/min)</td>
<td>2.2 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>LVDP (cm H₂O)</td>
<td>142 ± 6</td>
<td>132 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE (n=6). HR, heart rate; CFR, coronary flow rate; LVDP, left ventricular developed pressure.