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In vivo cardioprotection by S-nitroso-2-mercaptopropionyl glycine

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Abstract

The reversible S-nitrosation and inhibition of mitochondrial complex I is a potential mechanism of cardioprotection, recruited by ischemic preconditioning (IPC), S-nitrosothiols, and nitrite. Previously, to exploit this mechanism, the mitochondrial S-nitrosating agent S-nitroso-2-mercaptopropionyl glycine (SNO-MPG) was developed, and protected perfused hearts and isolated cardiomyocytes against ischemia-reperfusion (IR) injury. In the present study, the murine left anterior descending coronary artery (LAD) occlusion model of IR injury was employed, to determine the protective efficacy of SNO-MPG in vivo. Intraperitoneal administration of 1 mg/kg SNO-MPG, 30 min. prior to occlusion, significantly reduced myocardial infarction and improved EKG parameters, following 30 min. occlusion plus 2 or 24 hr. reperfusion. SNO-MPG protected to the same degree as IPC, and notably was also protective when administered at reperfusion. Cardioprotection was accompanied by increased mitochondrial protein S-nitrosothiol content, and inhibition of complex I, both of which were reversed after 2 hr. reperfusion. Finally, hearts from mice harboring a heterozygous mutation in the complex I NDUSF4 subunit were refractory to protection by either SNO-MPG or IPC, suggesting that a fully functional complex I, capable of reversible inhibition is critical for cardioprotection. Overall, these results are consistent with a role for mitochondrial S-nitrosation and complex I inhibition in the cardioprotective mechanism of IPC and SNO-MPG in vivo.

Keywords

Complex I; Preconditioning; Ischemia; Nitric Oxide; Reperfusion; Mitochondria

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Introduction

Ischemic preconditioning (IPC) is a cardioprotective event in which intermittent sub-lethal ischemia recruits several intracellular signaling mechanisms to protect against subsequent ischemia-reperfusion (IR) injury [1–3]. Mitochondria are key players in both the initiation of IPC signaling cascades, as well as the end-targets of protection [1,4–6]. Post-translational modifications to mitochondrial proteins (e.g. kinase mediated phosphorylation) have been widely recognized as playing an important role in IPC signaling [7], and more recently the mitochondrial formation of S-nitrosothiols (SNOs) has been proposed as a novel protective signaling mechanism in IPC [4,8–11].

S-nitrosation is a reversible nitric oxide (NO^{*}) dependent modification of thiols (e.g protein cysteines) resulting in formation of SNOs [12]. Many proteins with important roles in energy metabolism and Ca²⁺ homeostasis (both critical cardiomyocyte functions) have been identified as targets of S-nitrosation, including: L-type Ca²⁺ channels [13], ryanodine receptor [14], sarco/endoplasmic reticulum Ca²⁺ ATPase [10], caspases [15], hemoglobin [16], the 75 kDa subunit of complex I [8,10,17,18], thioredoxin [19], and the β -subunit of the F1-ATPase [10].

In agreement with the extensive literature suggesting a cardioprotective role for NO^{*} in IR injury [4,20,21], we previously showed that in Langendorff perfused hearts and isolated cardiomyocytes exposed to IPC, an elevation of mitochondrial SNOs was associated with the preservation of mitochondrial function during subsequent IR injury [9]. In order to exploit the potentially beneficial cardioprotective effects of mitochondrial S-nitrosation, the mitochondrial S-nitrosating agent S-nitroso-2-mercaptopyrionyl glycine (SNO-MPG) was developed [9] and was shown to mimic IPC-mediated protection against IR injury in both perfused hearts and cardiomyocytes, concurrent with S-nitrosation of mitochondrial proteins and reversible inhibition of complex I activity. These data are also in agreement with an emerging consensus that reversible inhibition of the mitochondrial respiratory chain is cardioprotective, as evidenced by the protective efficacy of a wide range of respiratory inhibitors [22–24]. Furthermore, it has recently been suggested that mitochondrial S-nitrosation and inhibition of complex I may underlie the cardioprotective efficacy of nitrite [25].

To date, cardioprotection by SNO-MPG has only been investigated in vitro, and the role of complex I remains unclear. Thus, the current study sought to investigate the effectiveness of SNO-MPG in vivo, using the popular murine left anterior descending coronary artery (LAD) occlusion model of IR injury. In addition the role of complex I was studied by employing a genetic model of complex I deficiency, the NDUF54^{+/-} mouse, which is deficient in the 18 kDa subunit of complex I, resulting a permanent 30% inhibition of cardiac complex I activity [26]. Summarizing the results, intraperitoneal delivery of SNO-MPG at 1 mg/kg, either prior to ischemia or at reperfusion, was cardioprotective in vivo. Both IPC and SNO-mediated cardioprotection were abrogated in perfused hearts from NDUF54^{+/-} mice. Together these data support a role for mitochondrial S-nitrosation and complex I inhibition in cardioprotection in vivo by IPC, S-nitrosothiols, and related therapies.

Materials & Methods

Animals, chemicals and reagents

Male C57BL6 mice (30–35g) were from Harlan (Indianapolis, IN). Mice with germ-line knockout of exon 5 of the complex I NDUF54 gene were generated to investigate the pathology of Leigh syndrome (an inherited metabolic disorder), as described elsewhere [26]. Animals were genotyped by tail biopsy PCR. Homozygous NDUF54^{-/-} mice exhibited embryonic lethality, whereas heterozygous NDUF54^{+/-} mice were viable with no overt cardiac phenotype

at 8 weeks (the age used in this study). These mice and their littermate controls were available on a very limited basis, such that infarct size after Langendorff perfusion was the only parameter measured. All mice were maintained in an AAALAC-accredited pathogen-free barrier facility with food and water available ad libitum. All procedures were in accordance with Guide for the Care and Use of Laboratory Animals (NIH Publication #85-23, 1996) and were approved by an Institutional Animal Committee for compliance with federal regulations (OLAW/PHS Assurance A3292-01). All chemicals were the highest grade obtainable from Sigma (St. Louis, MO) unless otherwise stated. SNO-MPG was freshly synthesized, purified and quantified as previously described [9], and sterile filtered prior to administration.

In-vivo ischemia-reperfusion

A published protocol was followed [27,28], with minor modifications. All procedures were performed under the sole light source of a white LED surgical light fitted with a UV-vis (400 nm cut off) filter. Prophylactic analgesia (1.5 mg/ml acetaminophen in drinking water) was provided 24 hr. prior to surgery. Acetaminophen does not impact on IR injury [29]. Mice were anesthetized with freshly prepared Avertin (2,2,2-tribromoethanol, 0.5 mg/kg injected intraperitoneally), then instrumented with a rectal thermistor probe connected to a heating blanket, and a 3-lead rodent electrocardiogram (Harvard Apparatus, Holliston MA). Temperature was maintained at 37°C and, following intubation, ventilation was provided at 100 cycles/min, 0.2 ml tidal volume, 100% FiO₂. A thoracotomy was performed and the heart exposed to visualize the left anterior descending (LAD) coronary artery, which was ligated 2 mm from its origin with 9-0 proline suture (Ethicon, Piscataway NJ). A length of PE-10 tubing was overlaid, and the suture tied around the tubing to occlude the artery for 30 min. Successful occlusion was confirmed by ST elevation and QRS widening on EKG, and the development of myocardial palor. After 30 min. the occlusion was released, which was confirmed by return of myocardial rubor. The chest was closed in two layers, the mouse removed from the ventilator, and recovery monitored for 2 hr. or 24 hr. Mortality during the procedure was <10%, was not different between experimental groups, and in most cases was caused by ventricular fibrillation during early reperfusion.

Experimental groups

Mice were divided into five experimental groups (Fig. 1) for the 2 hr. follow-up period: **(A)** IR plus vehicle (saline, 300 µl); **(B)** IR plus SNO-MPG (1 mg/kg) 30 min. prior to LAD occlusion; **(C)** IR plus SNO-MPG (1 mg/kg) at reperfusion; **(D)** IR plus 2-mercaptopyrionyl glycine (MPG, 1 mg/kg) 30 min. prior to LAD occlusion; **(E)** IR plus IPC (3 × 5 min. occlusion followed by 5 min. reperfusion) prior to the index IR injury. SNO-MPG was injected intraperitoneally in 300 µl sterile saline. Since infarct size measurement is a terminal procedure, a separate set of animals in groups A and B (designated **A*** and **B***) were used for 24 hr. reperfusion (Fig. 1). Asterisks in Fig. 1 indicate time-points where hearts were harvested for mitochondrial isolation. During preliminary testing, a high dose (10 mg/kg) of SNO-MPG caused 100% mortality during ischemia due to fibrillation, so 1 mg/kg was chosen for all subsequent studies presented herein.

Infarct size and area at risk

At the end of reperfusion mice were reanesthetized, the LAD reoccluded and 5% Evans blue dye injected via the LV apex, to delineate the area not at risk. To delineate infarct from live tissue, hearts were sliced transversely then stained in triphenyltetrazolium chloride (TTC, 1 % w/v in 100 mM phosphate buffer, pH 7.4) at 37°C for 20 min., followed by incubation in 10% formalin for 24 hr. to increase contrast. Slices were fixed between glass plates, scanned, and infarct (white) vs. live (red) vs. not at risk (blue) areas were quantified using free ImageJ software.

Cardiac mitochondrial isolation

A crude mitochondrial fraction was isolated in ice-cold buffer containing sucrose (300 mM), Tris-HCl (20 mM), EDTA (2 mM), DTPA (100 μ M), iodoacetamide (100 μ M), potassium hexacyanoferrate-III (4 mM), N-ethylmaleimide (10 mM) pH 7.35 @ 4°C. The latter 3 ingredients were added to preserve S-nitrosothiols [30]. Isolation buffer was injected via the LV apex to remove blood, and the heart was quickly excised and homogenized (Polytron) in 3 ml. isolation buffer. The homogenate was centrifuged at $700 \times g$ for 5 min. to pellet debris, then the supernatant centrifuged at $10,000 \times g$ for 5 min. to pellet a heavy membrane fraction containing mitochondria. Protein concentration was determined by the Folin-phenol method [31] against a standard curve constructed using bovine serum albumin. This rapid isolation protocol sacrifices a degree of mitochondrial purity in favor of S-nitrosothiol preservation.

Complex I activity assay and S-nitrosothiol analysis

Complex I was assayed spectrophotometrically in freeze-thawed (3 times) heart homogenates as rotenone-sensitive NADH oxidation at 340 nm [32]. Rates were normalized to the activity of the mitochondrial marker enzyme citrate synthase [32]. Mitochondrial pellets (1 mg protein) were analyzed by the biotin switch assay to determine S-nitrosothiol content [8,33]. Briefly, methyl-methanethiosulfonate (20 mM) was used to block free thiols, and SNO was labeled with biotin-HPDP (50 mM) (Pierce, Rockford IL) in the presence of 1 mM ascorbate. Proteins were separated by non-reducing SDS-PAGE followed by transfer to nitrocellulose. Equal protein loading was confirmed by Ponceau stain. Blots were developed using streptavidin-peroxidase (1:5000) and visualized by enhanced chemiluminescence reagents (GE Biosciences, Piscataway NJ). Consistent with a recent discovery [34], the specificity of the biotin-switch assay for S-nitrosothiols was confirmed by demonstrating an enhanced signal in the presence of copper (online supplement Figure S3).

Mouse heart perfusions

Heterozygous *NDUFS4*^{+/-} mice were anesthetized with freshly prepared Avertin (2,2,2-Tribromoethanol, 0.5 mg/kg injected intraperitoneally). The aorta was cannulated *in-situ* with a 22½ gauge needle filled with warm Krebs-Henseleit buffer (KH). The heart was rapidly transferred (<10 s.) to a perfusion apparatus, and retrograde (Langendorff) perfusion begun with 37°C KH gassed with 95% O₂ plus 5% CO₂, in constant flow mode (4 ml/min). *NDUFS4*^{+/-} and wild-type (WT) littermate control hearts were divided into 3 groups (Fig. 1): (F) IR injury comprising 25 min. global normothermic ischemia followed by 60 min. reperfusion; (G) IPC comprising 3 × 5 min. ischemia plus by 5 min. reperfusion, prior to index IR injury; (H) SNO-MPG treatment comprising infusion of 20 μ M SNO-MPG via a port immediately above the aortic cannula, for 20 min. prior to index IR injury [9]. At the end of each protocol the heart was cut into 5 transverse slices, and stained with TTC and fixed as described for *in vivo* hearts. In this global ischemia model the whole heart is the area at risk, thus Evans blue staining was not necessary.

Statistics

All infarct size data and EKG parameters were analyzed by ANOVA. Student's *t*-test was applied to some paired analyses. Appropriate numbers of experiments (N = 5–11) were performed to provide adequate power. Differences were considered significant at *p*<0.05.

Results

SNO-MPG and IPC protect vs. IR injury *in vivo*

To test the *in vivo* cardioprotective efficacy of SNO-MPG, the popular murine LAD artery occlusion model of IR injury was employed. The data in Fig. 2B show that under baseline IR

conditions (group **A**), after 2 hr. reperfusion a significant infarct developed (54 ± 4 % of the area at risk). Intraperitoneal injection of SNO-MPG (1 mg/kg) 30 min. prior to occlusion (group **B**) significantly decreased infarct size to 7 ± 1 %. From a more clinically relevant perspective, SNO-MPG (1 mg/kg) was also cardioprotective when delivered at reperfusion (group **C**), reducing infarct size to 15 ± 3 %. Administration of the same dose of MPG, the parent thiol from which SNO-MPG is derived (group **D**), did not significantly impact on infarct size, suggesting that the cardioprotective effect of SNO-MPG was not due to the antioxidant properties of MPG, which has been previously shown to be cardioprotective in vivo at much higher doses [35,36]. As a positive control, mice were also subjected to IPC (group **E**), which significantly decreased infarct size, to 8 ± 2 %. Thus, in vivo SNO-MPG protected to the same extent as IPC.

The protective effect of SNO-MPG pre-administration continued in mice subjected to a 24 hr. reperfusion period. While infarct size in controls (group **A***) grew to 68 ± 5 % at 24 hr., it remained low (9 ± 1 %) in the SNO-MPG treated mice (group **B***). The area at risk (AAR) was not significantly different between all experimental groups (Fig. 2C).

Effect of SNO-MPG and IPC on EKG parameters

IR injury causes severe disturbances in cardiac electrical activity, thus EKG was monitored throughout the IR protocols, and representative tracings are shown in Figs. 3A and 3B. Several characteristic changes occurred, including heart rate (HR) elevation during ischemia, increased amplitudes of both Q and R-waves, ST-segment elevation, and QRS widening [37, 38]. A schematic EKG tracing, indicating the position of the P, Q, R, S, and T waves, is shown in Fig. 3C for reference purposes. Numerical parameters calculated from EKG traces are shown in Table 1.

During the short ischemic cycles used to establish IPC, elevations in QRS duration and QT_{corr} were observed, but these changes were reversed during the short reperfusion cycles of IPC induction. In contrast, prolonged IR injury induced mostly irreversible changes in the EKG profile. Across all groups (**A–E**), HR was not significantly different prior to occlusion, but increased in all groups by ~10% during occlusion, indicating compensation for impaired contractility. This increase was sustained after 2 hr. reperfusion in all groups (**A–E**), and remained elevated in group **A*** at 24 hr. reperfusion but returned to baseline levels in group **B***.

PR interval (from the beginning of P wave to the beginning of Q wave) indicates the time for action potential to travel from the atria to the AV node and bundle of His, and was decreased by ~10% in all groups during occlusion. This decrease was sustained at 2 hr. reperfusion in all groups, and recovered to normal levels at 24 hr. reperfusion in groups **A*** and **B***, indicating no effect of any cardioprotective strategy on post-IR injury PR interval function.

QRS duration indicates the time for depolarization and partial repolarization of both ventricles, and exhibited a 2–3 fold widening during occlusion in all groups (**A–E**), indicating left or right bundle branch block. This widening was sustained in groups **A** and **D** at 2hr. reperfusion, but was significantly lower and closer to baseline levels in groups **B**, **C**, and **E**. Notably, as previously reported [39], the widening of QRS during ischemia was blunted in hearts exposed to IPC. At 24 hr. reperfusion, QRS duration was still elevated group **A*** but was completely returned to baseline level in group **B***. Thus, cardioprotective strategies (SNO-MPG or IPC) significantly improved post-IR injury QRS complex dimensions.

QT interval (from the beginning of Q wave to the end of T wave) represents the total time for depolarization and repolarization of the ventricles. QT_{corr} is a corrected version of QT which takes into account variations in heart rate, and is calculated as $[QT/(RR \text{ interval}/100)^{1/2}]$ [38].

QT_{corr} was elongated during occlusion in all groups (A–E), and remained elongated in groups A and D at 2 hr. reperfusion. In contrast, QT_{corr} became shorter at 2 hr. reperfusion in groups B, C, and E. At 24 hr. reperfusion, QT_{corr} remained elevated in group A*, and climbed slightly in group B* but remained below the peak attained during occlusion.

QRS duration has been proposed as an early indicator of myocardial infarct development [40,41], and post MI survival [42]. Consistent with this proposal, Fig. 3D shows a strong correlation ($r^2 = 0.594$) between infarct size and QRS duration at 2 hr. reperfusion, across all 5 experimental groups. Interestingly, no correlation was observed between infarct and QRS duration at 24 hr. of reperfusion ($r^2 = 0.107$, data not shown), suggesting QRS is useful only as an early prognostic marker. Overall, the EKG data indicate that SNO-MPG improved the post-IR recovery of cardiac electrical activity.

SNO-MPG and IPC S-nitrosate mitochondria

To check whether SNO-MPG or IPC mediated cardioprotection was accompanied by mitochondrial protein S-nitrosation, S-nitrosated proteins were visualized using the biotin-switch assay [8,33]. Fig. 4A shows that cardiac mitochondria isolated from SNO-MPG treated mice before LAD occlusion exhibited elevated levels of protein SNOs relative to untreated controls (lanes 1 vs. 2). A small increase in S-nitrosation was also observed in mitochondria from hearts exposed to IPC alone before LAD occlusion (lanes 3 vs. 4). The differences in protein SNO profile between SNO-MPG and vehicle-treated groups became more prominent following the onset of ischemia (lanes 5 vs. 6). Similarly, the same enhancement in S-nitrosation was seen in hearts subjected to IPC (lanes 7 vs. 8), i.e., ischemia increased the differences between the control and IPC groups. This is consistent with previous observations that IPC elevated SNO content in isolated perfused hearts [8,10], and is the first demonstration that IPC-mediated SNO formation occurs *in vivo*. Together these data suggest that treatments such as IPC or SNO-MPG prior to ischemia may *prime* the heart for S-nitrosation during ischemia.

In addition, since SNO-MPG was also cardioprotective when delivered at reperfusion, mitochondrial S-nitrosation was also examined under this condition (i.e., SNO-MPG delivery at reperfusion followed by mitochondrial isolation 5 min. later). The results (lanes 9 vs. 10) indicate that even with this short treatment time, a slight enhancement of S-nitrosation occurred. Finally, after 2 hr. reperfusion, the differences in mitochondrial protein SNO content found between SNO-MPG and vehicle pre-treated groups (lanes 5 & 6) were largely reversed (lanes 11 vs. 12).

Notably, all of the biotin-switch western blots appeared to show a degree of labeling under baseline conditions, with these same bands being enhanced by either SNO-MPG or IPC (as opposed to new bands appearing on the blots). This suggests that some endogenous S-nitrosation may exist, or that a sub-population of mitochondrial proteins is particularly susceptible to S-nitrosation. To characterize this mitochondrial S-nitrosation more thoroughly, proteomic studies were undertaken, using a novel 2D gel-compatible version of the biotin-switch assay. The results (online data supplement, Figs. S1 & S2, Table S1) showed a broad overlap between the proteins S-nitrosated in mitochondria from hearts subjected to IPC, and those S-nitrosated in mitochondria treated with SNO-MPG.

SNO-MPG reversibly inhibits complex I in-vivo

S-nitrosation of complex I has previously been linked to inhibition of its enzymatic activity in isolated systems such as perfused heart, cardiomyocytes and mitochondria [8,9,17,18]. Herein, complex I activity was measured in heart homogenates from the same hearts as used for SNO analysis in Fig. 4A. Prior to LAD occlusion, complex I was significantly inhibited in cardiac

mitochondria from SNO-MPG treated mice compared to controls (Fig. 4B, lanes 1 vs. 2). Interestingly, despite a small increase in S-nitrosation, no effect on complex I activity was observed in hearts exposed to IPC alone (lanes 3 vs. 4). Following the onset of ischemia, complex I activity remained lower in SNO-MPG treated mice, but not significantly so (lanes 5 vs. 6). Furthermore, the onset of ischemia resulted in a significant decrease in complex I activity in the IPC group, relative to mice subjected to LAD occlusion alone (lanes 7 vs. 8). Interestingly, ischemia alone did not significantly impact complex I activity in vehicle treated hearts (lanes 1 vs. 5), consistent with our previous report [32]. However, after both 5 min. and 2 hr. of reperfusion, complex I activity dropped significantly (lanes 1 vs. 9 and 1 vs. 11 respectively), suggesting impairment due to reperfusion injury [32,43]. The early impairment on complex I activity (after 5 min. reperfusion) was not affected by SNO-MPG delivery during this time (lanes 9 vs. 10). Contrastingly, in the SNO-MPG pre-treated mice, complex I activity recovered almost to control levels at 2 hr. reperfusion. Thus, SNO-MPG induced complex I inhibition, like mitochondrial S-nitrosation, appears to be reversible during the later stages of reperfusion.

Critical role for intact complex I in cardioprotection

To evaluate the effects of genetic alteration in complex I activity on cardioprotection, we employed a recently developed knockout mouse with deletion of the complex I NDUF54 subunit [26]. Cardiac mitochondria from NDUF54^{+/-} mice exhibited a 30% decrease in complex I activity [26]. Isolated perfused hearts from NDUF54^{+/-} and WT mice were subjected to 25 min. ischemia followed by 1 hr. reperfusion, revealing no difference in infarct size between the genotypes (Fig. 5). However, while both IPC and SNO-MPG diminished infarct size in WT hearts as expected, NDUF54^{+/-} hearts were refractory to the cardioprotective benefits of both IPC and SNO-MPG. These data suggest that an intact functional complex I is critical for cardioprotection mediated by S-nitrosation and by IPC.

Discussion

The main findings of this study are: (i) SNO-MPG protects hearts against IR injury in vivo and the magnitude of protection is similar to IPC; (ii) SNO-MPG and IPC induce S-nitrosation of similar mitochondrial proteins, and reversible complex I inhibition; (iii) NDUF54^{+/-} mice are refractory to the cardioprotective effects of IPC or SNO-MPG.

Previously we showed that SNO-MPG was cardioprotective in isolated perfused hearts and cardiomyocytes [9]. In addition we and others have shown that S-nitrosation of mitochondrial proteins occurs during IPC, and that the reversible inhibition of respiratory complex I appears to be an important event which may contribute to cardioprotection [4,9,10,22,44]. The studies performed herein suggest that the paradigm of cardioprotective mitochondrial S-nitrosation and reversible complex I inhibition [11] also translates to the in vivo situation. The mechanism of protection appears to be similar in vivo and in vitro, since complex I activity measurements (Fig. 4) showed that intraperitoneal administration of SNO-MPG was indeed capable of eliciting reversible cardiac complex I inhibition.

The molecular mechanisms linking mitochondrial S-nitrosation and complex I inhibition to cardioprotection remain to be elucidated, but several interesting candidate mechanisms have been proposed. First, S-nitrosation might protect critical thiol groups in mitochondrial proteins from irreversible oxidative damage [18]. Complex I contains many critical thiols, and the enhanced post-IR recovery of complex I enzymatic activity in SNO-MPG treated animals (Fig. 4B) supports this hypothesis. Second, elevation of NADH due to SNO-induced complex I inhibition may enhance the mitochondrial GSH/GSSG ratio and thus also the ability of mitochondria to detoxify peroxides via glutathione dependent pathways [45,46]. Third, a greater NADH/NAD⁺ ratio is a potent inhibitor of the mitochondrial permeability transition

pore, an important contributor to the pathology of IR injury [47,48]. Fourth, we recently proposed that reversible inhibition of the respiratory chain may facilitate a slower re-activation of mitochondrial metabolism at reperfusion (the “gradual wake up” hypothesis [4]) preventing a surge of electrons into the respiratory chain and thus preventing a burst of ROS generation and Ca^{2+} overload [49]. Fifth, it has been proposed that S-nitrosation may serve as a “gateway” modification to further thiol adduction by for example glutathione (i.e., glutathionylation) [50]. The role of glutathionylation in IPC overall is currently poorly understood, but ROS generation from complex I has recently been shown to be regulated by this modification, suggesting possible relevance [51].

While overall the importance of each of these mechanisms is unclear, it is notable that several reversible inhibitors of complex I are protective in models of IR injury, including rotenone [44], amobarbital [22], volatile anesthetics [24], ranolazine [52,53], and capsaicin [54,55] (for review see [4]). Thus, complex I inhibition is emerging as an important paradigm for cardioprotection.

Surprisingly, SNO-MPG administration at the moment of reperfusion also caused a decrease in post-IR infarct size. This protection was concurrent with a small increase in mitochondrial S-nitrosation, and while the mechanism of protection may also include transient inhibition of complex I during reperfusion, such inhibition was not significant (Fig. 4B). Similar protection was seen with administration of respiratory inhibitors at reperfusion [56], but it is perhaps more reasonable to propose that other non-mitochondrial mechanisms may prevail under such conditions. In support of this hypothesis, we showed that EKG parameters returned to normal after reperfusion in both the pre- and post- SNO treated groups (**B** and **C**), suggesting S-nitrosation may regulate the function of cardiac ion channels. Indeed, the inhibition of L-type Ca^{2+} channels due to S-nitrosation of the α_1 subunit has been demonstrated in both IPC- and GSNO-treated hearts [10]. This event decreases cardiomyocyte Ca^{2+} influx during the plateau phase of action potential, resulting in a shorter QT_{corr} interval, diminishing the risk of post-ischemic arrhythmias [57]. Table 1 shows that QT_{corr} duration was significantly reduced in SNO-MPG compared to vehicle-treated hearts during reperfusion. In addition to L-type Ca^{2+} channels, several other Ca^{2+} handling proteins are also regulated by S-nitrosation, including SERCA2a [10] and RyR2 [14]. S-nitrosation increases the activity of SERCA2a, which would stimulate sarcolemmal Ca^{2+} uptake and decrease the availability of Ca^{2+} for post-IR mitochondrial Ca^{2+} overload [49]. The role of SNO in the regulation of other important events of cardiac conductance, including Na^+ and K^+ channels and action potential transduction, is still unclear, but the effect of SNO-MPG on QRS duration at reperfusion (Table 1) suggests such regulation may exist. In support of a preconditioned S-nitrosation phenotype being induced by SNO-MPG, significant overlap was observed in the proteomic pattern of S-nitrosated proteins between IPC and SNO-MPG treated hearts (online data supplement).

A requirement for complex I in either IPC or SNO-MPG mediated cardioprotection was established herein via the use of a novel mouse model in which the $\text{NDUFS4}^{+/-}$ subunit of complex I was knocked out. As already mentioned, these mice exhibited elevated lactate levels, consistent with a mitochondrial metabolic defect [26]. Several important processes relevant to IR injury and cardioprotection are pH-sensitive, including the activity of the Na^+/H^+ exchanger [58], the mitochondrial PT pore [47], and ischemic post-conditioning [59,60]. Furthermore, another recently developed NDUFS4 knockout mouse exhibited an encephalomyopathy phenotype [61]. Thus, to avoid the confounding effects of systemic lactate levels and neuronal effects (e.g. altered sympathetic/parasympathetic activity) on cardiac function, isolated perfused hearts from these mice were analyzed.

The $\text{NDUFS4}^{+/-}$ and WT perfused heart studies showed that a 30% permanent inhibition of complex I activity did not protect against IR injury, measured by myocardial infarct size.

Therefore, while reversible complex I inhibition is associated with cardioprotection (Fig. 4) it appears that irreversible complex I inhibition does not confer any cardioprotective benefit.

Most strikingly, *NDUFS4*^{+/-} mouse hearts were refractory to cardioprotection by IPC and SNO-MPG (Fig. 5). Thus, functional complex I seems to be critically important for those cardioprotective stimuli which use complex I inhibition as a mechanism of protection. However, we cannot exclude the possibility that resistance to cardioprotection in the *NDUFS4*^{+/-} hearts may be due to other alterations which accompany the genetic manipulation of complex I in this model.

From a clinical perspective, it is known that cardiac complex I activity is decreased in many pathological situations, including diabetes and aging [62–64]. In addition, these same pathologies manifest a loss of cardioprotection in response to stimuli such as IPC or volatile anesthetics [65–71]. Thus, it may be speculated that the loss of cardioprotection in these situations may arise at least in part from the loss of complex I activity. In future, it will be interesting to investigate whether various cardiotoxic drugs such as doxorubicin, or diseases such as Parkinson's, both of which are known to impact cardiac complex I activity [72–74], may also result in loss of IPC mediated cardioprotection.

Naturally, the current findings also have implications for the human clinical applicability of reagents such as SNO-MPG. It would be expected that such agents may be ineffective in diabetics and aged populations, or other individuals with diminished complex I function. Another important aspect of S-nitrosothiol use in humans is toxicity, and in this regard we found that high doses of SNO-MPG (>10mg/kg), as well as decomposition of S-nitrosothiols promoted by direct high-intensity white lighting of the surgical site, both resulted in ventricular fibrillation and death during early reperfusion. Thus, the dosing of S-nitrosothiols as cardioprotective agents requires careful attention.

Furthermore, the studies herein only examined short-term SNO-MPG administration. It has been suggested that SNO accumulation in the brain is related to the development of Parkinson's disease [75,76], and in this regard S-nitrosothiols are similar to 3-nitropropionic acid, which is known to protect the heart against IR injury, but also causes striatal lesions related to Huntington's disease [23,77]. We did not find any SNO in brain homogenates 30 min. after intraperitoneal injection of 20 mg/kg SNO-MPG (limit of detection 1 pmol SNO/mg protein, data not shown), suggesting that SNO-MPG is unable to penetrate the hematoencephalic barrier. These data also suggest that S-nitrosothiols may be metabolized or carried in the blood. Indeed, hemoglobin (Hb) has been widely reported as a target of S-nitrosation [16], and it has been proposed that SNO-Hb is essential for hypoxic vasodilatation [78,79] (although this concept has recently been challenged by the generation of a Hb- $\beta_{93}\text{-Cys/Ala}$ mutant mouse [80]). Furthermore, it has been shown that administration of SNO-Hb is cardioprotective in IR injury [81]. Thus, the degree to which low-molecular weight SNOs such as SNO-MPG afford additional protection over that afforded by high-molecular weight blood-borne SNOs such as SNO-Hb, requires further investigation.

In conclusion, the results of the present study are consistent with the emerging paradigm that mitochondrial S-nitrosation and complex I inhibition are important in the cardioprotection afforded by IPC, and this paradigm extends to the *in vivo* situation. The forthcoming development of a variety of molecules which seek to exploit this cardioprotective mechanism (e.g. nitrite [25], which is about to enter clinical trials for myocardial infarction), may afford novel therapeutics for IR injury in humans.

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In vivo LAD occlusion **Langendorff perfused heart global ischemia** **Figure 1. Experimental Schemes**

Protocols for ischemia and reperfusion injury for the in vivo LAD occlusion model (upper panel) and isolated perfused heart model (lower panel). Schemes are labeled **A** through **H** as detailed in the methods. Where indicated by arrows, vehicle, SNO-MPG or MPG were administered by bolus injection to mice or by continuous infusion to perfused hearts. Asterisks indicate time points at which mitochondria were isolated for SNO and complex I analysis (Fig. 4).

Figure 2. Post-IR infarct size

Mice were subjected to IR injury as described in the methods, plus treatment with either SNO-MPG prior to ischemia, SNO-MPG at reperfusion, or IPC. Animals were stratified into seven groups (A, B, C, D, E, A*, B*), as defined in the methods. **(A):** Two representative formalin-fixed hearts (cross-sectional images at center of ventricles) from each experimental group, with dark blue indicating the area not at risk, red indicating live tissue, and pale/white indicating dead tissue (infarct). Numbers in parentheses above indicate the N for each group. **(B):** Quantitation of infarct size as a percentage of the area at risk for each experimental group. **(C):** Quantitation of the area at risk for each experimental group. In panels B and C, individual data points for each group are indicated on the left, with means $\pm$ SEM

indicated by the larger symbol on the right. N = 7–9 for all groups. * $p < 0.05$ vs IR alone (by ANOVA).

Figure 3. EKG parameters during IR injury. (A) and (B): Typical EKG tracings from groups A, A*, B, and B*, at time points indicated above the traces. (C): Typical expanded 3-lead EKG tracing from a normal mouse heart, with positions of the P, Q, R, S, T waves indicated. (D): Correlation between QRS duration (from Table 1) and infarct size (from Figure 2) for all experimental groups (A–E). Legend shows identity of different symbols, as per Fig. 2. Linear regression line through all points is shown. Each point represents data from a single mouse.

Figure 4. Mitochondrial protein S-nitrosation and complex I activity during in vivo IR injury ± SNO-MPG or IPC treatment

Crude mitochondrial fraction was prepared at times indicated in Figure 1, as described in the methods. Mitochondrial protein S-nitrosation was visualized by biotin-switch assay. **(A):** Representative biotin-switch western blots, in which immuno-reactive bands indicate S-nitrosated proteins. Due to offsets of experiment timing, and the short half-life of S-nitrosothiols, samples could only be compared in groups of two, and it was not possible to run all samples on the same gel at once. Thus, each pair of samples contains an appropriate control. Lanes are numbered 1–12 as detailed in the results text. **(B):** Complex I activity in heart homogenates under the same conditions as used for biotin-switch analysis. Data are means $\pm$ SEM, N=5. *p<0.05 between indicated groups. n.s. = not significant. Blots in panel A are representative of at least 3 independent experiments.

Figure 5. Infarct-size in WT vs. NDUFS4^{+/-} mouse hearts subjected to IR injury plus either IPC or SNO-MPG

Langendorff perfused hearts from WT (circles) or NDUFS4^{+/-} mice (triangles) were subjected to IR injury alone (white), IR with prior IPC (black) or IR with prior SNO-MPG infusion (gray), as detailed in the methods. Infarct size was quantified by TTC staining. Upper panels show 2 representative TTC stained hearts (cross-sectional images at center of ventricles) from each group, with live tissue stained red and dead tissue white. The graph below shows individual data points for each condition on the left, with means $\pm$ SEM on the right (enlarged symbols). * $p < 0.05$ vs. WT IR alone (by ANOVA). Groups F, G, H as defined in the methods and Figure 1 are indicated above. Area at risk is 100% in this global ischemia model.

Table 1

EKG parameters in mice subjected to IR injury

EKG parameters were measured at the time points indicated in the top row, and are defined in the results text. Groups are indicated in the left column.

Parameter		Pre-occlusion	End of Occlusion	2 hr. Reperfusion	24hr. Reperfusion
A	<i>IR</i>				
	HR (bpm)	454.6 ± 10.1	498.1 ± 14.6	518.9 ± 19.5	500.5 ± 38.5
	PR (ms)	44.9 ± 1.0	40.7 ± 1.1	40.9 ± 2.6	45.3 ± 4.4
	QRS (ms)	16.5 ± 0.5	51.1 ± 3.0	50.9 ± 3.2	36.8 ± 13.2
B	<i>SNO pre</i>				
	QT _{corr} (ms)	49.6 ± 1.0	66.2 ± 1.1	72.3 ± 3.2	71.0 ± 1.9
	HR (bpm)	416.3 ± 12.3	457.9 ± 11.9	466.9 ± 12.6	411.3 ± 23.4
	PR (ms)	45.5 ± 1.4	40.1 ± 1.2	39.7 ± 3.5	45.8 ± 3.0
C	<i>SNO post</i>				
	QRS (ms)	17.0 ± 0.7	41.7 ± 3.7	21.9 ± 1.6 [*]	19.0 ± 2.3
	QT _{corr} (ms)	48.3 ± 0.7	64.2 ± 2.0	53.5 ± 1.9 [#]	58.6 ± 2.6 [#]
	HR (bpm)	430.5 ± 17.1	510.0 ± 26.7	490.9 ± 23.3	-
D	<i>MPG pre</i>				
	PR (ms)	47.0 ± 1.8	42.4 ± 1.5	42.3 ± 1.3	-
	QRS (ms)	16.0 ± 1.0	55.3 ± 2.2	28.4 ± 4.5 [*]	-
	QT _{corr} (ms)	46.5 ± 0.8	67.0 ± 2.3	55.7 ± 1.8 [#]	-
E	<i>IPC</i>				
	HR (bpm)	414.3 ± 14.8	478.3 ± 14.7	461.9 ± 21.0	-
	PR (ms)	47.4 ± 2.3	41.6 ± 1.9	39.5 ± 2.2	-
	QRS (ms)	19.0 ± 1.1	45.8 ± 2.8	41.6 ± 5.2	-
	QT _{corr} (ms)	51.3 ± 1.3	69.4 ± 1.6	68.2 ± 2.2	-
	HR (bpm)	<i>see below</i>	423.1 ± 11.6	470.6 ± 23.5	-
	PR (ms)		42.3 ± 1.5	42.7 ± 2.8	-
	QRS (ms)		37.4 ± 6.2	21.0 ± 1.2 [*]	-
	QT _{corr} (ms)		66.8 ± 3.0	56.5 ± 1.2 [#]	-

Parameter	Baseline	1 st Isch'	1 st Rep'	2 nd Isch'	2 nd Rep'	3 rd Isch'	3 rd Rep'
HR (bpm)	413.4±17.2	461.4±20.7	450.4±18.1	416.9±34.5	441.9±15.5	437.0±14.4	435.4±15.5
PR (ms)	47.1±2.1	42.3±2.9	41.6±3.0	42.9±2.7	41.4±1.8	38.7±1.9	40.7±1.3
QRS (ms)	19.3±1.7	39.7±4.4 [†]	22.1±2.9	40.1±5.9 [†]	22.1±0.7	35.6±4.6 [†]	22.7±1.5
QT _{corr} (ms)	51.7±1.3	72.4±3.7 [‡]	58.3±2.0	75.6±2.3 [‡]	57.5±2.3	72.0±2.9 [‡]	56.7±1.8

Parameter	Baseline	1 st Isch'	1 st Rep'	2 nd Isch'	2 nd Rep'	3 rd Isch'	3 rd Rep'
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All data are means ± SEM, N = 7–9.

* p<0.05 for QRS duration, compared to IR alone (group A).

p<0.05 for QT_{corr}, compared to IR alone (group A). Data obtained during the short IR cycles used to induce IPC, are shown in the sub-table below.

† p<0.05 for QRS duration, compared to baseline condition in IPC group.

‡ p<0.05 for QT_{corr}, compared to baseline condition in IPC group.