Postconditioning leads to an increase in protein S-nitrosylation

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Role of nitric oxide-mediated S-nitrosylation in ischemic postconditioning

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Abstract

Previous studies have shown a role for nitric oxide and S-nitrosylation (SNO) in postconditioning (PostC), but specific SNO proteins and sites have not been identified in the myocardium after PostC. In this study, we examined SNO signaling in PostC using a Langendorff perfused mouse heart model. After 20 min of equilibrium perfusion and 25 min of global ischemia, PostC was applied at the beginning of reperfusion with six cycles of 10 seconds of reperfusion and 10 seconds of ischemia. The total period of reperfusion was 90 min. Compared to the ischemia/reperfusion (I/R) control, PostC significantly reduced post-ischemic contractile dysfunction and infarct size. PostC-induced protection was blocked by treatment with L-NAME (10 \( \mu \)mol/L, a constitutive NO synthase inhibitor), but not by either ODQ (10 \( \mu \)mol/L, a highly selective soluble guanylyl cyclase inhibitor) or KT5823 (1 \( \mu \)mol/L, a specific protein kinase G inhibitor). Two biotin switch based methods, two dimensional CyDye-maleimide difference gel electrophoresis (2D CyDye-maleimide DIGE) and SNO-resin-assisted capture (SNO-RAC) were utilized to identify SNO-modified proteins and sites. Using 2D CyDye-maleimide DIGE analysis, PostC was found to cause a 25% or greater increase in SNO of a number of proteins, which was blocked by treatment with L-NAME in parallel with the loss of protection. Using SNO-RAC, we identified 77 unique proteins with SNO sites after PostC. These results suggest that NO-mediated SNO signaling is involved in PostC-induced cardioprotection and these data provide the first set of candidate SNO proteins in PostC hearts.
Introduction

Ischemic postconditioning (PostC), the intermittent interruption of blood flow during the first minute of reperfusion, has been reported to effectively reduce myocardial ischemia-reperfusion (I/R) injury (30, 33, 34). Nitric oxide (NO) has been shown to be an important signaling molecule involved in PostC-induced cardioprotection (2, 20, 33). Early studies suggested that NO mediates PostC-induced protection through the classical soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) signaling pathway (1, 32). A specific sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3–a]quinoxalin-1-one (ODQ), was shown to block the PostC-mediated reduction in infarct size in a Langendorff perfused heart model of I/R injury (8, 32). However, Cohen et al recently reported that pharmacological postconditioning with a NO donor, S-nitroso-N-acetyl- D,L-penicillamine (SNAP), was not blocked by ODQ, suggesting that a sGC/cGMP-independent NO signaling is important in mediating PostC (3).

Besides activating the sGC/cGMP signaling pathways, NO can directly modify protein sulphydryl residues through protein S-nitrosylation (SNO), which has emerged as an important protein post-translational modification in cardiovascular signaling (14, 23) and cardioprotection (24, 27, 28). SNO exerts cardioprotection not only through modulating protein structure and function, but also by shielding the S-nitrosylated thiol(s) from irreversible oxidative modification upon reperfusion (13, 28).
The goal of this study was to determine if PostC leads to an increase in protein SNO and to identify specific proteins and sites that undergo SNO following PostC. Using a modified biotin switch method with CyDye-maleimide mono-reactive fluorescence dyes and a two dimensional difference gel electrophoresis (2D DIGE), we found that PostC significantly increased protein SNO, and this increase was prevented with L-NAME treatment. SNO-resin-assisted capture (SNO-RAC) was utilized to identify those SNO-modified proteins and sites (i.e., 8 unique SNO-modified proteins in I/R hearts, while almost 10-fold more proteins in PostC hearts). These data are consistent with the hypothesis that PostC exerts its cardioprotection via NO-mediated protein SNO.
Methods and Materials

Animals and compounds

Male C57BL/6J mice obtained from Jackson Laboratories (BarHarbor, ME) were used for all experiments. Mice were between 12 and 16 weeks of age at the time of experimentation. All animals received humane treatment in accordance with National Institutes of Health guidelines and the “Guiding Principles for Research Involving Animals and Human Beings.” This study was reviewed and approved by the Institutional Animal Care and Use Committee of the National Heart Lung and Blood Institute. All compounds were obtained from Sigma (St. Louis, MO). The dose of each compound was based on previous studies (25, 26).

Langendorff perfused mouse hearts and I/R-PostC protocol

After anesthesia with pentobarbital and anticoagulation with heparin, a thoracotomy was performed and the heart was quickly excised and placed in ice-cold Krebs-Henseleit buffer (in mmol/L: 120 NaCl, 11 D-glucose, 25 NaHCO₃, 1.75 CaCl₂, 4.7 KCl, 1.2 MgSO₄, and 1.2 KH₂PO₄). The aorta was cannulated and the heart was perfused with Krebs-Henseleit buffer (oxygenated with 95% O₂/5% CO₂ and maintained at pH7.4) in retrograde fashion at a constant pressure of 100 cm of water at 37°C. The perfusion was performed in the dark to prevent light-induced SNO decomposition. After equilibrium perfusion for 20 min, mouse hearts were subjected
to 25 min of no-flow global ischemia followed by 90 min of reperfusion. PostC was performed at the beginning of reperfusion with 6 cycles of 10 seconds of reperfusion and 10 seconds of ischemia. Each drug was infused during the first 7 min of reperfusion including the PostC period. Hearts that displayed a persistent irregular beating pattern after 20 min of reperfusion were excluded. The timing of drug administration is illustrated in the I/R-PostC protocol as shown in Figure 1.

**Cardiac contractile function and infarct size measurements**

A latex balloon connected to a pressure transducer was inserted into the left ventricle of Langendorff perfused mouse hearts to monitor left ventricular developed pressure (LVDP). LVDP was recorded and digitized using a PowerLab system (ADInstruments, Colorado Springs, CO). The rate pressure product (RPP = LVDP x heart rate) was used as an index of cardiac contractile function. The post-ischemic functional recovery was expressed as percentage of the pre-ischemic RPP during the equilibrium period. For measurement of myocardial infarct size, at the end of the 90 min of reperfusion, hearts were perfused with 1% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) and incubated in TTC at 37°C for 15 min, followed by fixation in 10% (w/v) formaldehyde. Infarct size was expressed as the percentage of the total cross-sectional area of the ventricles.
Total heart homogenate preparation

Total heart homogenate was prepared for 2D DIGE and SNO-RAC proteomic analyses, and sample preparations were carried out in the dark to prevent SNO decomposition. Each snap-frozen mouse heart was powdered on liquid nitrogen followed by homogenization with a tight-fitting glass Dounce homogenizer on ice in 1.5 ml homogenate buffer containing (in mmol/L): 300 sucrose, 250 Hepes-NaOH (pH7.8), 1 EDTA, 0.1 neocuproine (a copper chelating agent). An EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation, Indianapolis, IN) was added to the homogenate buffer just before use. Protein concentration of total heart homogenate was determined using the Bradford protein assay. Total heart homogenates were aliquoted in amber tubes, snap-frozen on dry ice, and stored at -80°C.

Identification of SNO proteins by 2D CyDye-maleimide DIGE

We used a modified biotin switch method (10) with CyDye maleimide mono-reactive sulphhydryl-reactive fluorescent dyes (GE Healthcare Life Sciences, Piscataway, NJ) to identify SNO proteins. After the CyDye-maleimide switch and 2D DIGE, each gel was scanned at the unique excitation/emission wave length of each dye using a Typhoon 9400 imager (GE Healthcare Life Sciences) at a resolution of 100 µm. Images from each gel were aligned using the two internal anchor spots and analyzed
with Progenesis Discovery software (Nonlinear Dynamics, Newcastle upon Tyne, UK). The gel was post-stained with SYPRO Ruby (Sigma) and the protein spots that corresponded to the CyDye-maleimide fluorescent intensity with 25% or higher difference were picked. The Ettan Spot Handling Workstation (GE Healthcare Life Sciences) was used for automated extraction of the selected protein spots followed by in-gel trypsin digestion. After sample extraction from the spot handling workstation, each sample was manually desalted using Millipore (Billerica, MA) C18 Ziptips following the manufacturer’s recommendation in preparation for liquid chromatography-tandem MS (LC-MS/MS) analysis.

SNO protein and site identification with SNO-resin assisted capture (SNO-RAC)

A modified version of the SNO-RAC protocol was developed to identify SNO-modified proteins and sites (5, 11, 12). All buffers were degassed before use with the SNO-RAC protocol to prevent oxidation of the thiopropyl sepharose resin (GE Healthcare, Piscataway, NJ). In brief, after blocking free sulfhydryl group with 20 mmol/L NEM, each total heart homogenate (0.25 mg) was loaded onto pre-equilibrated resin in the presence of 20 mmol/L Na-ascorbate, and incubated for 4 hrs at room temperature. After washing, resin-bound proteins were then subjected to trypsin digestion (sequencing grade modified; Promega) overnight at 37°C with rotation in the digestion buffer (in mmol/l): 50 NH₄HCO₃ and 1 EDTA. After washing, peptides were eluted for 30 min at room temperature in elution buffer (in mmol/L): 20
DTT, 10 NH4HCO3, and 50% methanol (v/v), followed by two volumes of H2O. All
fractions were combined and concentrated via speedvac. Samples were then
resuspended in 0.1% formic acid and cleaned with Millipore C18 Ziptips for
LC-MS/MS analysis.

**LC-MS/MS Analysis and database search**

LC-MS/MS was performed using an Eksigent nanoLC-Ultra 1D plus system (Dublin,
CA) coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific,
San Jose, CA) using CID fragmentation. Peptides were first loaded onto an Zorbax
300SB-C18 trap column (Agilent, Palo Alto, CA) at a flow rate of 6 ul/min for 6 min,
and then separated on a reversed-phase PicoFrit analytical column (New Objective,
Woburn, MA) using a short 15-min linear gradient of 5-40% acetonitrile for 2D gel
spots and 40-min gradient for SNO-RAC in 0.1% formic acid at a flow rate of 250
nl/min. LTQ-Orbitrap Elite settings were as follows: spray voltage 1.5 kV; full MS
mass range: m/z 300 to 2000. The LTQ-Orbitrap Elite was operated in a
data-dependent mode; *i.e.*, one MS1 high resolution (60,000) scan for precursor ions
followed by six data-dependent MS2 scans for precursor ions above a threshold ion
count of 500 with collision energy of 35%. The raw file generated from the LTQ
Orbitrap Elite was analyzed using Proteome Discoverer v1.3 software (Thermo Fisher
Scientific, LLC) using our six-processor Mascot cluster at NIH (v.2.4) search engine.
The following search criteria was set to: database, Swiss Institute of Bioinformatics
(Sprot_103112, 16573 sequences); taxonomy, mus musculus (mouse); enzyme, trypsin; miscleavages, 2; variable modifications, Oxidation (M), Deamidation (NQ), Acetyl (protein N-term), N-ethylmaleimide(C); MS peptide tolerance 20 ppm; MS/MS tolerance as 0.8 Da. Protein identifications were accepted based on two or more unique peptides with a false discovery rate (FDR) of 99% or higher. Raw mass spectrometry data for SNO sites identified via SNO-RAC in PostC hearts (Table 3) can be accessed at Peptide Atlas (http://www.peptideatlas.org/PASS/PASS00392).

Data analysis

Results are expressed as mean ± SE. Statistical significance was determined by one-way ANOVA followed by a post-hoc Bonferroni test.
Results

Ischemic PostC exerted a protective effect

We first confirmed that a protocol of PostC with 6 cycles of 10 seconds of reperfusion and 10 seconds of ischemia applied at the beginning of reperfusion after 25 min of global no-flow ischemia (Figure 1) was cardioprotective. As shown in Table 1 and Figure 2, PostC significantly increased the post-ischemic functional recovery, as the rate pressure product (RPP) recovery was 46.1±2.9% (n=14) in PostC hearts compared to 20.9±3.7% (n=9) in I/R-control hearts. The post-ischemic myocardial infarction was 33.8±2.4% (n=14), significantly smaller than I/R-control hearts (56.7±4.2%, n=9).

Reperfusion with SNAP protected the heart against I/R injury

Cohen et al (2010) recently demonstrated that infusion of an NO/SNO donor SNAP at reperfusion has a protective effect in an intact rabbit coronary artery occlusion and reperfusion model (3). To test whether an NO donor can mimic PostC in our Langendorff perfused mouse heart I/R model, we infused the NO donor, SNAP (10 μmol/L), into the perfusate during the first 7 min of reperfusion. As shown in Figure 2, treatment with SNAP upon reperfusion significantly improved cardiac post-ischemic functional recovery (40.0±3.4%, n=8), and decreased post-ischemic myocardial infarct size (36.8±2.7%, n=8).
PostC increased myocardial SNO

To test whether PostC increased SNO, PostC hearts were collected and snap frozen in liquid N\textsubscript{2} after 2 min of PostC plus 5 min of additional reperfusion and I/R-control hearts after 7 min of reperfusion. The total heart homogenate was prepared in the dark as described in the Methods and Materials. A modified biotin switch method using CyDye-maleimide mono-reactive fluorescence dyes and 2D DIGE proteomic analysis was carried out for SNO detection (25, 26). As shown in Figure 3, SNO proteins in I/R-control hearts were labeled by Cy3-maleimide (pseudo-colored in green) and PostC hearts were labeled with Cy5-maleimide (pseudo-colored in red). SNO protein spots showing a change of at least 25% or higher in PostC hearts compared to I/R-control were picked for identification via mass spectrometry. As shown in Table 2, PostC-treated hearts showed an increase in SNO for a number of proteins, and most of these SNO proteins were previously found in IPC hearts (25, 26), including aconitase, ATP synthase subunit α, creatine kinase S/M type, α-cardiac muscle actin, cytoplasmic malate dehydrogenase, electron transfer flavoproteins α/β, myosin light chain 1, and myoglobin.

L-NAME abolished the PostC-induced increase in protein SNO

To confirm that the changes in SNO observed in Figure 3 and Table 2 are involved in the protection associated with PostC, we performed additional experiments using
L-NAME to determine whether the inhibition of NOS with L-NAME, which has been previously shown to block PostC-induced protection, would also block the PostC-mediated increase in protein SNO. Langendorff-perfused mouse hearts were treated with 10 µmol/L L-NAME during the first 7 min of reperfusion. Compared to I/R-control, perfusion of non-PostC hearts with L-NAME had no significant effect on post-ischemic functional recovery (Table 1, Figure 2A) or infarct size (Figure 2B). However, treatment with L-NAME blocked PostC-induced cardioprotection (Figure 2). After confirming that PostC-induced protection was blocked by L-NAME, we compared the SNO level of PostC hearts with and without L-NAME treatment to determine whether the PostC-induced increase in SNO could be prevented by L-NAME. As shown in Table 2, PostC+L-NAME hearts have significantly lower SNO levels compared to PostC hearts, suggesting that inhibition of NOS with L-NAME prevented the PostC-induced increase in SNO.

**S-nitrosylation site identification in PostC hearts via SNO-RAC analysis**

Because of dynamic range issues and the limited amount of protein that can be loaded onto the 2D gel, the SNO proteins identified in 2D DIGE are biased toward detection of high-abundance proteins (Table 2). Furthermore, although 2D fluor-maleimide DIGE is useful in screening and quantifying SNO proteins, unfortunately, we have not been able to identify SNO sites with this method (12). Therefore, we utilized a SNO-RAC protocol to identify SNO proteins and sites in total heart homogenates. As
shown in Figure 4, SNO-RAC detected only 10 SNO peptides from 8 unique proteins in I/R hearts, while 91 SNO peptides from 77 proteins were identified in PostC hearts. More than half (37 out of 77) of those identified SNO proteins are mitochondrial, suggesting mitochondria are a major organelle targeted by PostC-mediated SNO. Most of these proteins contain only one SNO modified cysteine, while ~20% of them contain two or more SNO sites (Table 3).

ODQ or KT5823 treatment did not block PostC-mediated protection

In non-PostC hearts, perfusion with either ODQ (a sGC inhibitor) or KT5823 (a specific PKG inhibitor) during the first 7 min of reperfusion did not significantly affect post-ischemic functional recovery or infarct size (Figure 2). In contrast to L-NAME treatment, which abolished the protection of PostC, 10 μmol/L ODQ or 1 μmol/L KT5823 treatment did not block PostC-induced cardioprotection, i.e., post-ischemic RPP recovery was 40.2±2.2% (n=7) for PostC+ODQ and 43.5±3.0% (n=5) for PostC+KT5823, infarct size was 35.1±3.7% (n=7) for PostC+ODQ and 32.5±2.3% (n=5) for PostC+KT5823, which were comparable to the protective effect induced by PostC.
Discussion

NO signaling has been suggested to play an important role in PostC-induced protection. Inhibition of NOS by L-NAME has been shown to block protection in a variety of postconditioning models (18, 29, 33). Furthermore PostC was blocked by reducing agents such as N-acetyl-L-cysteine or 2-mercaptopropionylglycine (19), suggesting that a redox-sensitive mechanism is also involved in the protection afforded by PostC. In addition, a recent study has suggested that PostC prolongs early acidosis, and this would favor the formation of protein SNO (22). Therefore, all of these studies suggest a possible role for protein S-nitrosylation in PostC-induced cardioprotection.

The results contained herein provide the first demonstration that PostC leads to an increase in protein SNO. We further show that this PostC mediated increase in protein SNO is blocked with L-NAME, which also blocks the protective effects of PostC. Comparing the SNO proteins measured by SNO-RAC in PostC hearts (Table 3 in this paper) with the proteins that show SNO in IPC hearts (Table 1 from Kohr et al Circ Res 2011), we find that ~50% of those proteins that were SNO with IPC also show SNO with PostC (25, 26), suggesting that there might be a common set of proteins targeted by NO/SNO signaling with both IPC and PostC. Therefore, the PostC-induced increase in SNO may play a similar role as IPC in cardioprotection against I/R injury. For example, we have shown that IPC led to increase in SNO of the mitochondrial F1-ATPase subunit α. In this study, we also found that PostC induced
an increase in SNO of the mitochondrial F1-ATPase (Table 2). In addition, the
IPC-induced increase in SNO could shield critical cysteine residue(s) from further
oxidative damage upon reperfusion (13). Interestingly, a similar finding has been
reported in a recent study, in which cys294 of the mitochondrial F1-ATPase was
found to form a disulfide bond with another cysteine residue in dyssynchronous heart
failure, while cardiac resynchronization therapy leads to SNO of Cys294 and prevents
disulfide formation (31).

The sGC/cGMP/PKG signaling pathway has been suggested to mediate
PostC-induced cardioprotection (4, 15), and the main supportive evidence is that
inhibition of the cGMP-dependent signaling pathway with selective inhibitors such as
ODQ or KT5823 blocks the protection induced by PostC (8, 18, 32). However, a
recent study questioned the role of NO-mediated sGC/cGMP/PKG-dependent
signaling in PostC by demonstrating that the addition of SNAP, an NO donor, at
reperfusion produced protection that was not blocked by ODQ (3). In addition, a role
for cGMP-independent NO-induced cardioprotection against I/R injury has also been
demonstrated in studies using isolated cardiomyocytes (6, 9). In the present study,
treatment of perfused mouse hearts with either ODQ or KT5823 did not alter
post-ischemic functional recovery or infarct size in non-PostC hearts. However,
mouse hearts treated with either ODQ or KT5823 were still protected by PostC,
suggesting that blockade of the sGC/cGMP/PKG pathway does not abolish
NO-dependent PostC-induced cardioprotection. In addition, Methner et al have shown
that protection through postconditioning is unaffected by cardiomyocyte-selective
ablation of protein kinase G (16).

Cardioprotection similar to that obtained with PostC could be also achieved with
pharmacological agents given upon reperfusion, i.e., pharmacological
postconditioning. By using an intact I/R heart model, Cohen et al showed that SNAP,
an S-nitrosothiol agent, was protective when administered upon reperfusion (3). In
this study, we found that SNAP also elicits pharmacological postconditioning effects
in Langendorff perfused mouse hearts (Figure 2 and Table 1). A
mitochondria-targeted S-nitrosothiol agent, mito-SNO, has also been shown to be
protective when given at reperfusion in a recent study with an open-chest mouse I/R
model. Furthermore, the protection afforded by PostC or mito-SNO has been found to
be unaffected by cardiomyocyte-selective ablation of PKG, suggesting an important
role for SNO signaling in PostC (16). In addition, Penna et al showed that
pharmacological PostC by diazoxide induced mitochondrial protein S-nitrosylation
(21).

**Limitations and Perspective.** The data in this paper demonstrate that PostC
leads to an increase in SNO and myocardial protection. Further, the protection
afforded by PostC was abolished by L-NAME, suggesting a key role for NO/SNO
signaling in PostC-induced protection. However, treatment with either ODQ (a
specific sGC inhibitor) or KT5823 (a specific PKG inhibitor) did not block
PostC-induced protection, suggesting that NO-mediated protein SNO, rather than
activation of the sGC/cGMP/PKG signaling pathway, plays an essential role in PostC.

These results together with similar findings from IPC hearts (25) suggest that NO-mediated protein SNO plays a common protective role in the myocardium. These studies have identified a number of proteins that undergo SNO with PostC. However, future studies will be needed to demonstrate the functional impact of SNO on these protein targets and their specific role in PostC.
Acknowledgements

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List of abbreviations:

2D DIGE, two dimensional difference gel electrophoresis;
cGMP, cyclic guanosine monophosphate;
IPC, ischemic preconditioning;
I/R, ischemia/reperfusion;
L-NAME, L-NG-nitroarginine methyl ester;
NO, nitric oxide;
NOS, nitric oxide synthase;
ODQ, 1H-[1,2,4]oxadiazolo[4,3–a]quinoxalin-1-one;
PKG, protein kinase G;
PostC, postconditioning;
sGC, soluble guanylyl cyclase;
SNAP, S-nitroso-N-acetyl-D,L-penicillamine;
SNO, S-nitrosylation.
References


32. Yang X-M, Philipp S, Downey JM, and Cohen MV. Postconditioning’s protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3–kinase and guanylyl cyclase activation. Basic Res Cardiol 100: 57-63, 2005.


Figure 1. I/R and PostC protocol

Mouse hearts were Langendorff perfused with Krebs-Henseleit buffer (oxygenated with 95% O₂/5% CO₂ and maintained at pH 7.4) at a constant pressure of 100 cm of water at 37°C in the dark. After equilibrium perfusion for 20 min, mouse hearts were subjected to 25 min of no-flow ischemia. PostC (6 cycles of 10 sec of ischemia and 10 sec of reperfusion) was applied immediately upon reperfusion, followed by an additional 90 min of reperfusion. Drug administration is illustrated for each PostC or I/R protocol.

Figure 2. PostC-induced cardioprotection was dependent upon NO/SNO signaling.

(A) Post-ischemic left ventricular RPP functional recovery. (B) Infarct size, measured at the end of reperfusion by 1% TTC staining. Results are expressed as mean ± SE. Statistical significance was determined by one-way ANOVA followed by a post-hoc Bonferroni test, *, p<0.05; **, p<0.01 vs I/R-Control; #, p<0.05 vs I/R+ODQ; ##, p<0.01 vs I/R+KT5823. The number of animals in each group is indicated in the column.
Figure 3. PostC increased myocardial protein SNO.

*Top panels:* A representative 2D CyDye-maleimide DIGE gel from three independent experiments was scanned at each of the distinct wavelengths of the fluors, showing a pattern of protein SNO for that particular treatment group. *Bottom panel:* overlaid image of Cy3-maleimide (I/R control, green) *vs* Cy5-maleimide (PostC, red). Protein spots showing a change of at least 25% or higher in PostC hearts compared to I/R-control were picked for MS/MS analysis, and are listed in Table 2.

Figure 4. PostC increased total number of SNO-modified proteins and sites.

Total number of SNO-modified proteins (A) and sites (B) from SNO-RAC were identified via LC-MS/MS for I/R and PostC hearts (n=3 in each group). Protein identifications were accepted based on two or more unique peptides with a false discovery rate (FDR) of 99% or higher. Each protein/peptide was identified from at least 2 out of 3 SNO-RAC/LC-MS/MS proteomic analyses.
Table 1. Evaluation of cardiac contractile function in Langendorff perfused mouse hearts

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<th>(n)</th>
<th>BW (g)</th>
<th>FR (ml/min)</th>
<th>HR (bpm)</th>
<th>LVDP (cmH₂O)</th>
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<tr>
<td>ODQ+PostC</td>
<td>7</td>
<td>28.3±0.5</td>
<td>2.5±0.2</td>
<td>379±20</td>
<td>130±7</td>
<td>9.0±0.6</td>
<td>−5.7±0.4</td>
<td>2.0±0.2</td>
<td>334±10</td>
<td>57±4#</td>
<td>4.7±0.3</td>
<td>−3.9±0.3</td>
</tr>
<tr>
<td>I/R-KT5823</td>
<td>4</td>
<td>27.6±0.3</td>
<td>2.5±0.1</td>
<td>416±24</td>
<td>124±6</td>
<td>8.4±0.6</td>
<td>−5.6±0.3</td>
<td>1.9±0.1</td>
<td>314±11</td>
<td>39±5</td>
<td>3.9±0.3</td>
<td>−3.1±0.4</td>
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<tr>
<td>KT5823+PostC</td>
<td>5</td>
<td>27.6±0.6</td>
<td>2.4±0.1</td>
<td>408±21</td>
<td>127±5</td>
<td>8.5±0.4</td>
<td>−6.3±0.4</td>
<td>1.9±0.1</td>
<td>344±19</td>
<td>65±3$</td>
<td>5.5±0.3</td>
<td>−4.0±0.2</td>
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</tbody>
</table>

Note: Values are mean ± SE; (n), number of hearts; BW, body weight; FR, flow rate; HR, heart rate (beats per min, bpm); LVDP, left ventricular developed pressure; ±dp/dt, rates of pressure rise and fall, respectively. * p<0.05, ** p<0.01, vs I/R-Control; # p<0.05, vs I/R-ODQ; $ p<0.05, vs I/R-KT5823.
Table 2. Proteins identified by 2D CyDye-maleimide DIGE with increased SNO level in PostC hearts.

<table>
<thead>
<tr>
<th>Spots</th>
<th>Protein Name</th>
<th>Accession number</th>
<th>Mw (kDa)</th>
<th>Protein pI</th>
<th>SNO level (arbitrary ratio)</th>
<th>PostC vs I/R</th>
<th>PostC vs PostC+L-NAME</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>2-oxoglutarate dehydrogenase</td>
<td>Q60597</td>
<td>116.4</td>
<td>6.83</td>
<td>1.48 ± 0.15</td>
<td>1.40 ± 0.16</td>
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<td>2</td>
<td>Aconitate hydratase, mitochondrial</td>
<td>Q99KI0</td>
<td>85.4</td>
<td>8.08</td>
<td>1.50 ± 0.19</td>
<td>1.38 ± 0.14</td>
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<tr>
<td>3</td>
<td>Mitochondrial F1-ATPase subunit α</td>
<td>Q03265</td>
<td>59.7</td>
<td>9.22</td>
<td>1.47 ± 0.18</td>
<td>1.50 ± 0.13</td>
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<td>4</td>
<td>Creatine kinase S-type</td>
<td>Q6P8J7</td>
<td>47.8</td>
<td>8.64</td>
<td>1.45 ± 0.18</td>
<td>1.53 ± 0.14</td>
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<tr>
<td>5</td>
<td>Creatine kinase M-type</td>
<td>P07310</td>
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<td>6.58</td>
<td>1.51 ± 0.28</td>
<td>1.41 ± 0.11</td>
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<tr>
<td>6</td>
<td>α-Cardiac muscle actin</td>
<td>P68033</td>
<td>42.3</td>
<td>5.23</td>
<td>1.45 ± 0.17</td>
<td>1.44 ± 0.16</td>
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<tr>
<td>7</td>
<td>Malate dehydrogenase, cytoplasmic</td>
<td>P14152</td>
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<td>6.16</td>
<td>1.46 ± 0.15</td>
<td>1.49 ± 0.19</td>
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<tr>
<td>8</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>P16858</td>
<td>35.8</td>
<td>8.25</td>
<td>1.28 ± 0.04</td>
<td>1.35 ± 0.09</td>
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<tr>
<td>9</td>
<td>Malate dehydrogenase, mitochondrial</td>
<td>P08249</td>
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<td>8.68</td>
<td>1.57 ± 0.14</td>
<td>1.45 ± 0.08</td>
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<td>10</td>
<td>Electron transfer flavoprotein α</td>
<td>Q99LC5</td>
<td>35.0</td>
<td>8.62</td>
<td>1.54 ± 0.12</td>
<td>1.55 ± 0.14</td>
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<td>11</td>
<td>Electron transfer flavoprotein β</td>
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<td>1.46 ± 0.12</td>
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<tr>
<td>12</td>
<td>Myosin Light chain 1</td>
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<td>Myoglobin</td>
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<td>1.30 ± 0.05</td>
<td>1.31 ± 0.07</td>
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</tbody>
</table>

Note: Protein identifications were accepted based on two or more unique peptides with a false discovery rate (FDR) of 99% or higher and a correct molecular mass identification. SNO protein spots showed a change of at least 25% or higher in PostC hearts compared to I/R-control or PostC+L-NAME hearts (p<0.05, n=3 in each group).
### Table 3. S-nitrosylation sites identified via SNO-RAC proteomic analysis in PostC hearts

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>ID</th>
<th>Peptide Sequence</th>
<th>SNO-Cys</th>
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<tbody>
<tr>
<td><strong>Extracellular matrix &amp; Cell membrane</strong></td>
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<td>Galectin-1</td>
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<td>Long-chain fatty acid transport protein 1</td>
<td>Q60714</td>
<td>VGSCGFNSR</td>
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<tr>
<td>PDZ and LIM domain protein 5</td>
<td>Q8C151</td>
<td>ACTGSLNMTLQR</td>
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<tr>
<td>Protein-glutamine-gamma-glutamyltransferase 2</td>
<td>P21981</td>
<td>YSGCLETESNLK</td>
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<td><strong>Cytoplasm &amp; Cytoskeleton</strong></td>
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<td>6-phosphofructokinase, muscle type</td>
<td>P47857</td>
<td>LPLMECVQVTK</td>
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<tr>
<td>Alpha-enolase*</td>
<td>P17182</td>
<td>VNOIGSVTESLQACK</td>
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<td>Annexin A6</td>
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<td>Cytoplasmic dynein 1 heavy chain 1</td>
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<td>Destrin</td>
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<td>Dihydropyrimidinase-related protein 2</td>
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<td>E3 ubiquitin-protein ligase UBR4</td>
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<td>Glutaredoxin-1</td>
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<td>Heat shock protein HSP 90-β</td>
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<td>L-lactate dehydrogenase A chain</td>
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<td>Rab GDP dissociation inhibitor β</td>
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<td>Titin*</td>
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<td>Triosephosphate isomerase</td>
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<td>Tripartite motif-containing protein 72</td>
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<td><strong>Mitochondria</strong></td>
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<tr>
<td>2-oxoglutarate dehydrogenase</td>
<td>Q60597</td>
<td>ICEEAFTR</td>
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<tr>
<td>3-ketoacyl-CoA thiolase</td>
<td>Q8BTW1</td>
<td>YAAGSACIGGGGQGIALIQNTA</td>
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Acetyl-CoA acetyltransferase Q8QZT1 QATLGAFLPSTPCCTTVNK 116
ATP synthase subunit ε P56382 FSQICAK 19
Aconitate hydratase Q99K10 VGLIGSCTNNSYEDMGR 385
Aspartate aminotransferase P05202 VGFVVCK 295
Carnitine O-acetyltransferase P47934 IYGQACATYESASLR 449
Carnitine O-palmitoyltransferase 1 Q924X2 SCTNESAAVQAMMK 608
Citrate synthase Q9CZU6 LPCVAAK 211
Creatine kinase S-type Q6P87 GLSLPPAC 180
Cytochrome b-c1 complex subunit 1* Q9CZ13 LCTSATESEVTR 380
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex Q8BMF4 DVPLGAPLCIIVEK 290
Dynamin-1-like protein Q8K1M6 FATEYCNTEIGTAK 351
Electron transfer flavoprotein-ubiquinone oxidoreductase Q921G7 ASCDAQTYGIGLK 265
Glutathione S-transferase κ 1 Q9DCM2 LIENPTDAACK 176
Iron-sulfur cluster assembly 2 homolog Q9DCB8 LTDSCEQOR 56
Isocitrate dehydrogenase [NADP]* P54071 SSGFVVWACK 308
Isocitrate dehydrogenase [NAD] subunit α Q9D6R2 IEAACAFATIK 331
cSDFTEE1CR 359
Lactation elevated protein 1 Q3V384 VVQC LQK 100
Leucine-rich PPR motif-containing protein P08249 GVLPQEPQDCLK 89
eGVECSFVOŠK 275
Mitochondrial tRNA-specific 2-thiouridylase 1 Q9DA15 LCPDDICM 401
NADH-ubiquinone oxidoreductase 75 kDa subunit Q91VD9 LSVAQNCR 75
AVTEGAQAVEEPSCI 727
NADH-ubiquinone oxidoreductase chain 3 P03899 ANPYECFDPHTSSAŘ 39
Propionyl-CoA carboxylase α chain Q91ZA3 MADEAVCVGPAPTSK 107
Short/branched chain specific acyl-CoA dehydrogenase Q9DBL1 ASSTCQLTFENVK 261
Succinate dehydrogenase cytochrome b560 subunit Q9CZB0 SLCLGPTLIYSAK 107
Succinate dehydrogenase [ubiquinone] flavoprotein subunit Q8K2B3 TLNEADCATVPPAIR 654
Succinate-semialdehyde dehydrogenase Q8BFW0 NAGQTCVCSNR 328, 330
Succinyl-CoA ligase subunit α Q9WUM5 LIGPNCPGVINPGEČK 172, 181
Succinyl-CoA ligase subunit β Q9Z219 ICINOVFVCTER 158
Voltage-dependent anion-selective channel-1 Q60932 YQVDPDACKSAK 245
Voltage-dependent anion-selective channel-2* Q60930 CSGVEFSTSGSSNTDTGK 48
Voltage-dependent anion-selective channel-3 Q60931 cNITPTYČDLGK 8

Note: SNO cysteine residues (SNO-Cys) are labeled in upper case and underlined (C); Cysteine residues blocked by NEM are labeled in lower case (c). Protein identifications were accepted based on two or more unique peptides with a false discovery rate (FDR) of 99% or higher. *, SNO-modified proteins and peptides were also identified from 2 of 3 SNO-RAC/LC-MS/MS proteomic analyses.