S-Nitrosoglutathione Reductase Deficiency Enhances the Proliferative Expansion of Adult Heart Progenitors and Myocytes Post Myocardial Infarction

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Background—Mammalian heart regenerative activity is lost before adulthood but increases after cardiac injury. Cardiac repair mechanisms, which involve both endogenous cardiac stem cells (CSCs) and cardiomyocyte cell-cycle reentry, are inadequate to achieve full recovery after myocardial infarction (MI). Mice deficient in S-nitrosoglutathione reductase (GSNOR−/−), an enzyme regulating S-nitrosothiol turnover, have preserved cardiac function after MI. Here, we tested the hypothesis that GSNOR activity modulates cardiac cell proliferation in the post-MI adult heart.

Methods and Results—GSNOR−/− and C57Bl6/J (wild-type [WT]) mice were subjected to sham operation (n=3 GSNOR−/−; n=3 WT) or MI (n=41 GSNOR−/−; n=65 WT). Compared with WT, GSNOR−/− mice exhibited improved survival, cardiac performance, and architecture after MI, as demonstrated by higher ejection fraction (P<0.05), lower endocardial volumes (P<0.001), and diminished scar size (P<0.05). In addition, cardiomyocytes from post-MI GSNOR−/− hearts exhibited faster calcium decay and sarcomeric relaxation times (P<0.001). Immunophenotypic analysis illustrated that post-MI GSNOR−/− hearts demonstrated enhanced neovascularization (P<0.001), c-kit+ CSC abundance (P=0.013), and a ≥3-fold increase in proliferation of adult cardiomyocytes and c-kit+/CD45− CSCs (P<0.0001 and P=0.023, respectively) as measured by using 5-bromodeoxyuridine.

Conclusions—Loss of GSNOR confers enhanced post-MI cardiac regenerative activity, characterized by enhanced turnover of cardiomyocytes and CSCs. Endogenous denitrosylases exert an inhibitory effect over cardiac repair mechanisms and therefore represents a potential novel therapeutic target. (J Am Heart Assoc. 2015;4:e001974 doi: 10.1161/JAHA.115.001974)

Key Words: cardiovascular progenitor/stem cells • heart disease • heart regeneration • nitric oxide signaling • S-nitrosoglutathione reductase

The rate of myocardial renewal and regeneration in the adult mammalian heart is insufficient for recovery from damage.1,2 The mechanisms underlying this insufficiency remain highly controversial.1–3 Developmental studies in mice and human embryonic stem cells show that creation of new myocardium in mammals occurs through a multifaceted pathway that involves differentiation of cardiovascular progenitors and replication of mature cardiomyocytes.4,5 Both of these mechanisms are likely present in the postnatal mammalian heart to a limited degree,2,3,5 but their regenerative activity appears to be negatively regulated throughout adult life and are lost in the early postnatal period, possibly by mechanisms involving oxidative stress.6–12

Several pharmacologic and cell-based therapeutic agents improve the regenerative activity of postnatal cardiac progenitors and replicating cardiomyocytes.5,7,13–16 For example, infusion of thymosin–B4 into healthy mouse hearts enhances the cardiomyogenic capacity of adult epicardial progenitors after myocardial infarction (MI),15 whereas infusion of micro-RNAs 199a and 590 may selectively enhance cardiomyocyte cell-cycle reentry after MI.13 Furthermore, bone marrow–derived mesenchymal stem cells (MSCs)14 or growth hormone–releasing hormone receptor agonists17 enhance adult heart regeneration considerably by activating both cardiac progenitors and replicating cardiomyocytes.

GSNOR (alcohol dehydrogenase 3) regulates protein S-nitrosylation (SNO) turnover.18 In the adult heart, SNO...
modulates signaling pathways important for vasodilation, cardiomyocyte contraction, mitochondrial function, and Ca^2+ handling. Interestingly, studies in mouse and rat embryos show that while GSNOR is active during induction of the mammalian cardiogenic program, the developing heart exhibits the lowest GSNOR activity compared with other embryonic tissues. Moreover, mice deficient in GSNOR develop normally but are characterized by a high propensity for postnatal hepatocarcinogenesis (HCC). Remarkably, they also exhibit a profound capacity for repairing liver and heart injury. We therefore hypothesized that GSNOR may negatively regulate the regenerative activity of cardiac progenitors and cardiomyocytes in the post-MI adult heart.

To test this hypothesis, we analyzed the cardiovascular phenotype of GSNOR−/− and wild-type (WT) mice in response to MI. Here, we show that, in addition to improved revascularization and cardiomyocyte Ca^2+ handling, the loss of GSNOR in mice enhances the abundance and proliferative activity of adult cardiac progenitors, MSCs, and cardiomyocytes post MI. These findings have potentially important therapeutic implications because they suggest that GSNOR activity negatively regulates heart regeneration by suppressing proliferation of regenerative cardiovascular progenitors and cardiomyocytes.

Methods
This study was reviewed and approved by the University of Miami Institutional Animal Care and Use Committee and complies with all federal and state guidelines concerning the use of animals in research and teaching as defined by “The Guide for the Care and Use of Laboratory Animals” (National Institutes of Health, revised 2011).

Animals
The generation of GSNOR−/− mice has been reported. To eliminate/minimize genetic heterogeneity, GSNOR+/− mice were backcrossed for >10 generations into C57Bl6/J (WT) mice before GSNOR−/− mouse colonies were established at the University of Miami. Thus, our GSNOR−/− mice are considered to be on a C57Bl6/J background. Age-matched WT mice obtained from Jackson Laboratories were used as controls. Only male mice were used in this study. Mice received food and water ad libitum and were on a 12-hour light/dark cycle.

Experimental Model of MI
Three-month-old mice were anesthetized with isoflurane (2%) inhalation through endotracheal intubation. Body temperature was controlled during the entire procedure, and buprenor-
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Infarct size was quantified by using Image J, NIH, and Photoshop CS3 as previously described.31

Parafin sections while the animal was under sedation (2% isoflurane) were blocked with 10% normal donkey serum and goat anti-mouse IgG (Sigma-Aldrich) for 1 hour, sections were incubated with a primary antibody at 37°C for 1 hour, followed by the application of corresponding secondary antibody (Alexa dyes; Invitrogen) at 37°C for 1 hour. Omission of the primary antibodies on parallel sections was used as negative control. The following primary antibodies were used c-Kit (goat polyclonal; R&D systems), CD45 (rabbit polyclonal; Abcam), serine-10 phosphorylated histone-H3 (HP3; rabbit polyclonal; Abcam), anti-BrdU (biotinylated mouse monoclonal; Abcam), Aurora B kinase (Rabbit polyclonal, Abcam), activated caspase 3 (mouse monoclonal; BD), and tropomyosin (mouse monoclonal; Abcam). Nuclei were counterstained by incubation for 5 minutes with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). The total number of positively stained cells was quantified per zone observed and corrected per area of corresponding zone (mm²). All images were obtained with a fluorescent microscope (Olympus IX81; Olympus America Inc, Center Valley, PA) or a confocal laser-scanning module (LSM710; Carl Zeiss Microimaging).

In Vitro BrdU Cell Proliferation Assay

MSCs were plated in 96-well plates at the density of 1 x 10⁴ cells/well. BrdU incorporation was measured by using a BrdU proliferation assay kit (Millipore) according to the manufacturer’s protocol. Briefly, the cells were labeled with BrdU (1:500). Then, cells were fixed, air-dried, and incubated 1 hour with anti-BrdU monoclonal antibody (1:200). The cells were washed 3 times and then incubated with peroxidase-conjugated goat anti-mouse IgG (1:2000) at room temperature. Thereafter, 100 μL of peroxidase substrate was added to each well and incubated for 30 minutes in the dark. Absorbance at a dual wavelength of 450/550 nm was measured.

GSNOR Activity in c-Kit CSCs and Whole Heart Lysates

The GSNOR activity assay was performed as previously described.18,25 Briefly, heart tissues from WT and GSNOR⁻/⁻ mice at baseline and 8 weeks post MI were homogenized in a
cell lysis buffer (Cell Signaling). CSCs were isolated from WT and GSNOR−/− mice ventricles, expanded, and magnetically sorted for c-Kit according to a previously described method. The concentration of these samples was determined using a standard Bradford assay. Heart homogenate (100 µg/mL) was incubated with Tris-HCl (2 mmol/L, pH 8.0), EDTA (0.5 mmol/L), and NADH (200 µmol/L). The reaction was started by adding S-nitrosoglutathione (GSNO) (400 µmol/L), and activity was measured as GSNO-dependent NADH consumption at absorbance of 340 nm for 5 minutes.

**Statistics**

Data are shown as individual points and/or mean±SE. Statistical significance was determined by unpaired Student t test, Mann–Whitney test, 1-way or 2-way ANOVA, and Kruskal–Wallis test as appropriate, to meet test assumptions. When significant differences were found, Bonferroni or Dun’s multiple comparisons post-hoc tests were carried out, as appropriate. Survival data were analyzed using Kaplan–Meier survival curves and compared using the Wilcoxon and log-rank tests.

**Results**

**Cardiac Function is Improved While Cardiac Remodeling and Myocardial Infarct Size Are Reduced in GSNOR−/− Mice After MI**

To study the effect of GSNOR in heart function and in response to MI, we ligated a branch of the LCA of GSNOR−/− (n=41) and WT (n=65) mice and followed the animals using serial echocardiography. Additionally, 3 animals from each group underwent sham operations. Survival analysis revealed that, compared with WT mice, loss of GSNOR conferred a significant decrease in mortality in response to experimental MI (Figure 1A). Importantly, no differences in EF were recorded before surgery, suggesting that GSNOR−/− and WT mice have the same cardiac performance at baseline (Figure 1B). Induction of MI was accompanied by an equal reduction in EF in both mouse strains, and these changes were not different between groups during the first 4 weeks after MI (Figure 1B), indicating that the surgery was performed in a similar manner in both the GSNOR−/− and WT groups. However, at 8 weeks post MI, EF increased by ≈10% compared with 4 weeks earlier in GSNOR−/− mice and was significantly greater than in WT animals (Figure 1B and Table, P=0.048).

Consistent with previous reports, these changes in EF were accompanied by significant improvements in LV remodeling. GSNOR−/− and WT LV volumes during end-diastole (EDV) and end-systole (ESV) did not differ at baseline and underwent similar remodeling during the initial 4-week period after MI (Figure 1C and 1D). However, while LV remodeling continued to worsen in WT animals throughout the 8-week period of the study, expansion of EDV and ESV did not progress in GSNOR−/− mice after 4 weeks, and by 8 weeks, the EDV and ESV were significantly lower than in WT hearts (Figure 1C and 1D). Consistent with echocardiographic data, hemodynamic measurements at 8 weeks post MI showed an increase in EF in the GSNOR−/− group (Table); however, the developed pressure and end-systolic pressure were lower compared with WT mice. In addition, histologic quantification of scar volumes and scar perimeters at 8 weeks post MI demonstrated that GSNOR−/− hearts had significantly less fibrosis compared with WT (Figure 1E through 1G; P<0.05).

**GSNOR−/− Cardiomyocytes Have Faster Ca2+ Transient Decay**

Cardiac function depends on Ca2+ homeostasis, which is subject to intrinsic and extrinsic controls. Ca2+ transient amplitude (Δ[Ca2+]i) as well as its dynamics are important parameters for the activation of cardiac contractile proteins. Abnormalities in Ca2+ handling result in altered cardiac function. To test if better cardiac function in GSNOR−/− mice post MI was associated with improved Ca2+ handling, we quantified [Ca2+]i, and measured SL simultaneously in cardiomyocytes collected at 8 weeks post MI. GSNOR−/− cardiomyocytes consistently needed less time to reuptake Ca2+ from the cytosol (Figure 2A and 2B), resulting in faster sarcomeric relaxation (Figure 2C and 2D). Higher cytosolic free Ca2+ levels, due to inadequate Ca2+ reuptake or higher Ca2+ leak from sarcoplasmic reticulum, are associated with the lower cardiac contraction seen in heart failure.

**GSNOR−/− Hearts Exhibit Lower Adrenergic Signaling**

Sympathetic hyperactivity plays an important role in cardiac dysfunction in heart failure. In our study, GSNOR−/− mice post MI exhibited depressed sensitivity to ISO in both pressure-volume loop (Figure 3A) and in isolated cardiomyocyte studies (Figure 3B). WT mice responded to ISO treatment with elevated heart rates and cardiac function parameters during ISO treatment, while GSNOR−/− hearts did not exhibit any significant changes compared with steady state. Consistently, the contractile response to increasing ISO doses was shifted to the right in GSNOR−/− compared with WT cardiomyocytes, evidencing lower sensitivity.
Enhanced Proliferative Expansion of c-Kit+CSCs in Post-MI GSNOR⁻/⁻ Hearts

We and others previously showed that activation of endogenous cardiac progenitors is an underlying mechanism of postnatal heart regeneration in mammals. We therefore tested whether the number of adult c-Kit+CSCs in GSNOR⁻/⁻ was greater than in WT mouse hearts. Accordingly, immunohistochemistry against c-Kit and CD45 was performed in the hearts of healthy GSNOR⁻/⁻ and WT mice, as well as at 1 week, 1 month, and 2 months after experimental MI. In the absence of myocardial injury, both GSNOR⁻/⁻ and WT hearts had equivalent numbers of endogenous c-Kit+/CD45⁻ CSCs (Figure 4A). However, in response to MI, the abundance of c-Kit+/CD45⁻ CSCs increased significantly and this increase was greater in GSNOR⁻/⁻ compared with WT mice (Figure 4B). Importantly, compared with WT, GSNOR⁻/⁻ infarcted hearts had significantly more c-Kit+ CSCs throughout the 2-month follow-up period (Figure 4B). These findings strongly suggest that

Figure 1. GSNOR⁻/⁻ mice exhibit resilience to MI, have improved left ventricular function and less pathological remodeling at 8 weeks post MI. A, Kaplan–Meier plot illustrating significant differences in the survival of WT compared with GSNOR⁻/⁻ mice after experimental MI. B, Both WT and GSNOR⁻/⁻ mice have lower EF at 1 week post MI. WT EF remained low for the duration of the study, while GSNOR⁻/⁻ mice showed significant improvement in cardiac function. C and D, GSNOR⁻/⁻ mice exhibited left ventricular remodeling during diastole (C) and systole (D) compared with WT. E, Representative Masson’s trichrome staining of histological sections of WT and GSNOR⁻/⁻ hearts 8 weeks post MI. F, Infarcted WT hearts have more percentage of fibrosis area than sham groups and infarcted GSNOR⁻/⁻ hearts at 8 weeks post MI. G, Infarcted WT and GSNOR⁻/⁻ mice show increased fibrosis perimeter when compared with sham-operated animals 8 weeks post MI. WT hearts also demonstrate higher fibrosis perimeter compared with GSNOR⁻/⁻ hearts. A, Mean±SEM (n=65 WT, n=41 GSNOR⁻/⁻ mice). B through D, Mean±SEM (2-way ANOVA; n=15 WT, n=9 GSNOR⁻/⁻ mice at all time points; P= difference between WT and GSNOR⁻/⁻ mice). F and G, Individual values (1-way ANOVA; *P<0.05 compared with sham; #P<0.05 compared with MI). ANOVA indicates analysis of variance; EDV, volumes during end diastole; EF, ejection fraction; ESV, volumes during end systole; GSNOR, S-nitrosoglutathione reductase; MI, myocardial infarction; WT, wild-type.

DOI: 10.1161/JAHA.115.001974
expression of GSNOR in the infarcted myocardium suppresses the activity of endogenous c-Kit+ CSCs.

To elucidate whether the difference in the abundance of post-MI c-Kit+ CSCs was due to enhanced cell-cycle activity of GSNOR+/+ c-Kit+ CSCs, we primed infarcted GSNOR+/+ and WT mice with BrdU, a synthetic nucleoside that substitutes thymidine in newly synthesized DNA during the S-phase of the cell cycle and therefore provides a marker for tracking cell proliferation.38 One month later, heart tissues were collected and confocal immunofluorescence analysis against BrdU and c-Kit in the myocardium illustrated that the expansion in post-MI c-Kit+ CSCs in GSNOR−/− hearts was accompanied by a significantly higher rate of BrdU incorporation (Figure 4C and 4D; 10.9±4.99% of WT c-Kit+ CSCs compared with 15.9±3% of GSNOR−/− c-Kit+ CSCs were positive for BrdU, P=0.012). Thus, collectively these results suggest that, in response to myocardial damage, GSNOR activity negatively regulates heart regeneration by fostering important post-translational modifications to prevent proliferation and expansion of c-Kit+ CSCs.

Last, to elaborate whether the observed differences between GSNOR+/+ and GSNOR−/− c-Kit+ CSCs are directly or indirectly associated with GSNOR deficiency, we used an NADH-dependent GSNOR activity assay to gauge the enzymatic activity of GSNOR in culture-expanded c-Kit+ CSCs, purified from WT and GSNOR−/− hearts (Figure 4E).18,25 No change in the absorbance of NADH was detected in the absence of GSNO between the 2 CSC strains (Figure 4F). However, in the presence of GSNO, NADH oxidation produced a sharp decrease in absorbance in WT but not GSNOR−/− c-Kit+ lysates, over time (Figure 4F). These results indicate that GSNOR activity is present in GSNOR+/+ but not in GSNOR−/− c-Kit+ CSCs and, therefore, the differences observed in proliferative capacity may be directly associated with GSNOR deficiency.

### Table. Hemodynamic Measurement 8 Weeks After Left Anterior Descending Coronary Artery Ligation

| Parameters | WT (n=13) | GSNOR−/− (n=7) |
|------------|-----------|----------------|
| HR, bpm    | 448.1±17.43 | 392.3±20.83   |
| P_{dev}, mm Hg | 84.72±3.20  | 70.65±4.72*   |
| P_{es}, mm Hg | 13.69±1.26  | 11.49±3.39    |
| P_{es}, mm Hg | 91.17±3.38  | 75.64±6.12*   |
| EF, %      | 22.17±3.08  | 31.29±1.21*   |

Data are presented as mean±SEM. WT indicates wild-type; GSNOR, S-nitrosoglutathione reductase; HR, heart rate; P_{dev}, developed pressure; P_{es}, end-systolic pressure; EF, ejection fraction; MI, myocardial infarction.

*P<0.05 compared with WT after MI.

Figure 2. Relaxation mechanisms. GSNOR−/− and WT cardiomyocytes were stimulated at different frequencies (0.5 to 4 Hz). A, Tau, the time constant of [Ca^{2+}] decay (a measure of Ca^{2+} reuptake), and (B) the time to 90% of [Ca^{2+}] decay were measured and demonstrate that [Ca^{2+}] decay occurs faster in GSNOR−/− cardiomyocytes. C and D, Sarcomere relaxation (Tau [C] and time to 90% relaxation [D]) was improved in GSNOR−/− cardiomyocytes at all frequencies studied. Data are presented as mean±SEM (n=6 WT, n=6 GSNOR−/− mice at all time points). Two-way ANOVA; P<0.001 between WT and GSNOR−/− groups. ANOVA indicates analysis of variance; GSNOR, S-nitrosoglutathione reductase; WT, wild-type.
that replication of preexisting cardiomyocytes provides an additional mechanism for cardiomyogenesis in newborn\textsuperscript{9,11,12} and adult mammals\textsuperscript{10,14} we next asked whether, similar to c-Kit\textsuperscript{+} CSCs, GSNOR influences the cell-cycle activity in post-MI regenerative adult cardiomyocytes. Accordingly, we analyzed the expression of serine-10 phosphorylated histone H3 (Hp3) and Aurora-B kinase immunohistochemically to gauge the extent of cardiomyocyte mitosis in GSNOR\textsuperscript{−/−} and WT mice. Compared with WT, GSNOR\textsuperscript{−/−} mice exhibited a \(\approx 2.4\)-fold increase in the abundance of mitotic cardiomyocytes at 1 week after MI (Figure 5B through 5E).

In order to elucidate whether the enhanced mitotic activity in GSNOR\textsuperscript{−/−} cardiomyocytes yields newly formed cardiomyocytes in the infarcted myocardium, we studied the hearts of the GSNOR\textsuperscript{−/−} and WT mice that we had primed with BrdU after MI (see earlier). Confocal microscopy revealed that, 1 month after MI, GSNOR\textsuperscript{−/−} mice were characterized by 3-fold more BrdU\textsuperscript{+}/tropomyosin\textsuperscript{+} cardiomyocytes in the infarct zone compared with the WT controls (Figure 6A through 6C).

\(\text{S}\)-Nitrosylation influences apoptotic activity.\textsuperscript{39,40} We also investigated the possibility that the effects of enhanced cardiomyocyte mitosis were masked by increased apoptotic cell death by immunohistochemistry staining for cleaved caspase-3, a marker for both death receptor– and mitochondrial pathway–initiated apoptotic cell death.\textsuperscript{40} There were no significant differences in the numbers of apoptotic cardiomyocytes in 1-week post-MI GSNOR\textsuperscript{−/−} and WT mice (Figure 7A through 7C). Collectively, these results suggest that in response to myocardial damage, GSNOR activity does not affect cardiomyocyte apoptosis but, rather, negatively regulates heart regeneration through post-translational modifications that prevent cardiomyocyte proliferation.

Enhanced Neovascularization in Post-MI GSNOR\textsuperscript{−/−} Hearts

We previously showed that adult bone marrow–derived MSCs from GSNOR\textsuperscript{−/−} mice form fewer capillaries and exhibited less endothelial differentiation than WT MSCs in an in vivo Matrigel-plug assay.\textsuperscript{41} We also previously showed that, compared with WT, healthy GSNOR\textsuperscript{−/−} mice have higher coronary vascular density in their myocardium, which may partly explain their resistance to myocardial ischemia.\textsuperscript{21} To address whether GSNOR plays a role in the capacity of the myocardium to revascularize in response to MI, we analyzed the vascular density in GSNOR\textsuperscript{−/−} and WT mice 2 months after experimental MI. Quantitative immunofluorescence analysis for isolectin-B4, a marker for identifying angiogenic endothelium,\textsuperscript{17,42} demonstrated that, compared with WT, GSNOR\textsuperscript{−/−} mice were characterized by a significantly higher coronary vascular density in

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Figure 3. Adrenergic sensitivity. A, GSNOR\textsuperscript{−/−} mice (\(n=7\)) post MI have lower isoproterenol sensitivity during PV loop compared with WT mice (\(n=9\)). GSNOR\textsuperscript{−/−} mice do not exhibit changed heart parameters after 40 ng/kg per minute of isoproterenol injection into jugular vein. WT mice responded to isoproterenol infusion (1 way ANOVA). B, Dose-response to isoproterenol in GSNOR\textsuperscript{−/−} cardiomyocytes (\(n=6\)) is right-shifted compared with WT (\(n=6\)), exhibiting a reduced sensitivity to \(\beta\)-adrenergic stimulation, with conserved maximal contraction (nonlinear regression dose-response fit; \(P=0.0318\)). Data are presented as mean\(\pm\)SEM. ANOVA indicates analysis of variance; BL, Baseline; GSNOR, \(\text{S}\)-nitrosoglutathione reductase; MI, myocardial infarction; PRSW, preload recruitable stroke work; WT, wild-type.
the infarct and border zones (Figure 8). Furthermore, the presence of coronary vascular cells with BrdU incorporation in the myocardium of BrdU-primed GSNOR−/− mice 1 month after MI (Figure 8B) supported that post-MI coronary vascular cell proliferation contributed to the enhanced vascularity of the damaged myocardium. The increased vascularity suggests that endothelial cells rather than MSCs are primarily responsible for blood vessel formation in healthy21 and infarcted GSNOR−/− mouse hearts. Thus, these findings suggest that in response to myocardial damage, GSNOR activity negatively regulates heart regeneration, limiting revascularization of the infarcted myocardium.

Enhanced In Vitro Proliferative Expansion of MSCs in GSNOR−/− Mice

In addition to studying the cell-cycle activity in the post-MI heart, we also examined whether GSNOR loss influences the

Figure 4. Enhanced proliferative expansion of c-Kit+ CSCs in GSNOR−/− hearts post MI. A, At baseline, healthy adult WT and GSNOR−/− mice have an equivalent number of c-Kit+ CSCs (t test; P=0.5942). B, c-Kit+ CSC at 1 and 8 weeks post MI in the infarct, border and remote zones of WT and GSNOR−/− mice (2-way ANOVA; *P=0.013 between WT and GSNOR−/−; #P=0.03 within groups). C, One month after MI, GSNOR−/− mice have significantly more c-Kit+ CSCs in their hearts, compared with WT. In addition, significantly more c-Kit+ CSCs have incorporated BrdU in GSNOR−/− mice compared with WT, suggesting that GSNOR−/− c-Kit+ CSCs have an enhanced proliferative capacity after MI (Mann–Whitney test; *P=0.0016 and #P=0.012). D, Representative confocal photomicrographs of BrdU incorporation by cardiac c-Kit+ CSCs in WT and GSNOR−/− hearts, 1 month post MI. Two GSNOR−/− c-Kit+ CSCs are shown, one of which has incorporated BrdU (inset) whereas the other has not (arrow). E, Principle of the GSNOR activity assay. GSNOR catalyzes the reduction of GSNO to GSSG in the presence of NADH. Thus, the activity of GSNOR can be indirectly estimated, by spectrophotometrically monitoring NADH oxidation. F, GSNOR enzymatic activity in c-Kit+ CSCs. GSNOR activity is enriched in WT, but is absent in GSNOR−/− progenitors. Data are presented as individual data (A) or mean±SEM (B and C) or fold-change (E); WT (n=6) GSNOR−/− (n=5). *P=0.01 and #P=0.02. ANOVA indicates analysis of variance; BrdU, 5-bromodeoxyuridine; BZ, border zone; CSC, cardiac stem cell; GSNOR, S-nitrosoglutathione reductase; GSSG, glutathione disulfide (oxidized GSH); IZ, infarct zone; MI, myocardial infarction; NADH, reduced form of nicotinamide adenine dinucleotide (NAD); RZ, remote zone; WT, wild-type.
Figure 5. Enhanced mitotic activity of cardiomyocytes in $\text{GSNOR}^{-/-}$ hearts post MI. A, Absorption of NADH in whole-heart lysates from $\text{GSNOR}^{-/-}$ and WT mice, before and 8 weeks after the occurrence of MI. No NADH oxidation is recorded before the addition of GSNO in WT or $\text{GSNOR}^{-/-}$ lysates (time -2 to 0). Addition of GSNO (time 0), produces a sharp decrease in the absorbance of NADH in WT (black lines), but not in $\text{GSNOR}^{-/-}$ (red lines) heart lysates, before and after MI. These results indicate that the adult myocardium is enriched in GSNOR activity before and after MI, and that GSNOR activity is diminished in the pre- and post-MI $\text{GSNOR}^{-/-}$ heart. B and C, Confocal immunofluorescence analysis of cardiomyocyte mitosis, based on the nuclear expression of Hp3, in WT (B) and $\text{GSNOR}^{-/-}$ (C) hearts, 1 week post MI. D, Quantification of Hp3+ cardiomyocytes reveals that, compared with WT, $\text{GSNOR}^{-/-}$ hearts are characterized by a 3-fold increase in cardiomyocyte mitosis, 1 week post MI (t test; *$P=0.01$). E, Representative confocal immunofluorescence image illustrating expression of Aurora-B kinase, a marker of cytokinesis (arrow), at the cleavage furrow of a mitotically dividing $\text{GSNOR}^{-/-}$ cardiomyocyte, 1 week post MI. Values are mean±SEM. cTn-T indicates cardiac troponin T; GSNOR, S-nitrosoglutathione reductase; MI, myocardial infarction; NADH, reduced form of nicotinamide adenine dinucleotide (NAD); WT, wild-type.
proliferative activity of MSCs. MSCs are present in a wide variety of adult tissues, including the heart, and they are thought to support adult tissue homeostasis. They are also an important component of various adult stem cell niches, and we recently showed that GSNOR regulates their fate-choice decisions. Additionally, we and others have shown that MSCs comprise an important cell population for the diagnosis and treatment of cardiovascular disease. However, because MSCs are a heterogeneous population with a diverse immunophenotype, their direct in vivo identification is technically challenging. We therefore followed the approach of isolating and expanding MSCs in vitro from adult GSNOR<sup>−/−</sup> and WT mice, to perform our study. In vitro BrdU proliferation assay demonstrated that, similar to cardiac progenitors and cardiomyocytes, GSNOR<sup>−/−</sup> MSCs were characterized by a significantly higher proliferative activity compared with WT MSCs (Figure 9).

**Discussion**

The major new findings of this study are that the profound resilience to cardiac injury of mice with homozygous deletion of GSNOR involves an enhanced capacity of both c-Kit<sup>+</sup> cardiac progenitors and mature cardiomyocytes to proliferate in response to MI. We also report that loss of GSNOR augments the capacity of the adult myocardium to turn over new coronary vessels and revascularize in response to ischemic damage. Furthermore, in agreement with the findings that GSNOR<sup>−/−</sup> mice are characterized by an expanded hematopoietic stem cell pool, we now show that the GSNOR<sup>−/−</sup> bone marrow is also enriched in MSCs with superior in vitro proliferative activity.

**S-Nitrosylation/Denitrosylation of Proteins and Cell-Cycle Activity**

Dynamic nitrosylation/denitrosylation of cysteine thiols (SNOs) has emerged as a ubiquitous post-translational modification system throughout biology. Similar to other post-translational modification systems (ie, phosphorylation, methylation, acetylation), SNOs regulate the expression and function of most, if not all, main classes of protein, including several key proteins controlling mammalian cell differentiation and cell-cycle activity. Tumor suppressor proteins of the retinoblastoma gene product (Rb) and p53 are directly and/or indirectly regulated post-translationally via SNOs.

It is intriguing that the heart and bone marrow are not the only adult tissues that appear to exhibit an augmented proliferative phenotype in response to injury in GSNOR<sup>−/−</sup> mice. Recently, a higher propensity for HCC was documented in these animals, although defective DNA damage repair rather than excessive hepatocellular proliferation pathways due to SNO were primarily suggested to drive progression to malignancy. However, because in these studies cell-cycle analyses were limited to the immunohistochemical expression of a single cellular proliferation marker, Ki-67, in normal hepatocytes (which may or may not have been the cellular source of HCC), cell-cycle–based mechanisms of HCC cannot be excluded. For example, clonal analysis of BrdU incorporation by WT and GSNOR<sup>−/−</sup> hepatocytes and hepatic progenitors (during, before, and after the occurrence of HCC)
Figure 7. Quantification of cardiomyocyte apoptosis between WT and GSNOR−/− hearts post MI. A and B, Immunofluorescent images of cleaved caspase-3 (Casp. 3), a marker of apoptotic cell death, in WT (left panel) and GSNOR−/− (right panel) hearts, 1 week post MI. C, Quantification of activated Casp. caspase-3+ cardiomyocytes. Compared with baseline, the numbers of apoptotic cardiomyocytes expressing activated caspase-3 are dramatically increased within 1 week post MI. However, no significant differences are detected in apoptotic cardiomyocytes between WT and GSNOR−/− mice. (Kruskall–Wallis, *P=0.0002). Values are mean±SEM. AF indicates autofluorescence; GSNOR, S-nitrosoglutathione reductase; MI, myocardial infarction; WT, wild-type.

Figure 8. Enhanced neovascularization in post MI GSNOR−/− hearts. A, Representative immunofluorescent images of isolectin-IB4 in WT (left panel) and GSNOR−/− (right panel) hearts, 2 months post MI. B, BrdU incorporation in coronary vascular cells of a GSNOR−/− heart, demonstrates the presence of newly regenerated coronary vessels 1 month post MI. C, Quantification of isolectin-IB4+ vessels demonstrates that GSNOR−/− mice are characterized by a significantly enhanced vascular density compared with WT (1-way ANOVA, *P<0.0001). Values are mean±SEM. ANOVA indicates analysis of variance; BrdU, 5-bromodeoxyuridine; BZ, border zone; GSNOR, S-nitrosoglutathione reductase; IZ, infarct zone; MI, myocardial infarction; RZ, remote zone; WT, wild-type.
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GSNOR* Nitrosylation/Denitrosylation of Proteins in Cardiomyocyte Function

S-Nitrosylation/Denitrosylation of Proteins and Cardiomyocyte Function

GSNOR metabolizes SNOs and its absence results in increased abundance of S-nitrosylated proteins. Important substrates of SNO are found in the heart, and they control excitation-contraction coupling. Our study expands on the knowledge that GSNOR*−/− cardiomyocytes have faster Ca2+ decay and relaxation compared with WT cardiomyocytes post MI. Ca2+ cycling and myofilament responsiveness in cardiomyocytes underlie myocardial contractility. Thus, any alteration in intracellular Ca2+ handling is reflected as a change in heart performance. The myocardial relaxation is critical for proper diastolic function. This property is mainly governed by the affinity of myofilament for Ca2+ and by Ca2+ reuptake. It has been suggested that S-nitrosylation of certain sarcomeric proteins desensitizes myofilaments to Ca2+; however, the effect of hyper-S-nitrosylation of proteins involved in cytosolic Ca2+ removal and its cardioprotective role after myocardial injury is still unclear. Our data suggest that the cytosolic Ca2+ reuptake mechanism is preserved in GSNOR*−/− cardiomyocytes post MI and may be associated with improved sarcomplasmic reticulum Ca2+-ATPase activity.

S-Nitrosylation/Denitrosylation of Proteins in Response to Tissue Damage

In addition to its potential role as a post-translational regulator of cell-cycle activity in the injured adult heart, post-translational modification by SNOs modulate the expression and function of several proteins associated with damage response pathways, including NF-κB (nuclear factor κ light-chain enhancer of activated B cells), Nrf2 (nuclear factor erythroid-derived 2), and Hif-1α (hypoxia-inducible factor 1α). Of particular interest is the regulation of Hif-1α, 1 of the 2 subunits of the Hif-1 basic helix-loop-helix-PAS domain transcription factor. Under normal oxygen conditions, Hif-1α is expressed in the heart in an NF-κB-dependent manner but becomes degraded due to oxygen-dependent hydroxylation by prolyl hydroxylases, which mark the protein to be captured by the von Hippel–Lindau protein and undergo ubiquitination and proteasomal degradation. Under low oxygen conditions, the activity of hydroxylases is inhibited and Hif-1α is stabilized. However, GSNOR*−/− hearts are characterized by an enhanced Hif-1α transcriptional activity and, even under normal oxygen conditions, the protein becomes stabilized due to enhanced S-nitrosylation. This novel mechanism of Hif-1α stabilization is thought to underlie the enhanced vasculogenic phenotype of GSNOR*−/− mice.

Importantly, Puente et al recently reported that the rapid transition from an hypoxic to an oxygen-rich cardiac microenvironment during the first week of postnatal life in mice induces cell-cycle exit of cardiomyocytes through the production of reactive oxygen species (ROS) and DNA damage response. Thus, whether SNO-mediated stabilization of Hif-1α, resilience to ROS, and/or a defective DNA damage response mechanism could also underlie the enhanced proliferative phenotype we documented in adult GSNOR*−/− mouse hearts remains to be further investigated.

Figure 9. In vitro proliferation of WT and GSNOR*−/− MSCs measured by BrdU cell proliferation assay. Two-way ANOVA; *P<0.0001 between groups and *P<0.0005 between WT and GSNOR*−/− MSCs. n=3. Values are mean±SEM. ANOVA indicates analysis of variance; BrdU, 5-bromodeoxyuridine; GSNOR, S-nitrosoglutathione reductase; MSC, mesenchymal stem cell; WT, wild-type.
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Involved in heart failure progression.56 We showed lower ISO conditions are associated with pathophysiologic mechanisms after MI. Brum et al showed that chronic hypersympathetic independent effects of GSNOR in the heart.

DOI: 10.1161/JAHA.115.001974

Although the capacity of GSNOR−/− mice to do better after MI has been previously described,21 little is known about the mechanisms underlying this effect. Lima et al,21 suggested a potential mechanistic explanation by demonstrating that, at baseline, the uninjured, normoxic GSNOR−/− hearts are invested with an expanded coronary vascular bed, due to the SNO-mediated stabilization of HIF1α. In addition, we now report that GSNOR deletion produces an augmented proliferative activity in regenerative coronary vessels, cardiomyocytes, MSCs, and c-Kit+ cardiac progenitors in response to MI. Furthermore, we show that GSNOR−/− cardiomyocytes exhibit improved Ca2+ handling homeostasis and reduced adrenergic sensitivity due to their resilience to ROS.22 Based on the findings by Puente et al,11 it is intriguing to hypothesize that such resilience to ROS may also explain the enhanced cell turnover we documented in the GSNOR−/− hearts. Notably, although the degree of cardiac cell proliferation may appear insufficient to account for the post-MI functional recovery observed in the GSNOR−/− hearts (7.4±1.7% versus 2.7±0.7% BrdU+ cardiomyocytes in the infarct zones of GSNOR−/− and WT mice, respectively; Figure 6), 2 recent studies reported that enhancing the degree of cardiomyocyte proliferation to a similar level may efficiently trigger heart regeneration in mice.57,58 Thus, collectively, the findings presented by us and others21,22 suggest that the mechanism by which GSNOR deletion confers cardioprotection is multifaceted, ranging from regulating damage response pathways to cardiomyocyte contractility and cardiac cell-cycle reentry. However, the relative contribution of each mechanism to tissue regeneration and functional recovery in the post-MI GSNOR−/− hearts remains to be determined.

In summary, our study shows augmented cardiac regenerative pathways after MI in mice with a targeted deletion of GSNOR, an enzyme that governs protein nitrosylation/denitrosylation. Both cardiac precursor cell abundance and myocyte cell cycle activity are augmented in the GSNOR−/− hearts after MI. Thus, collectively, our findings have therapeutic implications for the treatment of heart disease because they reveal novel pathways by which nitroso-redox balance influences cardiac repair in the adult mammal.

Sources of Funding

This study was funded by National Institutes of Health (NIH) grants (awarded to Dr Hare) R01 HL107110 and R01 HL094849. Dr Hare is also supported by the NIH grants R01 HL110737, R01 HL084275, and 5UM HL113460 and by grants from the Starr Foundation and the Soffer Family Foundation. Drs Bellio and Cao are supported by awards from the American Heart Association. Dr Kulandavelu was supported by an award from the Canadian Institutes for Health Research.

Disclosures

Drs Hare and Hatzistergos disclose a relationship with Vestion that includes equity, board membership, and consulting. Vesion did not contribute funding to this study. Dr Hare discloses equity in Kardia and a grant from Biocardia.

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