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S-Nitrosoglutathione Reductase Is Essential for Protecting the Female Heart from Ischemia-Reperfusion Injury

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Abstract

Rationale: Protein *S*-nitros(yl)ation (SNO) has been implicated as an essential mediator of nitric oxide-dependent cardioprotection. Compared to males, female hearts exhibit higher baseline levels of protein SNO and associated with this, reduced susceptibility to myocardial ischemia-reperfusion (I/R) injury. Female hearts also exhibit enhanced *S*-nitrosoglutathione reductase (GSNO-R) activity, which would typically favor decreased SNO levels as GSNO-R mediates SNO catabolism.

Objective: Since female hearts exhibit higher SNO levels, we hypothesized that GSNO-R is an essential component of sex-dependent cardioprotection in females.

Methods and Results: Male and female wildtype mouse hearts were subjected to ex vivo I/R injury with or without GSNO-R inhibition (N6022). Control female hearts exhibited enhanced functional recovery and decreased infarct size vs. control males. Interestingly, GSNO-R inhibition reversed this sex disparity, significantly reducing injury in male hearts, and exacerbating injury in females. Similar results were obtained with male and female GSNO-$R^{-/-}$ hearts using ex vivo and in vivo models of I/R injury. Assessment of SNO levels using SNO-resin assisted capture revealed an increase in total SNO levels with GSNO-R inhibition in males, whereas total SNO levels remained unchanged in females. However, we found that while GSNO-R inhibition significantly increased SNO at the cardioprotective Cys39 residue of NADH dehydrogenase subunit 3 in males, SNO-ND3 levels were surprisingly reduced in N6022-treated female hearts. Since GSNO-R also acts as a formaldehyde dehydrogenase, we examined post-ischemic formaldehyde levels and found that they were nearly 2-fold higher in N6022-treated female hearts compared to non-treated hearts.

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DISCLOSURES

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METHODS

The authors declare that all supporting data are available within the article and its online supplementary files.

Please see the Online Supporting Document available at http://circres.ahajournals.com for all methods related to this study.

Importantly, the mitochondrial aldehyde dehydrogenase 2 activator, Alda-1, rescued the phenotype in GSNO-R$^{-/-}$ female hearts, significantly reducing infarct size.

Conclusions: These striking findings point to GSNO-R as a critical sex-dependent mediator of myocardial protein SNO and formaldehyde levels, and further suggest that different therapeutic strategies may be required to combat ischemic heart disease in males and females.

Keywords

S-nitrosylation; formaldehyde; reactive oxygen species; sex differences; N6022; nitric oxide; ischemia reperfusion injury; ADH5; Animal Models of Human Disease; Basic Science Research; Cell Signaling/Signal Transduction; Ischemia; Proteomics

INTRODUCTION

Reversible cysteine oxidation plays a critical role in reducing myocardial injury from ischemia-reperfusion (I/R), which is a major cause of death among males and females in the US. Nitric oxide (NO) has also been shown to be an important component of cardioprotection.[1–3] Taken together, this suggests a potential role for protein *S*-nitros(yl)ation (SNO) in cardioprotection.[4, 5] SNO is a specific and reversible cysteine thiol modification resulting from the covalent attachment of a NO moiety, with diverse effects on protein activity, stability, protein-protein interactions, and cellular localization.[4] Myocardial SNO homeostasis is primarily mediated by the actions of constitutive NO synthase (NOS) isoforms, endothelial NOS (eNOS) and neuronal NOS, and via *S*-nitrosoglutathione reductase (GSNO-R).[6] Originally characterized as an alcohol dehydrogenase (ADH5), GSNO-R also functions as a reductase that metabolizes *S*-nitrosoglutathione (GSNO),[7] which is an important bioavailable reserve for NO in the cell that remains in equilibrium with SNO proteins. GSNO-R also acts as a formaldehyde dehydrogenase by catalyzing the oxidation of S-hydroxymethylglutathione,[8] the spontaneous reaction product of formaldehyde and glutathione. Importantly, we and others have demonstrated that myocardial SNO protein levels increase with various cardioprotective stimuli,[9–17] thus implicating SNO as a critical stress response that serves to acutely protect the heart against I/R injury. Chronic upregulation of protein SNO levels, as occurs with the genetic deletion of GSNO-R, is also associated with protection in male hearts.[18, 19] Recent findings from our group further suggest that protein SNO plays a key role in protecting the female heart from I/R injury.[17, 20]

Pre-menopausal women have a reduced risk for developing cardiovascular disease compared to age-matched men.[21–23] Pre-clinical studies from our group and others have demonstrated an intrinsic, NO-dependent cardioprotective mechanism in female hearts, with females exhibiting enhanced functional recovery and smaller infarcts vs. males.[20, 24–26] Prior work has also shown that protection from I/R injury is lost in female hearts following ovariectomy,[26, 27] suggesting a role for estrogen. In further support of this NO-dependent protection, we recently demonstrated that female hearts exhibit enhanced PI3K-Akt signaling, increased eNOS expression and activation, and higher protein SNO levels compared to male hearts.[17, 20] We also found that GSNO-R activity was significantly enhanced in female hearts at baseline vs. males,[17, 20] which should favor reduced SNO

levels in female hearts. This finding suggests that GSNO-R may play a protective role in female hearts, perhaps by mitigating the potential for excessive protein SNO accumulation and/or nitrosative stress. Indeed prior studies demonstrate GSNO-R-mediated protection in sepsis and pulmonary hypertension.[6, 28] However, little is known with regard to the role of GSNO-R in female myocardial I/R injury.

In the current study, we utilized a specific and potent GSNO-R inhibitor (N6022) to assess the role of GSNO-R in I/R injury in male and female hearts.[29] N6022 is currently in clinical trials for the treatment of asthma and cystic fibrosis, and has been well tolerated in humans and animals.[7] We found that GSNO-R inhibition or genetic deletion, led to sex-disparate effects using ex vivo and in vivo models of myocardial I/R injury, yielding protective effects in males, but unexpectedly worsening injury in females. The beneficial and injurious effects of GSNO-R inhibition resulted, in part, from differential changes in myocardial protein SNO signaling, ROS production, and formaldehyde accumulation.

RESULTS

Acute GSNO-R inhibition alters the myocardial response to I/R injury.

The genetic deletion of GSNO-R is known to be protective in male hearts subjected to in vivo I/R injury,[18, 19] but female hearts have not been examined. Thus, we sought to determine the effect of GSNO-R inhibition on the response to I/R injury in female hearts. To avoid compensatory adaptation with the use of genetically modified mice, we initially utilized the GSNO-R inhibitor N6022 to acutely block enzyme activity.[29] We used a Langendorff-perfused heart model of I/R injury to avoid the confounding effects of circulating hormones (i.e., estrogen) and/or GSNO on the response to I/R injury. Sex-dependent cardioprotection was assessed in C57Bl/6J wildtype (WT) mouse hearts under control conditions (i.e., without N6022) (Fig. 1a). Initial hemodynamic measurements indicated no differences in baseline left ventricular developed pressure (LVDP) or heart rate between WT male and female hearts (Online Table I). After 20 minutes of global ischemia and 60 minutes of reperfusion, contractile function was significantly impaired in male and female hearts, but post-ischemic functional recovery was significantly higher in female hearts (Fig. 1b) and infarct size was reduced (Fig. 1c).

Male and female WT hearts were also perfused with N6022 (100 nmol/L or 10 μmol/L) for 15 minutes prior to the onset of ischemia and for five minutes at the start of reperfusion (Fig. 1a). Interestingly, baseline heart rate was significantly decreased in N6022-treated female hearts (Online Table I); baseline cardiac function in male hearts was unaffected by N6022. After 20 minutes of ischemia and 60 minutes of reperfusion, male hearts exhibited a dose-dependent improvement in post-ischemic functional recovery with N6022 (Fig. 1d); infarct size showed a similar dose-dependent decrease with N6022 (Fig. 1e). In female hearts, however, we found that GSNO-R inhibition abrogated sex-dependent protection and exacerbated injury, with female hearts exhibiting a dose-dependent decrease in post-ischemic functional recovery (Fig. 1f) and increased infarct size (Fig. 1g) with N6022. Treatment of WT male and female hearts with vehicle control (DMSO) had no effect on the response to I/R injury (data not shown). These results suggest that GSNO-R is a requisite component for reducing I/R injury in females, but not in males (Fig. 1h-1i).

Genetic deletion of GSNO-R alters the myocardial response to I/R injury.

Experiments were repeated in male and female mice with a genetic deletion of GSNO-R (GSNO-$R^{-/-}$; Online Fig. I). The phenotype of these mice has been well-characterized in previous studies.[6, 30] Initial hemodynamic measurements in Langendorff-perfused hearts revealed no differences in baseline LVDP or heart rate between male and female, WT and GSNO-$R^{-/-}$ mice (Online Table I). A more thorough evaluation of baseline cardiac function using echocardiography revealed subtle differences in function between male and female, WT and GSNO-$R^{-/-}$ mice (Online Table II), but these differences are not likely to impact the response to myocardial I/R injury. These results agree with findings in prior studies.[6, 30] Additionally, we did find a significant difference in weight, with male GSNO-$R^{-/-}$ mice weighing significantly less than male WT mice (Online Table II).

Consistent with the effects of acute GSNO-R inhibition, we found that male GSNO-$R^{-/-}$ hearts showed a significant reduction in infarct size compared to WT using our Langendorff-perfused heart model of I/R injury (Fig. 2a). Female GSNO-$R^{-/-}$ hearts, on the other hand, showed a substantial and significant increase in infarct size compared to WT (Fig. 2b). As such, infarct size was reduced by more than 60% in male GSNO-$R^{-/-}$ hearts, while infarct size was increased by more than 30% in female GSNO-$R^{-/-}$ hearts (vs. respective WT). Treatment of male and female GSNO-$R^{-/-}$ hearts with N6022 had no effect on the response to I/R injury (data not shown), confirming inhibitor specificity.

We also utilized an in vivo model of myocardial I/R injury to further examine the loss of GSNO-R. Male and female, WT and GSNO-$R^{-/-}$ mice were subjected to left anterior descending coronary artery (LAD) occlusion for 45 minutes, followed by 24 hours of reperfusion. Consistent with our ex vivo results (Fig. 2a-2b) and those reported previously,[18, 19] we found that male GSNO-$R^{-/-}$ hearts showed a significant reduction in infarct size compared to WT, while female GSNO-$R^{-/-}$ hearts showed a significant increase in infarct size compared to WT (Fig. 2c-f). Plasma cTnI levels were also decreased in male GSNO-$R^{-/-}$ vs. WT and increased in female GSNO-$R^{-/-}$ vs. WT (Fig. 2i and 2j), but these differences were not statistically significant. Area at risk was not different between groups (Fig. 2c and 2d).

GSNO-R inhibition increases protein SNO levels in male hearts.

We examined the effect of GSNO-R inhibition on protein SNO levels at baseline and following I/R injury in male and female WT hearts using SNO-RAC.[9, 15] To increase confidence, all reported SNO protein identifications were detected in at least two of three hearts per group. Consistent with the findings in our prior studies,[17, 20] WT female hearts exhibited nearly two-fold more SNO protein identifications at baseline vs. WT males (Figs. 3a-3b, Online Tables III and IV). N6022 perfusion for 15 minutes substantially increased the number of SNO proteins identified in WT male hearts (Figs. 3a-3b, Online Table V), but had essentially no effect on total SNO protein identifications in WT female hearts (Figs. 3a-3b, Online Table VI). Following I/R injury, SNO protein levels decreased in all groups (Fig. 3d-3e, Online Tables VII-X), which is consistent with our previous study. However, the post-I/R decrease in SNO protein levels was partly impaired in N6022-treated male and female

hearts, suggesting that N6022 does impact SNO protein levels in both male and female hearts.

We noted a strong association between the degree of I/R-dependent injury in both male and female, N6022-treated and non-treated hearts (Fig. 1), and pre-ischemic levels of SNO at Cys39 of NADH dehydrogenase subunit 3 (ND3). This cysteine has been shown to be a critical mitochondrial redox switch for NO-dependent protection from myocardial I/R injury by reducing ROS generation from complex I that can occur via reverse electron transport.[31] Label-free peptide quantification was used to assess SNO-ND3 levels. We found that N6022 induced a significant increase in pre-ischemic SNO-ND3 levels in male hearts, but significantly decreased pre-ischemic SNO-ND3 levels in female hearts (Fig. 3c). After reperfusion, we were no longer able to detect differences in SNO-ND3 levels (Fig. 3f). These data suggest that enhanced SNO-ND3 may contribute to intrinsic protection in female hearts and N6022-mediated protection in male hearts.

GSNO-R inhibition suppresses post-ischemic ROS production.

Since SNO-ND3 was reported to augment complex I-mediated ROS generation,[32] we examined the impact of N6022 on post-ischemic H_2O_2 production in male and female hearts. Consistent with prior studies,[17, 27] post-ischemic H_2O_2 production was significantly decreased in control WT female hearts vs. males (Figs. 4a-4b), possibly via enhanced SNO-ND3 levels. Perfusion with N6022 prior to I/R injury substantially reduced post-ischemic H_2O_2 production in WT male and female hearts (Figs. 4a-4b). This decrease in post-ischemic ROS production is consistent with the N6022-mediated increase in SNO-ND3 levels in male hearts. However, since N6022 reduced SNO-ND3 levels in female hearts, the N6022-mediated reduction in post-ischemic ROS production likely occurs through a different mechanism.

N6022 inhibits the GSNO-R-mediated metabolism of GSNO and formaldehyde in the heart.

GSNO-R is reported to be the major enzyme responsible for the NADH-dependent reduction of GSNO in the heart and other organs.[6] To confirm GSNO-R inhibition with N6022, WT male and female hearts were perfused with or without N6022, and assayed for GSNO-R activity by monitoring NADH consumption using GSNO as a substrate. N6022 treatment completely blocked GSNO metabolism in WT male and female hearts (Figs 5a-5b). The assay was repeated with male and female heart homogenates from GSNO-$R^{-/-}$ mice treated with or without N6022, but GSNO-R activity was not detected. Additionally, since liver shows high GSNO-R expression and activity,[6] we utilized liver homogenate as a positive control. Pre-incubation of mouse liver homogenate with N6022 for 15 minutes was sufficient to significantly inhibit GSNO metabolism (Online Fig. IIa).

GSNO-R is also known to act as a formaldehyde dehydrogenase by metabolizing S-hydroxymethylglutathione.[8] We assayed total formaldehyde dehydrogenase activity in homogenates from WT male and female hearts perfused with or without N6022, and monitored NADH absorbance. We detected very little FDH activity at baseline in male and female hearts using formaldehyde as a substrate, especially compared to purified FDH enzyme as a positive control (data not shown), but GSNO-R inhibition nonetheless induced a

consistent decrease in NADH absorbance (Fig. 5c-5d). A similar decrease in NADH absorbance was observed in male and female GSNO-R$^{-/-}$ hearts compared to WT controls, suggesting that GSNO-R inhibition or genetic deletion may impair formaldehyde metabolism in the heart.

Although GSNO and *S*-hydroxymethylglutathione are the primary substrates of GSNO-R, GSNO-R is also capable of acting as an alcohol dehydrogenase.[7] We assayed total ADH activity using isopropanol as a substrate. As a positive control for the assay, we utilized purified enzyme (data not shown) and total liver homogenate (Online Fig. IIb). Pre-incubation of mouse liver homogenate with N6022 for 15 minutes had no appreciable effect on total ADH activity in the liver, suggesting that GSNO-R contributes minimally to total liver ADH activity with isopropanol as a substrate. In whole heart homogenates, we detected very little activity in WT male and female hearts, and GSNO-R inhibition or genetic deletion had no further effect on total ADH activity in the heart (Online Fig. IIc-IId).

GSNO-R inhibition increases post-ischemic free formaldehyde levels in female hearts.

Since N6022 had a significant impact on formaldehyde dehydrogenase activity (Figs. 5c-5d), baseline formaldehyde levels were examined in male and female WT and GSNO-R$^{-/-}$ hearts using a commercially available kit (Sigma-Aldrich). WT female hearts had nearly 2-fold more free formaldehyde at baseline vs. WT males (Fig. 6a). In addition, although male and female GSNO-R$^{-/-}$ hearts had slightly elevated free formaldehyde levels vs. WT controls, a 15 minute perfusion with N6022 was not sufficient to acutely increase free formaldehyde levels in either male or female hearts. Post-ischemic free formaldehyde levels were also assessed, and we no longer detected a difference in free formaldehyde levels between male and female WT hearts (Fig. 6b). However, free formaldehyde levels were significantly elevated in N6022-treated female hearts, and in male and female GSNO-R$^{-/-}$ hearts. N6022-treated male hearts did not show an increase in post-ischemic free formaldehyde compared to non-N6022-treated male hearts. These results suggest that free formaldehyde levels may contribute to the detrimental impact of N6022 treatment during I/R injury in female hearts.

Reactive aldehydes can be produced during I/R injury from lipid peroxidation resulting from elevated ROS.[33] Therefore, we examined 4-hydroxynonenal (4-HNE) levels, a marker of lipid peroxidation, to determine if this reaction may be the source of formaldehyde. N6022-treated male hearts and male GSNO-R$^{-/-}$ hearts showed elevated post-ischemic 4-HNE levels compared to control male hearts (Online Fig. IIIa-IIIb). However, post-ischemic 4-HNE levels did not change in N6022-treated female hearts or female GSNO-R$^{-/-}$ hearts (Online Fig. IIIa-IIIb). These data suggest that lipid peroxidation is not the source of formaldehyde in the post-ischemic female heart.

Aldehyde dehydrogenase 2 activation reduces I/R injury in female GSNO-R$^{-/-}$ hearts.

We attempted a rescue experiment using Alda-1 to activate mitochondrial aldehyde dehydrogenase 2 (ALDH2), which can metabolize formaldehyde.[34] To determine whether post-ischemic formaldehyde accumulation contributes to the injury observed in female hearts with GSNO-R ablation, male and female, WT and GSNO-R$^{-/-}$ hearts were perfused with Alda-1 (20 μmol/L) for 10 minutes prior to the onset of ischemia and for 10 minutes at

the start of reperfusion (Fig. 7a). Alda-1 significantly reduced infarct size in WT male hearts, but did not confer an additional infarct-sparing benefit to WT female hearts (Fig. 7b), which is consistent with prior reports.[27] Nevertheless, Alda-1 significantly reduced infarct size in female GSNO-R$^{-/-}$ hearts (Fig. 7b), suggesting that excess mitochondrial formaldehyde may exacerbate I/R injury in female hearts with the disruption of GSNO-R. Alda-1 provided no additional benefit to male GSNO-R$^{-/-}$ hearts. Alda-1 also normalized post-I/R formaldehyde levels in male and female GSNO-R$^{-/-}$ hearts (Online Fig. IV).

Formaldehyde does not compete with SNO for the modification of common cysteine residues.

Formaldehyde not only reacts with the amine group on the side chain of lysine, but it can also react with thiols[35–37] and thus, may compete with SNO for the modification of common cysteine residues. We used a fluorescent maleimide tag to label-free thiols in homogenized hearts treated with the *S*-nitros(yl)ating agent GSNO or formaldehyde to test this hypothesis. As expected, GSNO reduced the fluorescent signal at all concentrations tested in male and female heart homogenates, thus indicating thiol modification (Fig. 8a-8b). Formaldehyde treatment did not yield a statistically significant decrease in fluorescence (Fig. 8a-8c), suggesting that cysteine may not be the preferred target of formaldehyde. In addition, to determine if GSNO and formaldehyde compete to modify common cysteine residues, we treated samples for 15 minutes with GSNO followed by formaldehyde and vice versa. GSNO significantly suppressed the fluorescent signal in male and female hearts regardless of whether it was applied before or after formaldehyde treatment (Fig. 8d-8e), suggesting that formaldehyde likely does not directly compete with SNO for the modification of common cysteine residues.

DISCUSSION

Herein we report for the first time that disruption of GSNO-R induces sex-disparate effects during myocardial I/R injury, in part by abrogating sex-dependent protection in females. Although male hearts undoubtedly benefit from N6022-mediated inhibition of GSNO-R during I/R injury, the impact of GSNO-R inhibition on the response to injury in female hearts is clearly detrimental (Fig. 1). Similar sex-disparate effects were also apparent in hearts from GSNO-R$^{-/-}$ mice using ex vivo and in vivo models of I/R injury (Fig. 2). These results are consistent with prior in vivo work from the male heart,[18, 19] but to our knowledge, are the first to demonstrate that GSNO-R inhibition exacerbates I/R injury in female hearts.

GSNO-R is a major metabolizer of GSNO in the heart,[6] and consistent with the inhibition of GSNO metabolism, we found that N6022 increased total pre-ischemic SNO protein levels, but only in male hearts (Fig. 3). Female hearts exhibited higher baseline SNO protein levels compared to males, which is consistent with our prior studies,[17, 20] but total SNO protein levels remained unchanged with N6022 (Fig. 3). However, post-ischemic SNO levels were enhanced in N6022-treated male and female hearts compared to non-treated controls, suggesting that N6022 does affect SNO protein levels in male and female hearts. In addition, we found a strong association between pre-ischemic SNO-ND3 levels (at Cys39) and the

degree of I/R injury (Fig. 3c), such that control female and N6022-treated male hearts showed higher SNO-ND3 levels and less injury, while control male and N6022-treated female hearts showed lower SNO-ND3 levels and more injury. SNO at Cys39 of ND3 was reported to reduce post-ischemic ROS production,[38] and consistent with these results, we found decreased post-ischemic ROS production in control female and N6022-treated male hearts vs. control males (Fig. 4). However, N6022-treated female hearts also showed a reduction in ROS production, which may occur independently from SNO-ND3 levels. Given these results, we investigated the effect of GSNO-R inhibition on its reductase and dehydrogenase functions.

GSNO-R plays a critical role in redox biology by acting as a reductase to regulate reactive nitrogen species and as a dehydrogenase to regulate reactive aldehyde species, all while recycling glutathione and NADH.[39] We performed GSNO-R activity assays to confirm N6022-mediated inhibition of reductase activity in male and female hearts, but unexpectedly found that N6022 significantly impaired GSNO-R-mediated formaldehyde dehydrogenase activity (Fig. 5). Consistent with this impairment, post-ischemic formaldehyde levels were increased by nearly two-fold in N6022-treated female hearts and GSNO-$R^{-/-}$ hearts vs. other treatment groups (Fig. 6). Taken together, these results suggest that endogenous formaldehyde production may contribute to the pathology of I/R injury. Formaldehyde can be endogenously produced through multiple pathways, including serine oxidation and via demethylation reactions.[40, 41] Serine oxidation by myeloperoxidase may serve as a potential source of formaldehyde during I/R injury.[42] We examined a role for formaldehyde-induced toxicity by using Alda-1 to enhance the activity of ALDH2, which has been shown to play a role in cardioprotection,[27, 43] in part, by metabolizing reactive aldehydes like formaldehyde.[44] Interestingly, we found that Alda-1 reduced infarct size in female GSNO-$R^{-/-}$ hearts (Fig. 7), further supporting a role for formaldehyde in the pathology of myocardial I/R injury.

Although formaldehyde is a natural byproduct of cellular metabolism,[45] formaldehyde is a reactive electrophile with known toxicological implications. Formaldehyde can form adducts with DNA, lipids, and proteins,[45] in part via the modification of cysteine residues.[35, 46] As such, formaldehyde has the potential to compete with SNO for the modification of cysteines. However, our results suggest that formaldehyde-induced toxicity is likely not occurring through the modification of cysteine residues (Fig. 8). Overall, our results support a protective mechanism in male hearts whereby the loss of GSNO-R enhances protein SNO levels and decreases post-ischemic ROS production by increasing SNO-ND3 levels. Female hearts, on the other hand, experience increased injury with the loss of GSNO-R, despite a general decrease in post-ischemic ROS production. This injury likely occurs through the N6022-mediated reduction in SNO-ND3 levels and via the cytotoxic accumulation of formaldehyde.

GSNO-R, I/R injury and the female heart.

Epidemiological studies and preclinical models support the existence of an intrinsic, NO-dependent cardioprotective mechanism in pre-menopausal female hearts that reduces susceptibility to myocardial I/R injury.[20, 24–26] In prior studies, we found that WT female hearts exhibited higher levels of phospho-Akt, increased baseline eNOS expression and

activation (via Ser1177 phosphorylation), enhanced NO_x production, and higher SNO protein levels, which we propose contributes to intrinsic protection from I/R injury.[17, 20] In the current study, we again detect enhanced SNO protein levels at baseline in female hearts (Fig. 3) and protection from I/R injury vs. males (Fig. 1). We have also found GSNO-R activity to be enhanced at baseline in female hearts vs. males,[17, 20] suggesting that GSNO-R plays a protective role in female hearts. Indeed, this appears to be the case, as GSNO-R inhibition or genetic deletion exacerbated I/R injury in female hearts (Figs. 1 and 2). Although total pre-ischemic SNO protein levels did not change in N6022-treated female hearts (Fig. 3), we did find that SNO-ND3 levels were significantly decreased with N6022 treatment in female hearts (Fig. 3), suggesting that SNO-ND3 may be a critical component of cardioprotection in females that is downregulated with GNSO-R inbition.[47] We also noted that post-ischemic SNO protein levels were elevated in N6022-treated female hearts, so enhanced SNO of other protein targets may also play a role. Excessive protein SNO has been shown to contribute to disease pathogenesis with neurodegenerative conditions, neuromuscular atrophy and sepsis.[48–50]

GSNO-R inhibition or genetic deletion in female hearts showed the highest levels of post-ischemic free formaldehyde (Fig. 6), suggesting that formaldehyde may also contribute to the detrimental impact of GSNO-R disruption in female hearts. Formaldehyde is produced endogenously,[40, 41] and can be detoxified by GSNO-R.[7] GSNO-$R^{-/-}$ mice also exhibit increased susceptibility to formaldehyde-induced toxicity.[51] ALDH2 is also important for metabolizing formaldehyde and other reactive aldehydes. Lagranha e*t al.* suggested that the phosphorylation and activation of ALDH2 contributes to intrinsic cardioprotection in females,[27] and ALDH2 activation with Alda-1 has been shown to reduce ischemic injury in male hearts.[52] Activation of ALDH2 with Alda-1 reduced I/R injury in female GSNO-$R^{-/-}$ hearts, further supporting a role for formaldehyde in I/R injury. Formaldehyde is a reactive electrophile that is cytotoxic and can modify cysteine residues.[35, 46] However, our results suggest that formaldehyde does not directly compete with SNO for the modification of common cysteine residues (Fig. 8). Nonetheless, formaldehyde appears to be important, yet an unexplored component of I/R injury in female hearts. However, it is not clear why formaldehyde would preferentially affect female hearts (vs. males), but female hearts do appear to produce higher baseline levels of formaldehyde (Fig. 6), and thus may be more vulnerable to increases in formaldehyde with the loss of GSNO-R.

GSNO-R, I/R injury and the male heart.

Recent work from our group and others has shown that SNO protein levels increase in male hearts with many different forms of cardioprotective stimuli, ranging from ischemic pre- and post-conditioning to the use of pharmacologic agents.[9–15, 53–55] These protective effects can be blocked with the SNO-specific reducing agent ascorbate,[12] but not with soluble guanylate cyclase or PKG inhibition.[11, 55–57] Previous studies have reported that the genetic deletion of GSNO-R, which leads to increased myocardial SNO levels, [6, 18, 58] reduced myocardial infarct size in male mice subjected to coronary ligation.[18, 19] In a study by Lima *et al.*,[18] the protection observed in male GSNO-$R^{-/-}$ hearts was attributed to increased capillary density resulting from the SNO-induced, normoxic stabilization of hypoxia-inducible factor 1-α. Our results are in agreement with prior studies as we demonstrate significant

cardioprotective effects with the N6022-mediated inhibition or genetic deletion of GSNO-R in WT male hearts subjected to ex vivo and in vivo models of I/R injury (Fig. 1 and 2). Since N6022 increased myocardial SNO protein levels in males (Fig. 3), our results provide further support for the protective role of enhanced SNO protein levels in I/R injury, at least in male hearts. Although Lima *et al.* ultimately concluded that enhanced capillary density was responsible for cardioprotection in male GSNO-R$^{-/-}$ mice,[18] changes in capillary density are unlikely to play a role in our model of protection resulting from acute, ex vivo GSNO-R inhibition. As such, the SNO modification of additional target proteins like ND3 (Fig. 3), may be more important in our model of acute cardioprotection with N6022. With regard to formaldehyde levels in male hearts, we no longer detected a male-female difference in formaldehyde levels after I/R injury (Fig. 6), suggesting that formaldehyde levels may change during ischemia and the initial minutes of reperfusion. WT male hearts also benefited from ALDH2 activation (Fig. 7), but it is not clear why elevated formaldehyde levels did not have a detrimental impact on male GSNO-R$^{-/-}$ hearts since formaldehyde levels were elevated (Fig. 6). Compensatory adaptation may likely play a role.

Clinical ramifications of N6022 in the heart.

The results of our studies have important clinical implications. Although a number of pharmacologic compounds for inducing cardioprotection have shown great promise in pre-clinical models of I/R injury, these compounds failed to demonstrate a reduction in infarct size in recent clinical trials.[59–62] There are a number of factors that may influence the success of these trials, including advanced age in the human population and/or concurrent pathology,[63–66] but our results suggest that sex must also be considered when assessing cardioprotective strategies. These results further suggest that different therapeutic strategies may be required to combat ischemic heart disease in males and females. In addition, it is important to note that while most GSNO-R inhibitors are being developed to enhance protein SNO levels, the sex-dependent accumulation of cytotoxic levels of formaldehyde may yield unintended consequences in certain organ systems.

Conclusions.

In summary, we demonstrate a novel, sex-dependent role for GSNO-R in myocardial I/R injury. In the male heart, GSNO-R does not appear to be necessary for reducing I/R injury, as GSNO-R inhibition or genetic deletion yielded an improvement in post-ischemic functional recovery and a reduction in infarct size by enhancing SNO protein levels, namely at Cys39 of ND3, and reducing ROS production at the onset of reperfusion. In female hearts, however, GSNO-R is a requisite for protection, as GSNO-R inhibition reduced post-ischemic functional recovery and increased infarct size. Although GSNO-R inhibition reduced post-ischemic ROS production in female hearts, differential changes in protein SNO signaling (i.e., reduced SNO at Cys39 of ND3) and increased formaldehyde levels appear to work in tandem to drive injury in female hearts with GSNO-R inhibition. As such, the sex disparate effects of GSNO-R inhibition provide important mechanistic insight into the regulation of SNO protein and formaldehyde levels during I/R injury in male and female hearts, and identify formaldehyde as a novel reactive species in the context of I/R injury.

These results further suggest that different strategies may be required for the treatment of ischemic heart disease in males and females.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

ADH	alcohol dehydrogenase
ALDH2	aldehyde dehydrogenase 2
CypD	cyclophilin D
DMSO	dimethyl sulfoxide
EDV	end diastolic volume
ESV	end systolic volume
EF	ejection fraction
eNOS	endothelial nitric oxide synthase
FDH	formaldehyde dehydrogenase
FDR	false discovery rate
FS	fractional shortening
GSNO	nitrosoglutathione reductase
GSNO-R	*S*-nitrosoglutathione reductase
HR	heart rate
IA	iodoacetamide
IVSD	inter-ventricular septal thickness at end diastole
I/R	ischemia-reperfusion
LC-MS/MS	liquid chromatography-tandem mass spectrometry

LVDP	left ventricular diastolic pressure
LVEDD	left ventricle chamber diameter at end diastole
LVESD	left ventricle chamber diameter at end systole
LVIDd	left ventricular internal diameter at end diastole
LVIDs	left ventricular internal diameter at end systole
LV mass	left ventricle mass
m/z	mass to charge ratio
NADH	nicotinamide adenine dinucleotide
ND3	NADH dehydrogenase subunit 3
NEM	N-ethylmaleimide
NO	nitric oxide
NOS	nitric oxide synthase
PSM	peptide spectrum match
PWTED	posterior wall thickness at end diastole
RPP	rate pressure product
RWT	relative wall thickness
SNO	*S*-nitros(yl)ation
TTC	tetrazolium tetrachloride
WT	wildtype

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NOVELTY AND SIGNIFICANCE

What Is Known?

- The enzyme - *S*-nitrosoglutathione reductase (GSNO-R) indirectly regulates the cellular levels of protein *S*-nitros(yl)ation (SNO) and formaldehyde.
- Increased SNO protein levels and the genetic deletion of GSNO-R have been shown to reduce infarct size in male hearts subjected to ischemia-reperfusion injury (I/R).
- In comparison with male hearts, female hearts have increased SNO protein levels and GSNO-R activity, and exhibit endogenous, nitric oxide-dependent protection from I/R injury.

What New Information Does this Article Contribute?

- Inhibition or genetic deletion of GSNO-R led to sex-disparate effects on myocardial I/R injury.
- In male hearts, the loss of GSNO-R reduced I/R injury, in part, through increased SNO protein levels and decreased production of reactive oxygen species (ROS).
- In female hearts, the loss of GSNO-R exacerbated I/R injury, in part, through increased formaldehyde production.

Sex-dependent differences in myocardial pathophysiology are emerging as a critical area of research. In this study we examined the role in GSNO-R in male and female hearts during I/R injury. We found that genetic deletion of GSNO-R or inhibition of the enzyme using a specific and potent inhibitor (N6022), led to sex disparate effects in both in vivo and ex vivo models of myocardial I/R injury. In males, loss of GSNO-R reduced damage, but in females cell death in the myocardium was increased.. In male hearts, the loss of GSNO-R increased total SNO protein levels and reduced the post-ischemic production of ROS, in part, through the SNO-induced modification of mitochondrial proteins. In female hearts, GSNO-R loss did not alter SNO protein levels, but increased post-ischemic levels of formaldehyde. The Alda-1-induced activation of aldehyde dehydrogenase 2 to scavenge excess formaldehyde, rescued the injurious phenotype in female hearts lacking GSNO-R. These results identify formaldehyde as a novel mediator of myocardial I/R injury in female hearts and suggest that GSNO-R is an essential component of cardioprotection in females. These findings emphasize that different therapeutic strategies may be required to treat ischemic heart disease in males and females.

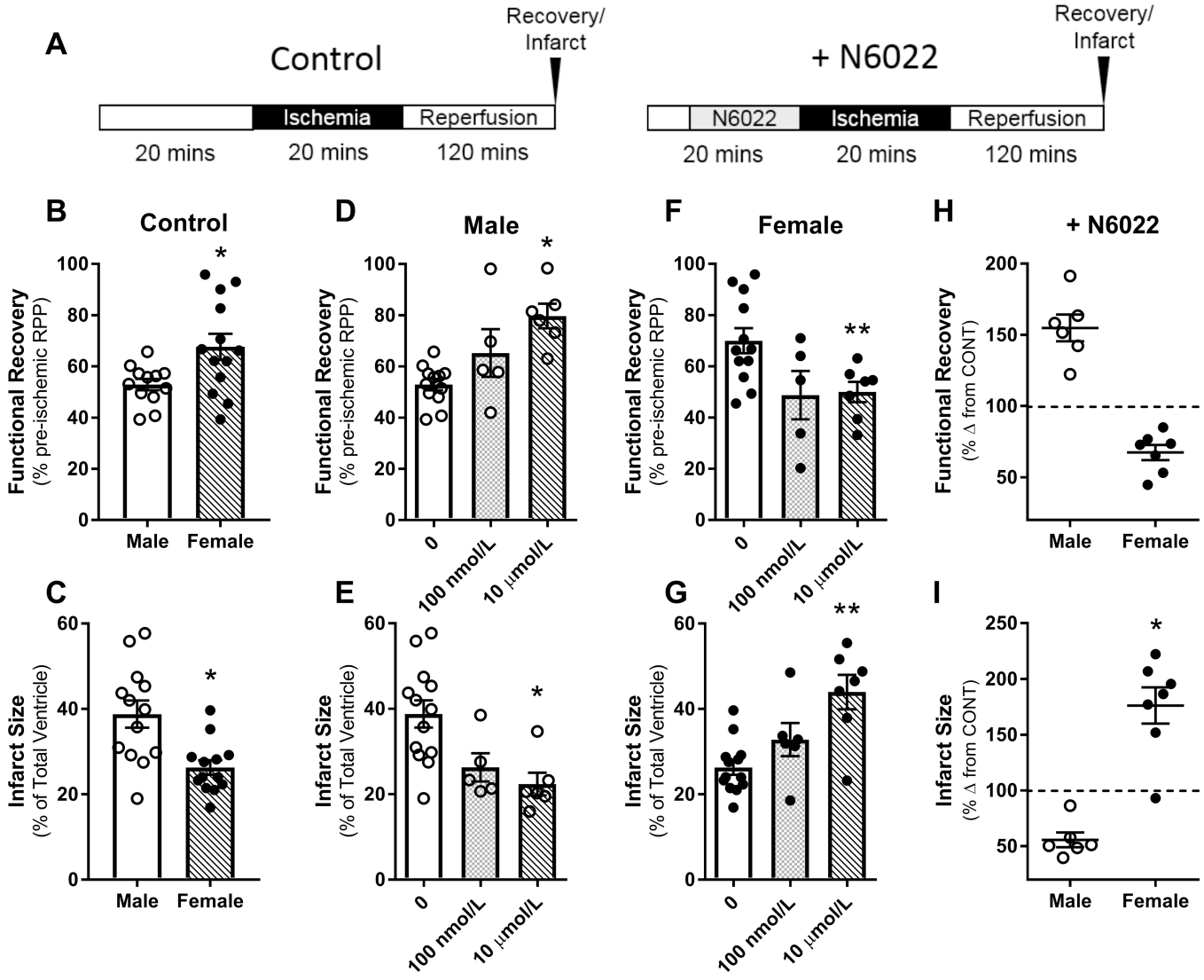

Figure 1. GSNO-R inhibition induces protection in male hearts, but exacerbates injury in female hearts.

(**A**) Hearts were perfused with or without the GSNO-R inhibitor N6022 for 15 minutes, and then subjected to 20 minutes of ischemia and 120 minutes of reperfusion; N6022 was also present during the first five minutes of reperfusion. (**B**) Post-ischemic functional recovery and (**C**) infarct size in control male and female WT hearts (n = 12–13 hearts/group; *p<0.05 vs. control male). (**D**) Post-ischemic functional recovery and (**E**) infarct size in control and N6022-perfused male hearts (n = 5–12 hearts/group; *p<0.05 vs. control male). (**F**) Post-ischemic functional recovery and (**G**) infarct size in control and N6022-perfused female hearts (n = 5–13 hearts/group; **p<0.05 vs. control female). (**H**) Percent change in post-ischemic functional recovery and (**I**) infarct size with 10 μmol/L N6022 treatment vs. respective control male or female heart (n = 6–7 hearts/group; *p<0.05 vs. N6022-treated male).

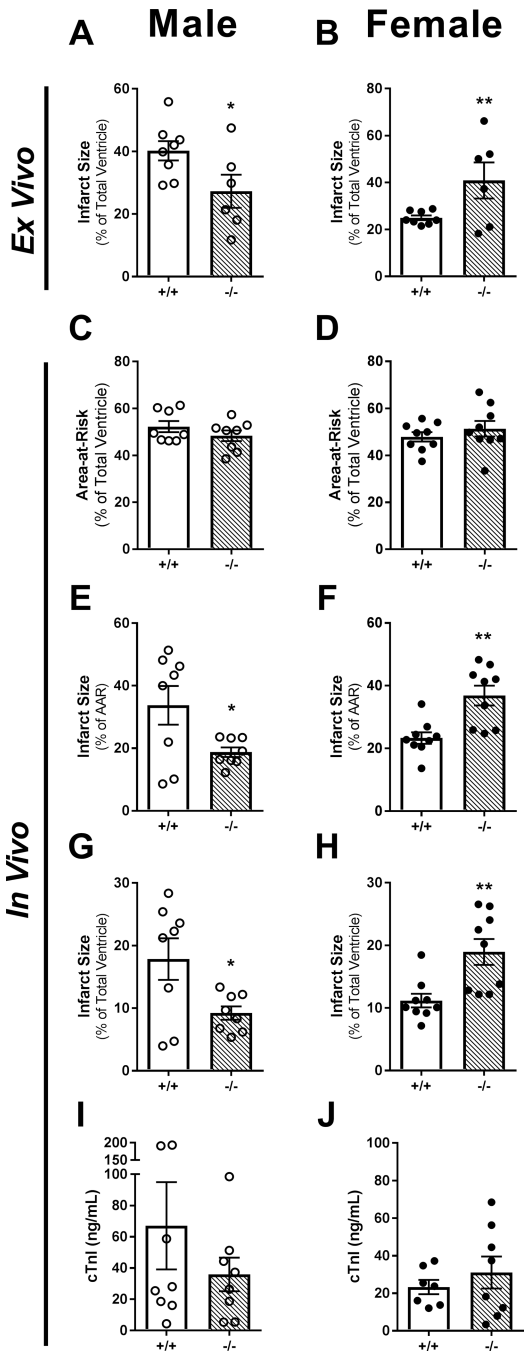

Figure 2. Genetic deletion of GSNO-R induces protection in male hearts, but exacerbates injury in female hearts.

(**A, B**) Infarct size from Langendorff-perfused male (**A**) and female (**B**) WT (+/+) and GSNO-$R^{-/-}$ (−/−) hearts (n = 6–8 hearts/group; *p<0.05 vs. male WT, **p<0.05 vs. female WT). (**C-J**) Area-at-risk (AAR) (**C, D**), infarct size (% of AAR) (**E, F**), infarct size (% of total ventricle) (**G, H**), and plasma cTnI levels (**I, J**) from male and female WT and GSNO-$R^{-/-}$ hearts subjected to LAD occlusion surgery (n = 8–9 mice/group; *p<0.05 vs. male WT, **p<0.05 vs. female WT).

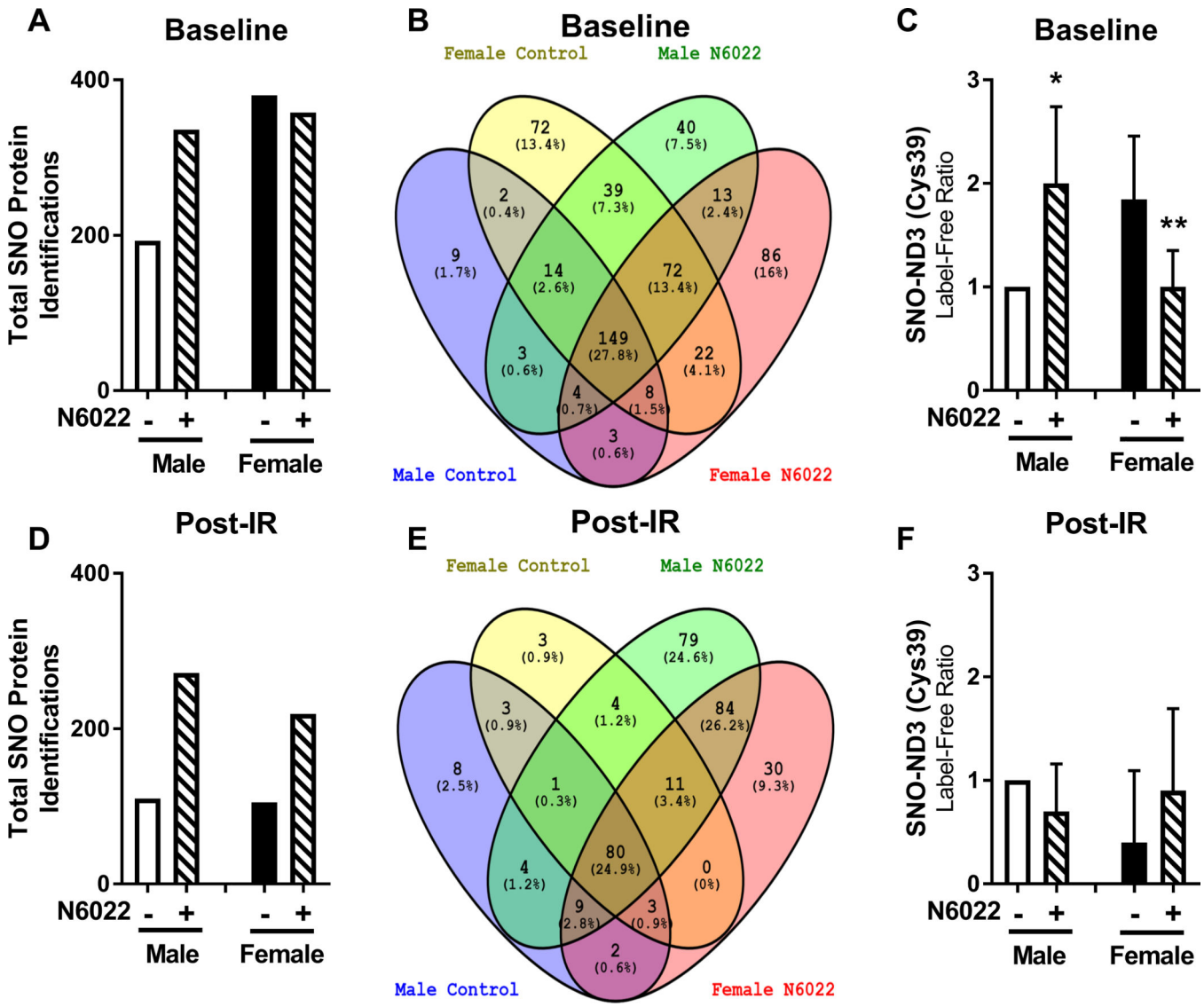

Figure 3. SNO protein levels increase in male hearts with GSNO-R inhibition, but not in female hearts.

(**A-F**) Total number of SNO protein identifications from control and N6022-treated male and female WT hearts at baseline (**A**) with Venn diagram (**B**), and post-I/R (**D**) with Venn diagram (**E**) as assessed via SNO-RAC in tandem with LC-MS/MS (n = 3 hearts/group; FDR: 1%). Note: All SNO protein identifications were detected in at least two of three hearts/group; these numbers represent the total number of SNO proteins identified in each group, so statistics were not performed. (**C, F**) SNO-ND3 levels from control and N6022-treated male and female WT hearts at baseline (**C**) and post I/R (**F**) as assessed via spectral counting (n = 3 hearts/group; *p<0.05 vs. control male, **p<0.05 vs. control female).

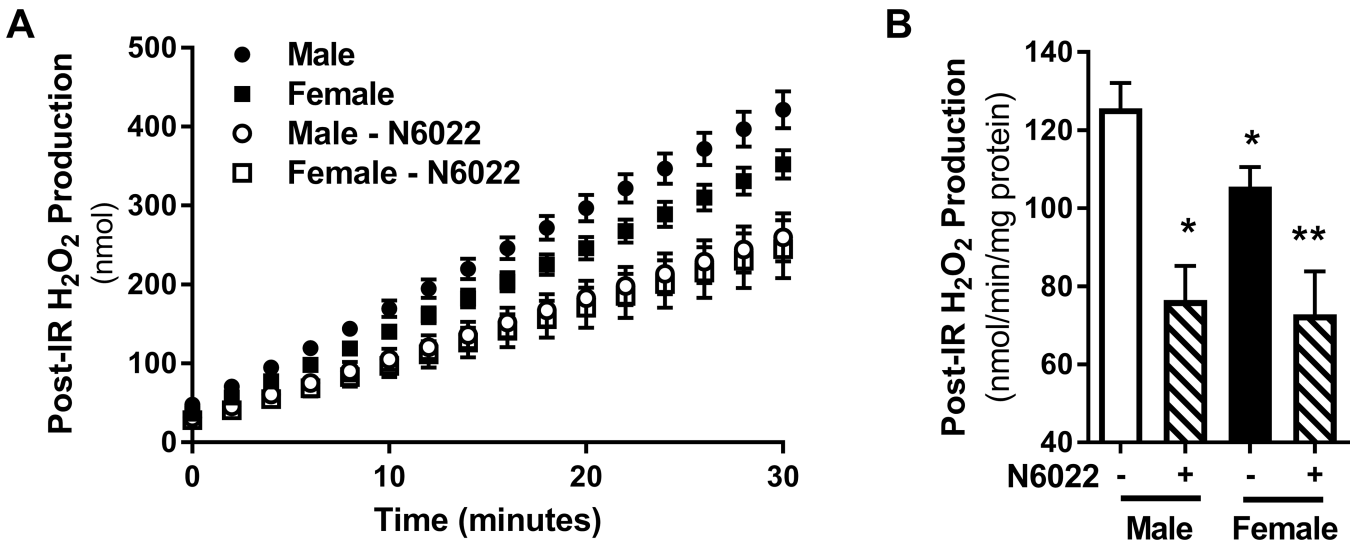

Figure 4. N6022 suppresses post-ischemic ROS production in male and female hearts.

(**A**) Hydrogen peroxide production over time and (**B**) the rate of hydrogen peroxide production in post-ischemic control and N6022-treated male and female WT hearts (n = 5 hearts/group; *p<0.05 vs. control male, **p<0.05 vs. control female).

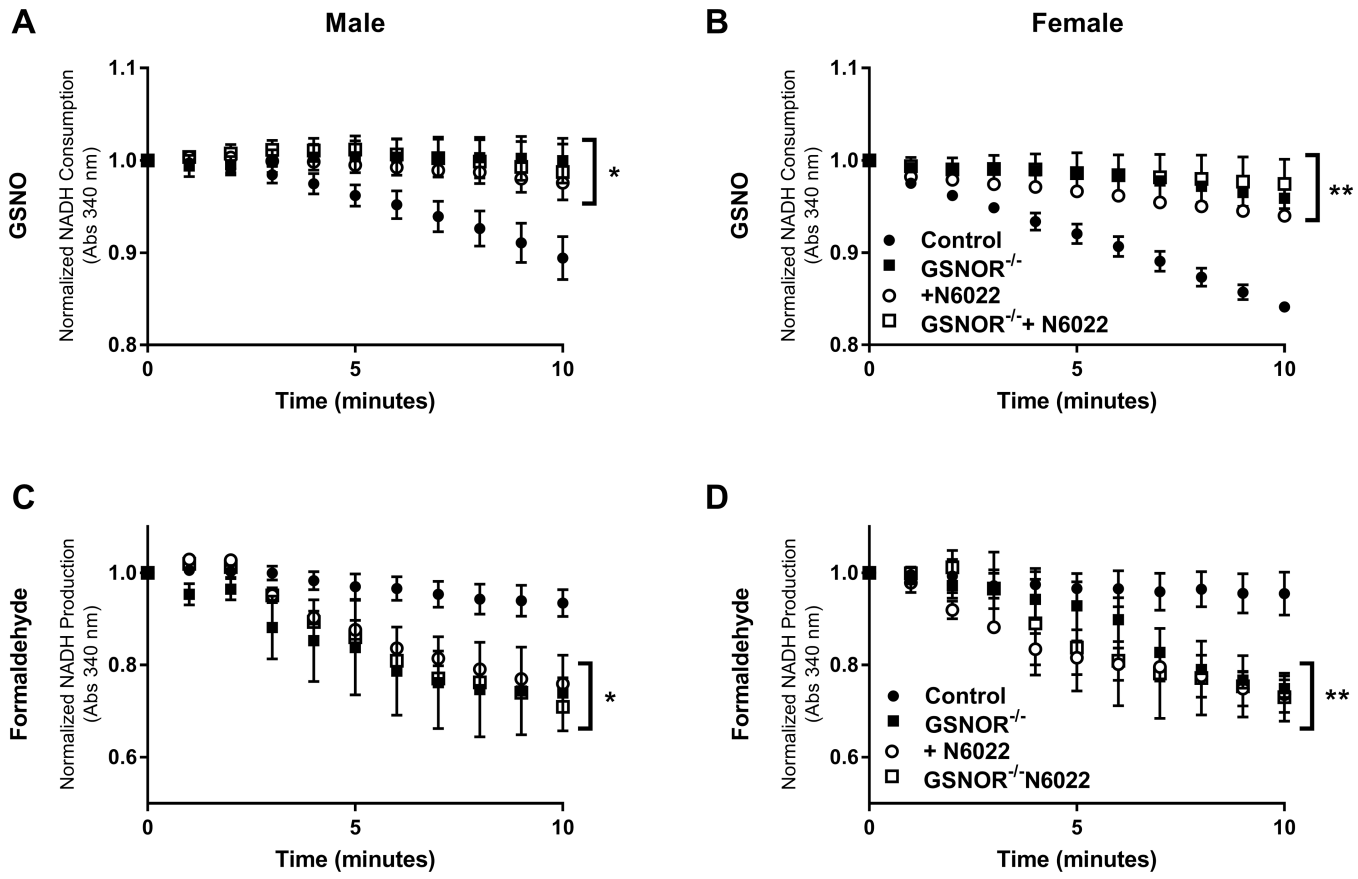

Figure 5. N6022 inhibits GSNO-R activity in male and female hearts.

(**A-D**) GSNO-R activity in whole heart homogenates from control and N6022-treated male and female, WT and GSNO-R$^{-/-}$ hearts measured via NADH consumption with GSNO as a substrate (**A-B**), and via NADH production with formaldehyde as a substrate (**C-D**) (n = 3 hearts/group; *p<0.05 vs. control male, **p<0.05 vs. control female).

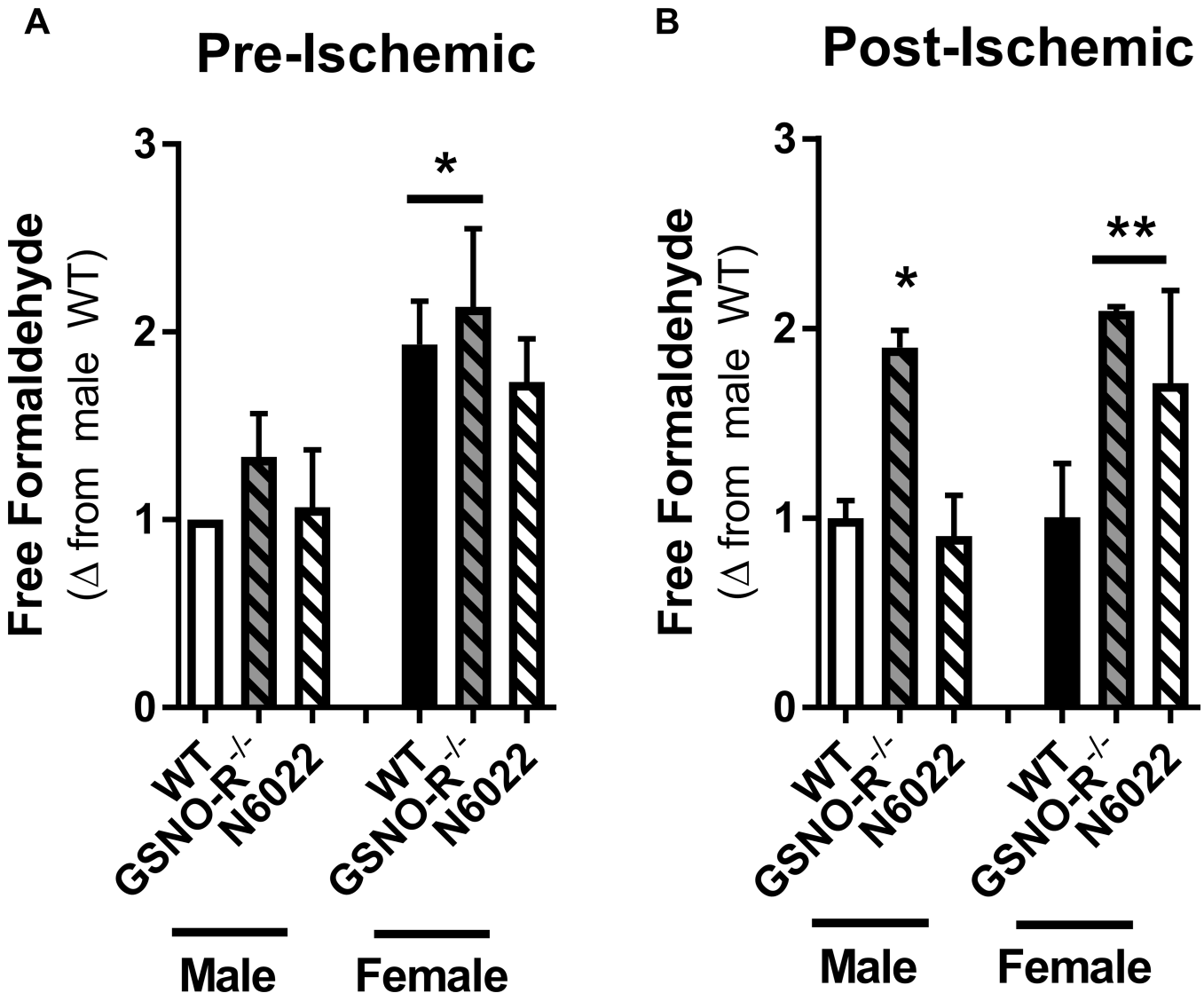

Figure 6. GSNO-R inhibition increases post-ischemic free formaldehyde levels in female hearts, but not in males.

(A) Pre-ischemic and **(B)** post-ischemic free formaldehyde levels assessed in control, N6022-treated and GSNO-$R^{-/-}$ male and female hearts (n = 3 hearts/group; *p<0.05 vs. WT male, **p<0.05 vs. WT female).

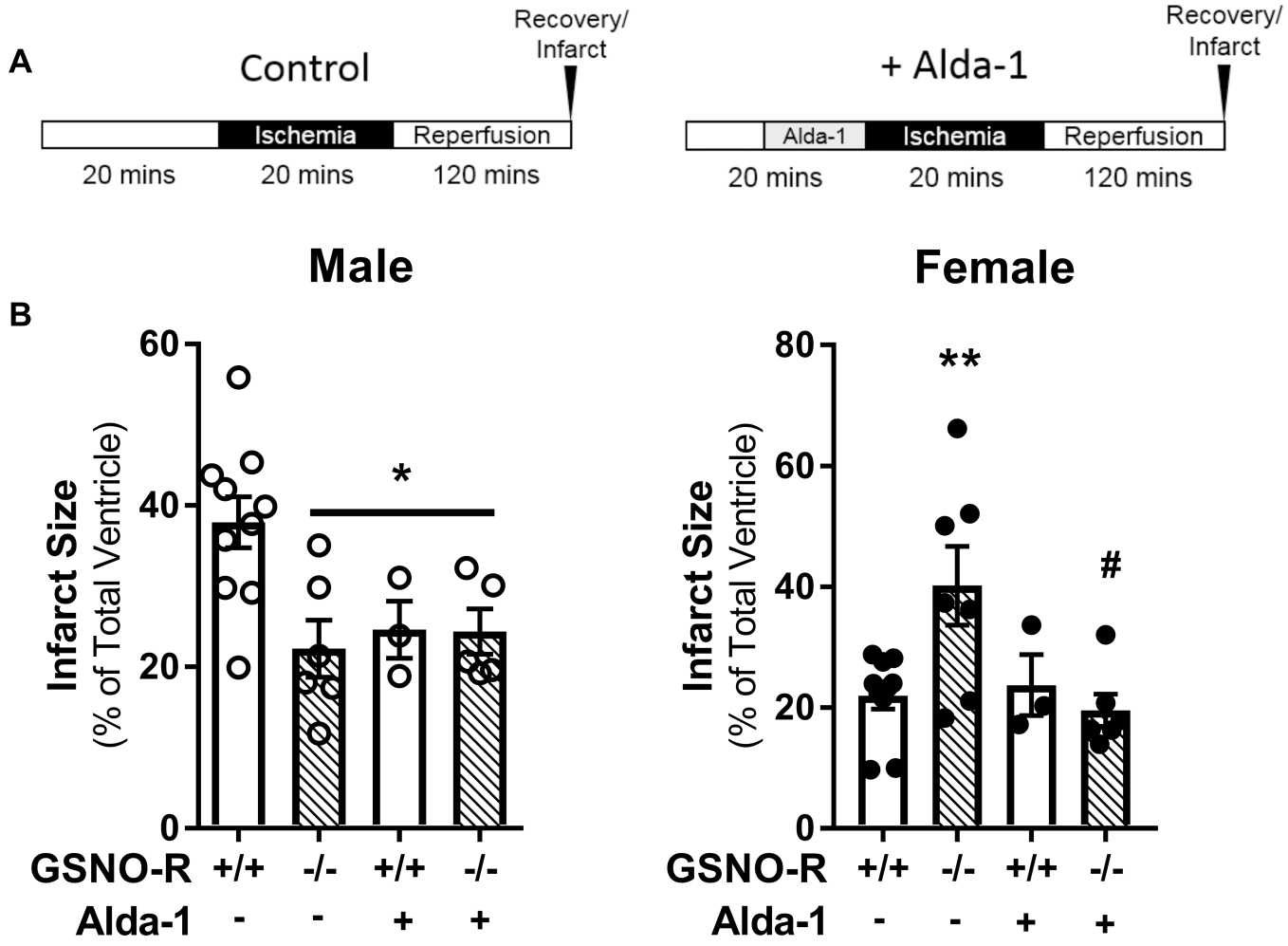

Figure 7. ALDH2 activation reduces infarct size in GSNO-R$^{-/-}$ female hearts.

(**A**) Hearts were perfused with or without the ALDH2 activator Alda-1 (20 μmol/L) for 10 minutes, and then subjected to 20 minutes of ischemia and 120 minutes of reperfusion; Alda-1 was also present for the first 10 minutes of reperfusion. (**B**) Infarct size in control and Alda-1-perfused male and female, WT and GSNO-R$^{-/-}$ hearts (n = 3–10 hearts/group; *p<0.05 vs. control male, **p<0.05 vs. control female, $^{\#}$p<0.05 vs. GSNO-R$^{-/-}$ female).

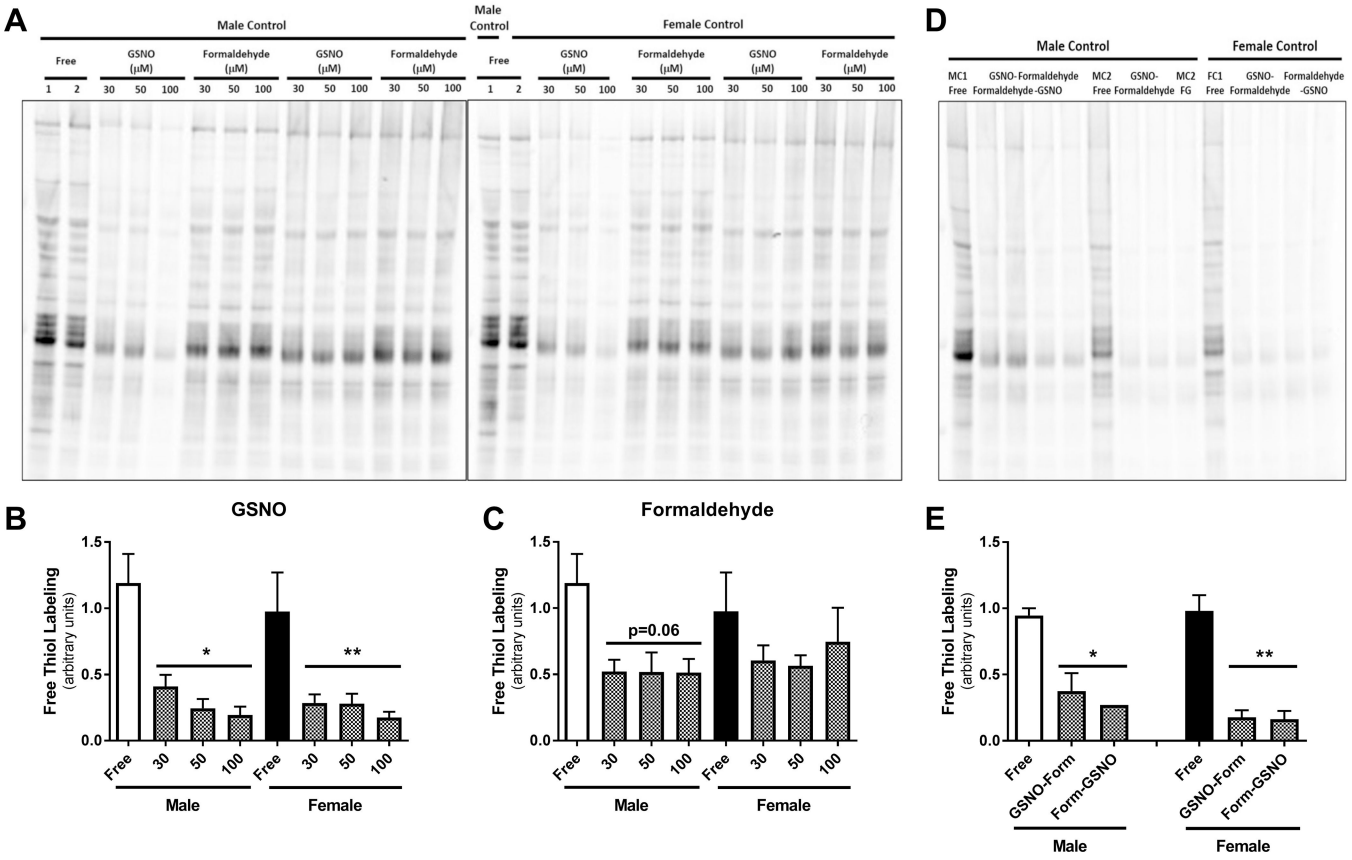

Figure 8. Formaldehyde does not compete with SNO for the modification of common cysteines.

(**A-E**) Free thiols were labeled with fluorescent maleimide tags in whole heart homogenates from WT males and females before and after treatment with 30, 50, 100 μmol/L GSNO or formaldehyde (**A-C**) (n = 3–4 hearts/group) or 50 μmol/L GSNO for 15 minutes, then 50 μmol/L formaldehyde for 15 minutes, and vice versa (**D, E**) (n = 2–3 hearts/group). *p<0.05 vs. free thiol male, **p<0.05 vs. free thiol female. Note: gel-to-gel variability was normalized with free thiol male sample (MC1).