Reduction of cardiomyocyte S-nitrosylation by S-nitrosoglutathione reductase protects against sepsis-induced myocardial depression

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Sips PY, Irie T, Zou L, Shinozaki S, Sakai M, Shimizu N, Nguyen R, Stamler JS, Chao W, Kaneki M, Ichinose F. Reduction of cardiomyocyte S-nitrosylation by S-nitrosoglutathione reductase protects against sepsis-induced myocardial depression. Am J Physiol Heart Circ Physiol 304: H1134–H1146, 2013. First published February 15, 2013; doi:10.1152/ajpheart.00887.2012.—Myocardial depression is an important contributor to morbidity and mortality in septic patients. Nitric oxide (NO) plays an important role in the development of septic cardiomyopathy, but also has protective effects. Recent evidence has indicated that NO exerts many of its downstream effects on the cardiovascular system via protein S-nitrosylation, which is negatively regulated by S-nitrosoglutathione reductase (GSNOR), an enzyme promoting denitrosylation. We tested the hypothesis that reducing cardiomyocyte S-nitrosylation by increasing GSNOR activity can improve myocardial dysfunction during sepsis. Therefore, we generated mice with a cardiomyocyte-specific overexpression of GSNOR (GSNOR-CMTg mice) and subjected them to endotoxemic shock. Measurements of cardiac function in vivo and ex vivo showed that GSNOR-CMTg mice had a significantly improved cardiac function after lipopolysaccharide challenge (LPS, 50 mg/kg) compared with wild-type (WT) mice. Cardiomyocytes isolated from septic GSNOR-CMTg mice showed a corresponding improvement in contractility compared with WT cells. However, systolic Ca2+ release was similarly depressed in both genotypes after LPS, indicating that GSNOR-CMTg cardiomyocytes have increased Ca2+ sensitivity during sepsis. Parameters of inflammation were equally increased in LPS-treated hearts of both genotypes, and no compensatory changes in protein expression levels (37). Therefore, we hypothesized that protein nitrosylation might be responsible for the detrimental cardiac effects of sepsis (10, 33), leading to the hypothesis that NOS2-derived NO also has protective effects.

NO can regulate myocardial function via two major downstream signaling pathways. On the one hand, NO can activate soluble guanylate cyclase (sGC) (23), leading to increased production of the second messenger cyclic guanosine monophosphate (cGMP). On the other hand, numerous reports indicate that NO exerts many, if not most, of its effects by reversibly binding to specific protein thiol residues, leading to the formation of S-nitrosothiols (22, 52). A recent proteomic study identified as many as 951 unique proteins that can be S-nitrosylated in the heart (29), demonstrating its potential importance in regulating cardiac function. An important determinant of the level of protein S-nitrosylation is the activity of denitrosylating enzymes. So far, two enzymatic systems have been identified that specifically catalyze the metabolism of S-nitrosothiols. One system is based on the reduction of thioredoxin by thioredoxin reductase (5), and the second system relies on the reduction of S-nitrosylated glutathione (GSNO) by GSNOR reductase (GSNOR). GSNOR controls the cellular levels of protein S-nitrosylation by influencing the equilibrium between GSNO and protein S-nitrosothiols (36). Mice deficient for GSNOR (GSNOR-KO mice) were found to have increased levels of S-nitrosylated hemoglobin in red blood cells but were otherwise indistinguishable from control animals during basal conditions (37).

We have recently shown that mice lacking the alpha1 isoform of sGC have a worse outcome after endotoxin challenge, associated with a more severe cardiac depression (9). On the other hand, GSNOR-KO mice were shown to have increased tissue damage and mortality in mouse models of sepsis, associated with a significant accumulation of protein S-nitrosylation levels (37). Therefore, we hypothesized that protein S-nitrosylation might be responsible for the detrimental cardiac effects of the increased nitrosative stress that is observed during sepsis (15, 27), while sGC activation has beneficial effects. To specifically dissect the role of protein S-nitrosylation in the heart, we generated mice with cardiomyocyte-
specific overexpression of GSNOR and studied their cardiac phenotype during endotoxic shock.

METHODS

Mice. All animal procedures were performed in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Cardiomyocyte-specific GSNOR-overexpressing (GSNOR-CMTg) mice were generated by pronuclear injection of a transgenic construct (Fig. 1A) into C57BL/6 zygotes at the Boston Area Diabetes Endocrinology Research Center. Wild-type (WT) littermates were used as controls for GSNOR-CMTg mice. WT mice on the C57BL/6N background were obtained from Charles River Laboratories (Wilmington, MA) and were used as controls for GSNOR-deficient (GSNOR-KO) mice (37). Age-matched male mice 2–4 mo old were used throughout the study.

To generate an endotoxin-induced model of sepsis, *E. coli* lipopolysaccharide (LPS, serotype 0111:B4, Sigma) was injected intraperitoneally into mice. For comparisons of WT and GSNOR-CMTg mice, an LPS dose of 50 mg/kg body wt was used, and for experiments with GSNOR-KO mice 10 mg/kg was injected. Physiological saline solution was injected into control animals. For in vivo measurements of cardiac function, mice received prewarmed saline solution (0.03 ml/g body wt) at 30 min and at 5 h after LPS challenge to ensure adequate intravascular volume.

Echocardiography. In vivo cardiac function was evaluated 3 days before and 6 h after vehicle or LPS administration by transthoracic echocardiography as described previously (47, 60). Mice were lightly anesthetized with ketamine (20 mg/kg), and images were collected by transthoracic echocardiography using a 13.0-MHz linear probe (Vivid 7; GE Medical System, Milwaukee, WI). M-mode images were obtained from a parasternal short-axis view at the midventricular level with a clear view of the papillary muscle. Left ventricular (LV) internal diameters at end-diastole and end-systole were measured, and the fractional shortening, ejection fraction, and heart rate were calculated on an EchoPAC workstation (GE Healthcare, Wauwatosa, WI).

Isolated heart function. Functional parameters were measured in hearts isolated from mice 6 h after vehicle or LPS challenge using a Langendorff setup as described previously (49). Mice were heparinized and anesthetized with 60 mg/kg pentobarbital. After excision of
the heart, the aorta was cannulated immediately and hearts were perfused at a constant pressure of 70 mmHg on the Langendorff apparatus with a modified Krebs-Henseleit buffer containing (in mmol/l) 118.5 NaCl, 25 NaHCO3, 11 glucose, 4 KCl, 1.2 MgSO4, 1.2 KH2PO4, 1 pyruvate, 1.8 CaCl2, gassed and equilibrated with 95% O2-5% CO2 at 37°C. The LV pressure was measured using a fluid-filled balloon inserted into the LV, containing the tip of a Millar SPR-671 pressure transducer (ADInstruments, Colorado Springs, CO). Coronary flow rate was measured using an NI in-line flow probe and a T106 flowmeter (Transonic Systems, Ithaca, NY). Coronary flow rate and LV pressure were constantly measured, and heart rate, LV developed pressure, and rate of LV pressure change were calculated from the LV pressure signal using a Powerlab 8/30 data-acquisition system and Chart Pro software (ADInstruments). Isolated cardiomyocytes. LV myocytes were dissociated from hearts of vehicle- and LPS-treated mice as described previously (24), with some modifications to improve cell viability and function (48). In brief, mice were heparinized and deeply anesthetized with pentobarbital sodium (60 mg/kg), and the heart was quickly excised and cannulated via the aorta. Hearts were then perfused in the Langendorff mode for 3–4 min at 37°C with perfusion buffer containing (in mmol/l) 25 HEPES (pH 7.4), 130 NaCl, 5.4 KCl, 0.5 MgCl2, 0.33 NaH2PO4, 22 glucose, 0.4 ethylene glycol tetraacetic acid, and 50 µM insulin. Next the heart was digested for 9–10 min by supplementing the perfusion buffer with 0.3 mM Ca2+, 0.6 mg/ml collagenase B, 0.6 mg/ml collagenase D, 0.06 mg/ml trypsin, and 0.06 mg/ml protease XIV. The digested ventricles were then shielded, filtered through a 300-µm mesh, and further incubated in fresh enzyme solution for 15–20 min at 37°C. Then 2 mg/ml bovine serum albumin was added and the Ca2+ concentration was stepwise increased to 1.8 mM. Freshly isolated cells were used for experiments. Sarcomere shortening and intracellular Ca2+ were measured as described previously (20, 24). Cardiomyocytes were loaded with 1.5 µmol/l fura-2/acetoxymethyl ester (Invitrogen, Carlsbad, CA) in Tyrode buffer for 20 min at 25°C and were subsequently observed in a light-sealed chamber using a Nikon TS100 inverted microscope (Nikon Instruments, Melville, NY). Cardiomyocytes were superfused with Tyrode buffer at 37°C and paced at 1–4 Hz by a MyoPacer Field Stimulator (IonOptix, Milton, MA). A dual-excitation spectrofluorometer (Hyperswitch Light Source, IonOptix) was used to record photon live count of fura-2 fluorescence emission at 505 nm elicited from exciting wavelengths at 340 and 380 nm. Intracellular Ca2+ levels were recorded as the 340/380 nm ratio using IonWizard software (IonOptix). Sarcomere length was simultaneously recorded by a video motion detector system (Ionoptix), used to analyze images obtained with a CCD video camera (Javelin Electronics, Torrance, CA). Measurements of sarcomere shortening and intracellular Ca2+ levels recorded at a pacing frequency of 2 Hz were used for “phase-plane” analysis and calculation of the intracellular Ca2+ level at 50% relaxation as described in Refs. 51 and 58.

**Immunocytochemistry.** Cardiomyocytes were isolated as described above, with the exception that 2 mmol/l ATP was added together with the bovine serum albumin after enzymatic digestion. Next, CaCl2 was added stepwise to increase final Ca2+ concentration to 1 mmol/l. Isolated cardiomyocytes were plated on laminin-coated glass coverslips in Hanks’ Medium 199 (Gibco, Carlsbad, CA) supplemented with 10 mmol/l 2,3-butanediene monoxime, 10 U/ml penicillin, 10 µg/ml streptomycin, and 2 mmol/l ATP. After 1–2 h incubation at 37°C in 95% O2-5% CO2, plating medium was gently aspirated, and cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then washed in PBS and permeabilized in 0.5% Triton X-100 for 10 min at room temperature. Next, cells were washed and incubated in 0.1 mol/l glycine for 2 × 5 min. Then cells were blocked in 10% goat serum for 1 h at room temperature, followed by overnight incubation with primary antibody against GSNOR (1:50, Proteintech) at 4°C. Next, cells were washed and incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:500, Molecular Probes) for 1 h at room temperature in the dark. After washing, cells were incubated with Alexa 647-phalloidin (1:60, Molecular Probes) for 15 min at room temperature. Cardiomyocytes were counterstained with Hoechst 33342 (0.5 µg/ml) and mounted in ProLong Gold antifade reagent. Fluorescent imaging was visualized using a Nikon A1 Confocal Laser Microscope System with the NIS Elements software package (Nikon Instruments, Melville, NY).

**Immunoblot analysis.** Hearts were harvested from vehicle- and LPS-treated mice, and protein extracts were prepared using lysis buffer containing (in mmol/l) 50 Tris-HCl (pH 7.4), 150 NaCl, 0.1 EDTA, 1% Triton X-100, 0.1% SDS, and 1:100 each of phosphatase inhibitor cocktails 2 and 3 and protease inhibitor cocktail (all from Sigma-Aldrich, St. Louis, MO). After centrifugation for 10 min at 14,000 g, supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a Mini-PROTEAN System (Bio-Rad, Hercules, CA) and transferred to PVDF membranes. Membranes were blocked in 1% ECL Prime Blocking Reagent (GE Healthcare) for 1 h and then probed overnight at 4°C with primary antibodies against GSNOR (1:10,000, Proteintech, Chicago, IL), β-tubulin (1: 5,000, Cell Signaling Technology, Danvers, MA), NOS2 (1:5,000, Millipore, Billerica, MA), phospholamban (PLB) phosphorylated at Ser26 (1:5,000, Upstate Group LLC, Charlottesville, VA), PLB phosphorylated at Thr17 (1:5,000, Badera, Leeds, UK), total PLB (1:15,000, Pierce Biotechnology, Rockford, IL), cardiac troponin I (cTnI) phosphorylated at Ser223/225 (1:5,000, Cell Signaling Technology), total cTnI (1:10,000, Millipore), caspase-3 (1:5,000, Cell Signaling Technology), pan-actin (1:5,000, Cell Signaling Technology), and NOS1 (1:5,000, BD Transduction Laboratories, San Jose, CA). Bound antibodies were detected with a horseradish peroxidase (HRP)-linked antibody directed against rabbit IgG (1:50,000, Cell Signaling Technology) or against mouse IgG (1:100,000, Thermo Fisher Scientific, Rockford, IL) and were visualized using chemiluminescence with the ECL Advance kit (GE Healthcare, Piscataway, NJ). Densi-

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**Table 1. Primers used for RT-PCR analysis of mRNA levels**

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<td>GCT GGA ATT ACC GGG GCT</td>
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<td>Bax</td>
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<td>AAT TCG CCG GAG ACA CTC G</td>
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<td>GCC GTG TAG ATG TGG TAC AAG GA</td>
</tr>
<tr>
<td>ICAM-1</td>
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<td>AGG ATO AGG TGG TGG CCT AC</td>
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<tr>
<td>XO</td>
<td>ATG TGC ACA GGG ACT GCA CC</td>
<td>TGT GCT GAC GAA GAC TGG CAT</td>
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metric analysis of the results was performed with NIH Image software (version 1.47).

Biotin switch for protein S-nitrosylation assessment. A modified biotin switch method, adapted from Refs. 25 and 17, was used to assess the level of protein S-nitrosylation. Hearts were isolated from mice 6 h after vehicle or LPS challenge, and were perfused after aorta cannulation with PBS containing (in mmol/l) 1 EDTA, 1 diethylenetriamine pentaacetic acid (DTPA), and 0.1 neocuproine. Heart samples were then homogenized in HEN buffer [containing (in mmol/l) 250 HEPES (pH 7.7), 1 EDTA, and 0.1 neocuproine] supplemented with 1 mmol/l DTPA, 1% Triton X-100, and 1:100 protease inhibitor cocktail (Sigma). After 10 min centrifugation at 10,000 g, supernatants were diluted to a protein concentration of 0.5 mg/ml in blocking buffer consisting of HEN buffer supplemented with 30 mmol/l methylmethanethiosulfonate (MMTS) and 2.5% SDS. This solution was incubated for 30 min at 50°C under N2 gas with frequent vortexing to block all free thiols. Following acetone precipitation to remove excess MMTS, protein pellets were dissolved in HEN buffer with 0.5% SDS. Next, 20 mmol/l sodium L-ascorbate, and 0.2 mmol/l N-[6-(biotin-amido)hexyl]-3′-((2-pyridyldithio)propionamide (biotin-HPDP) were added to specifically reduce S-nitrosated bonds and label the newly formed free thiols residues with biotin. For negative controls sodium L-ascorbate was omitted to determine the level of aspecific biotin labeling. After incubation of this labeling mixture for 2 h in the dark at room temperature, proteins were precipitated by addition of ice-cold acetone. Protein pellets were washed to remove excess biotin-HPDP label, and the level of biotin incorporation was assessed by nonreducing immunoblot analysis using HRP-linked goat anti-biotin antibody (1:100,000, Sigma).

GSNOR activity measurements. The rate of GSNOR-dependent NADH consumption was measured to assess GSNOR activity levels in tissue homogenates, based on previously described methods (36, 57). Cardiac samples were homogenized in a buffer containing (in mmol/l) 50 Tris-HCl (pH 8.0), 150 NaCl, 1 EDTA, 0.1% Triton X-100, and 1:100 protease inhibitor cocktail (Sigma). After centrifugation for 10 min at 10,000 g, samples were diluted to a protein concentration of 0.1 mg/ml in reaction buffer containing 20 mmol/l Tris-HCl (pH 8.0), 0.5 mmol/l EDTA, and incubated with 75 μmol/l NADH with or without 100 μmol/l GSNO. NADH consumption was monitored by fluorescence spectrofluorometry with excitation at 340 nm and emission at 460 nm. GSNOR activity was defined as the rate of NADH decrease in samples incubated in the presence of GSNO minus the rate of NADH consumption without GSNO.

In a separate assay of GSNOR-dependent protein denitrosylation, cardiac homogenates prepared as above were diluted to 1 mg/ml in reaction buffer containing (in mmol/l) 20 Tris-HCl (pH 8.0), 0.5 EDTA, 0.5 DTPA, and 0.1 neocuproine. Duplicate samples were incubated with 100 μmol/l GSNO for 5 min in the dark at room temperature. Half of the samples were then further incubated with 100 μmol/l each of NADH and NADPH (essential cofactors of GSNOR and glutathione reductase, respectively) for 20 min at room temperature in the dark, while the other half of the samples were incubated with vehicle. The samples were then precipitated with ice-cold acetonitrile to remove excess GSNO and cofactors, and the protein pellets were resuspended in biotin switch blocking buffer and further processed for detection of protein S-nitrosylation as described above.

Quantitative PCR. Specific mRNA levels were measured by real-time polymerase chain reaction (RT-PCR) as described previously (6). Total RNA was extracted from cardiac tissue using the illustra RNAspin Mini Kit (GE Healthcare), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). RT-PCR reactions were performed in a Realplex2 system (Eppendorf, Haupauge, NY), using 18S ribosomal RNA for normalization. Primer sequences used for RT-PCR are indicated in Table 1.

cGMP concentration measurements. Cardiac cGMP levels were measured as described previously (49). In brief, cardiac tissue was homogenized in 1 ml ice-cold 10% trichloroacetic acid, and the protein pellet obtained after centrifugation was saved to measure original protein concentration. The supernatants were extracted with water-saturated ether and dried by vacuum centrifugation. After resuspending the samples in assay buffer, cGMP concentration was determined using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s recommendations. The obtained results were normalized to the protein concentration in the original sample.

Thioredoxin reductase activity. Cardiac tissue samples were homogenized in HEN buffer containing 0.5% NP-40 and 1:100 protease inhibitor cocktail (Sigma). After centrifugation at 10,000 g for 15 min, supernatants were assayed for thioredoxin reductase activity by measuring the rate of aurothiomalate-inhibitable 5,5′-dithio-bis(2-dinitrobenzoic acid) reduction using the Thioredoxin Reductase Assay Kit from Cayman Chemical according to the manufacturer’s recommendations.

Statistics. All data are presented as means ± standard error of the mean (SE), and the number of samples for each experimental group is indicated for each experiment. Data were analyzed using the Prism 5 software package (GraphPad Software, La Jolla, CA). Statistical differences among groups were evaluated using the appropriate test as indicated in the text. P values smaller than 0.05 were considered significant.
RESULTS

Cardiomyocyte-specific overexpression of GSNOR. A transgenic construct containing the coding sequence of human GSNOR driven by the α-myosin heavy chain promoter (Fig. 1A) was used for generating GSNOR-CMTg mice. Transgenic founder mice were identified by genomic PCR analysis of their offspring (Fig. 1, B and C). Characterization of these GSNOR-CMTg mice indicated a significant overexpression of GSNOR in cardiac tissue (Fig. 1, D and E). Immunohistochemistry confirmed localization of the GSNOR transgene protein to cardiomyocytes in cardiac tissue sections (not shown). Staining of isolated cardiomyocytes demonstrated a striated pattern of intracellular GSNOR, colocalizing with the Z-disc pattern of sarcomeric actin staining (Fig. 1F).

Cardiac homogenates of GSNOR-CMTg mice had significantly higher GSNOR activity than WT and GSNOR-KO samples (Fig. 2A). Stimulation of GSNOR activity in vitro by incubating cardiac protein homogenate with NADH and NADPH, essential cofactors for GSNOR and glutathione reductase, respectively, resulted in a much more efficient removal of GSNO-induced protein S-nitrosylation in GSNOR-CMTg samples than in WT samples (Fig. 2B).

GSNOR improves cardiac function after endotoxin challenge. In vivo assessment of cardiac function using echocardiography showed no difference between male WT and GSNOR-CMTg mice at baseline. Six hours after intraperitoneal administration of 50 mg/kg LPS, WT mice showed a severe myocardial dysfunction characterized by significant decreases in fractional shortening and ejection fraction, and an increase in LV end-systolic diameter. GSNOR-CMTg mice on the other hand displayed a significantly improved cardiac function compared with WT mice after LPS injection (Fig. 3). Heart rates were decreased after LPS challenge but did not differ between genotypes. These results indicate that cardiomyocyte-specific GSNOR overexpression can protect against sepsis-induced LV dysfunction.
In support of this observation, hearts isolated from LPS-challenged GSNOR-CMTg mice also showed improved parameters of cardiac contractility measured in a Langendorff perfusion setup, compared with LPS-treated WT hearts (Fig. 4). Coronary flow rates were similar in both genotypes indicating that the improved inotropy in LPS-treated GSNOR-CMTg hearts was not the result of increased substrate availability.

To further confirm the cardioprotective role of GSNOR, we investigated the phenotype of GSNOR-KO mice. Consistent with our findings in GSNOR-CMTg mice, we found a more severe cardiac dysfunction in mice lacking GSNOR than in WT mice after challenge with 10 mg/kg LPS (Fig. 5). A lower dose of LPS was used in this experiment because 50 mg/kg LPS already caused a very severe cardiac phenotype in WT mice and any additional detrimental effects of the loss of endogenous GSNOR function would not be detectable at this dose.

Septic GSNOR overexpressing cardiomyocytes have improved sensitivity to Ca\(^{2+}\). Cardiomyocytes isolated from WT mice 6 h after LPS challenge showed a significant decrease in contractility, correlating with decreased transient systolic Ca\(^{2+}\) release, compared with cells isolated from vehicle-treated WT mice. GSNOR-overexpressing cardiomyocytes on the other hand showed significantly improved sarcormere shortening after LPS compared with WT cells, which is in agreement with the improved GSNOR-CMTg cardiac function observed in vivo and ex vivo. Importantly, however, the amplitude of intracellular Ca\(^{2+}\) transients was similarly depressed in LPS-treated cardiomyocytes of both genotypes (Fig. 6, A–C, and Table 2). These results suggest that after endotoxin challenge the myofilament of GSNOR-CMTg cardiomyocytes had a higher sensitivity to Ca\(^{2+}\) than the myofilament of WT cardiac myocytes. Cell shortening and Ca\(^{2+}\) transients were similar at basal conditions. Diastolic Ca\(^{2+}\) levels however were lower in GSNOR-CMTg than in WT cardiomyocytes both after vehicle and LPS administration at pacing frequencies of 1 and 2 Hz. LPS administration also caused a small decrease in diastolic Ca\(^{2+}\) levels in WT cells at 4 Hz (Table 2).

To measure cardiomyocyte Ca\(^{2+}\) sensitivity, we performed a “phase-plane” analysis, mapping intracellular Ca\(^{2+}\) concentration against sarcomere length. Plotting these data results in a counterclockwise trajectory, where the descending portion of
the relaxation phase provides a dynamic index of myofilament Ca\(^{2+}\) sensitivity (51, 58). We observed that this relaxation trajectory was shifted to the left in both genotypes after LPS challenge, but most importantly also in LPS-treated GSNOR-CMTg cardiomyocytes compared with WT cells after LPS (Fig. 6D). To quantify this relative change in Ca\(^{2+}\) sensitivity, we calculated the intracellular Ca\(^{2+}\) concentration at 50% sarcomere relaxation, which was decreased in both genotypes after LPS treatment. We confirmed that the Ca\(^{2+}\) level at 50% relaxation was significantly lower in GSNOR-CMTg than in WT cells after endotoxin challenge (Fig. 6E), consistent with an increased Ca\(^{2+}\) sensitivity in GSNOR-CMTg compared with WT cardiomyocytes during sepsis.

Next, we investigated the phosphorylation status of phospholamban (PLB), which is an important determinant of sarco/endoplasmic reticulum ATPase (SERCA) activity and conse-

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**Fig. 6.** GSNOR-overexpressing cardiomyocytes have higher sensitivity to Ca\(^{2+}\). A: tracings of sarcomere shortening and intracellular Ca\(^{2+}\) transients [measured as the fluorescence ratio (F) of fura-2 excitation at 340 and 380 nm] obtained at a 2-Hz pacing frequency in cardiomyocytes isolated from WT and GSNOR-CMTg (TG) mice 6 h after vehicle or LPS (50 mg/kg) challenge. SL, sarcomere length. Quantification of percentage sarcomere shortening (B) and peak amplitude of intracellular systolic Ca\(^{2+}\) release (C) at different pacing frequencies. D: phase-plane plot of SL vs. intracellular Ca\(^{2+}\) concentration of cells paced at 2 Hz. E: intracellular Ca\(^{2+}\) levels at 50% sarcomere relaxation as an index of cardiomyocyte Ca\(^{2+}\) sensitivity (N = 4–6 mice per group, 10–14 cardiomyocytes were analyzed per mouse; *P < 0.05, **P < 0.01, ***P < 0.001 between genotypes; ##P < 0.01, ###P < 0.001 vs. vehicle treatment in the same genotype by Bonferroni posttest after repeated-measures 2-way ANOVA). Quantification of levels of phospholamban (PLB) phosphorylation at the Ser\(^{16}\) and Thr\(^{17}\) residues (F and G) and cardiac troponin I (TnI) phosphorylation at the Ser\(^{22}\) and Ser\(^{23}\) residues (H) assessed using Western blot analysis of cardiac samples obtained 6 h after administration of vehicle or LPS (50 mg/kg) (N = 4–6 per group; #P < 0.05 and ##P < 0.01 between vehicle and LPS treatments by Bonferroni posttest after 2-way ANOVA).
not altered by LPS or GSNOR overexpression. These results with the decreased Ca$^{2+}$
levels of myofilament Ca$^{2+}$ sensitivity, on the other hand showed a small increase after LPS in GSNOR-CMTg hearts, but importantly no differences were detected between the genotypes (Fig. 6H). Total PLB and cTnI protein expression levels were not altered by LPS or GSNOR overexpression. These results indicate that the increased Ca$^{2+}$ sensitivity we observed in septic GSNOR-CMTg cardiomyocytes cannot be explained by changes in the phosphorylation status of cTnI.

**GSNOR-CMTg mice have similar levels of cardiac inflammation as WT mice after LPS.** To investigate the effect of cardiac GSNOR overexpression on tissue inflammation, we performed quantitative PCR analysis on heart samples obtained from WT and GSNOR-CMTg mice 6 h after vehicle or LPS challenge. Cardiac mRNA levels of interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1), markers of tissue inflammation, as well as the anti-inflammatory cytokine IL-10, were significantly higher in LPS-treated samples than in control samples, but no differences were found between genotypes (Fig. 7, A–C). The reactive oxygen species producing enzyme xanthine oxidase (XO) as well as the antioxidant enzymes heme oxygenase-1 (HO-1) and manganese superoxide dismutase (MnSOD) were also significantly upregulated in cardiac samples from LPS-treated mice, to similar levels in both genotypes (Fig. 7, D–F). These observations suggest that tissue inflammation as well as oxidative stress and antioxidant defense were increased to a similar extent in WT and GSNOR-CMTg hearts after LPS challenge.

In addition, apoptotic signaling pathways were not different between WT and GSNOR-CMTg hearts at 6 h after 50 mg/kg LPS challenge. The ratio of expression levels of the antiapoptotic Bcl2 to proapoptotic Bax was significantly lower after LPS challenge, but there was no significant difference between both genotypes (Fig. 7G). On the other hand, no cleaved caspase 3 fragments were detected in either genotype at this time point (Fig. 7H).

**Cardiac NOS levels and thioredoxin reductase activity are not affected by GSNOR overexpression.** LPS administration caused a strong increase in cardiac protein and mRNA levels of NOS2, the inducible NO synthase that is expressed during inflammation. This induction of NOS2 expression was not different between WT and GSNOR-CMTg hearts (Fig. 8, A–C). We found that cardiac mRNA levels of the constitutively expressed NOS1 were lower in GSNOR-CMTg than in WT samples both after vehicle and LPS (Fig. 8D). Nevertheless, NOS1 protein levels were similar in WT and GSNOR-CMTg hearts and decreased to the same extent after LPS challenge in both genotypes (Fig. 8E). In addition, cardiac mRNA levels of NOS3 were not different between genotypes at baseline and similarly decreased after LPS treatment (Fig. 8F). Correlating with increased NOS2 expression, cardiac intracellular cGMP levels were higher in WT hearts from mice challenged with LPS compared with vehicle-treated mice. However, cGMP concentrations were not different between WT and GSNOR-CMTg hearts (Fig. 8G). These data suggest that cardiac inflammatory NOS2 induction, constitutive NOS1 and NOS3 expression, and sGC-dependent signaling pathways are not affected by GSNOR overexpression.

We also investigated possible compensatory changes in the thioredoxin system, the other major cellular denitrosylation mechanism, in response to increased cardiac GSNOR expression and activity in GSNOR-CMTg mice. We measured the activity levels of thioredoxin reductase (TrxR), the enzyme responsible for recycling oxidized thioredoxin, and found no differences in TrxR activity between cardiac samples from WT and GSNOR-CMTg mice after vehicle or LPS challenge (Fig. 8H), suggesting that the activity of this denitrosylation mechanism is not affected by increased GSNOR activity.

Taken together, these results suggest that no major compensatory changes in NO production systems or in the parallel

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<th>Parameters of contractility and Ca$^{2+}$ handling as measured in cardiomyocytes isolated from WT and GSNOR-CMTg mice 6 h after vehicle or LPS (50 mg/kg) administration</th>
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<td>Diastolic Ca$^{2+}$ level, F/F0</td>
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<td>Systolic Ca$^{2+}$ release, ΔF/F0</td>
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Data are presented as mean ± SE of N = 4–6 mice per group (10–14 cardiomyocytes were analyzed per mouse). Freq. pacing frequency. *P < 0.05, **P < 0.01, ***P < 0.001 between genotypes; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. vehicle treatment in the same genotype by Bonferroni posttest after repeated-measures 2-way ANOVA.
thioredoxin-dependent denitrosylation system have occurred in GSNOR-overexpressing cardiac tissue.

Cardiac S-nitrosylation is decreased in GSNOR-CMTg hearts after LPS challenge. We analyzed the protein S-nitrosylation status of cardiac samples from mice subjected to vehicle and LPS challenge using the biotin switch method. Total biotin labeling (of proteins) was significantly lower in GSNOR-CMTg samples compared with WT samples after LPS challenge (Fig. 9, A and B), corresponding with an increased denitrosylation capacity in GSNOR-CMTg hearts. We observed a prominent immunoreactive band in all GSNOR-CMTg samples at 35 kDa, correlating with the transgenic GSNOR protein (Fig. 9C), which is expressed at high levels, as shown in Fig. 1. Therefore we excluded proteins of 35 kDa and below from the measurements for quantitative comparison of WT and GSNOR-CMTg cardiac protein S-nitrosylation levels.

DISCUSSION

This report describes the generation of a novel transgenic mouse strain, which overexpresses the denitrosylating enzyme GSNOR specifically in cardiomyocytes. These genetically modified mice allow us to study the effects of reducing endogenous protein S-nitrosylation in normal cardiac signaling mechanisms as well as in myocardial disease. Although a critical role of NO-dependent protein S-nitrosylation in cardiovascular signaling has been suggested, whether or not enhancement of denitrosylation capacity counteracts the potentially negative impact of S-nitrosylation in cardiomyopathy was unknown. Our experiments demonstrate that increased protein denitrosylation protects against the development of sepsis-induced myocardial dysfunction.

The α-myosin heavy chain promoter transgene construct used to generate the GSNOR-CMTg mice leads to high levels of GSNOR overexpression in cardiomyocytes. The transgenic GSNOR protein at least partially colocalizes with sarcomeric actin, most likely in the Z-discs where phalloidin staining is most intense (2). The sarcomeric Z-disc is a subcellular structure where many important signaling pathways governing cardiac contractility converge (19), including the NO-generating enzyme NOS3 (54). In addition, NOS2 has also been
localized to contractile fibers in cardiomyocytes (7). As such, the spatial distribution of the transgenic GSNOR protein allows it to potentially have an important functional impact on NO-dependent modulation of the contractile machinery of the cardiomyocyte. This subcellular distribution of overexpressed GSNOR closely mimics the expression pattern of endogenous GSNOR, which has been colocalized with the ryanodine receptor in T-tubules (4). The T-tubule network has been shown to closely interdigitate with the sarcomeric Z-discs in cardiomyocytes (18).

Our measurements in sedated mice, isolated hearts, and isolated cardiomyocytes all indicate that cardiomyocyte-specific overexpression of GSNOR improves cardiac contractility in the endotoxin-induced sepsis model. Taken together with the previously observed improvement of cardiac function in the same model of sepsis using mice lacking NOS2 (55), these results suggest that NOS2 induction by LPS leads to myocardial dysfunction by increasing protein S-nitrosylation. Although some reports have described an inhibitory effect of S-nitrosylation on sGC function (13, 46), our data suggest that GSNOR overexpression does not directly affect NO-dependent sGC stimulation. Therefore, specifically increasing the cellular denitrosylation capacity could potentially improve the outcome of septic cardiomyopathy without affecting the potentially protective effects of NO-sGC signaling (9). This strategy might have important therapeutic benefits compared with total NO synthesis blockade, which increased mortality in patients with septic shock (39).

To determine the mechanisms responsible for the preserved myocardial function in GSNOR-CMTg mice, we studied Ca2+/H11001 handling in isolated cardiomyocytes obtained from mice 6 h after LPS challenge. We observed that LPS challenge markedly depressed intracellular systolic Ca2+/H11001 transients in WT cardiac myocytes, associated with decreased PLB phosphorylation levels. Lower levels of PLB phosphorylation cause a stronger inhibition of SERCA activity resulting in decreased sarcoplasmic reticulum Ca2+ reuptake, which contributes to decreases in systolic Ca2+ release. Similar results have been reported previously (26), leading to the hypothesis that the decrease in systolic Ca2+ transients causes myocardial dysfunction in WT mice. Nevertheless, we observed that Ca2+
transients in GSNOR-CMTg cardiomyocytes and PLB phosphorylation levels were depressed to a similar extent after in vivo LPS challenge as in WT cardiomyocytes. Taken together, these observations suggest that the GSNOR-mediated improvement in cardiac function during sepsis is not mediated by prevention of the sepsis-induced decrease in SERCA function.

Our observation that GSNOR-CMTg cardiomyocytes have a similar decrease in systolic Ca\(^{2+}\) transients as WT cells after endotoxin challenge, while their contractility is significantly improved, suggests that GSNOR-CMTg myofilaments have a higher sensitivity to Ca\(^{2+}\). This hypothesis was confirmed by our calculation of the intracellular Ca\(^{2+}\) concentration at 50% relaxation of sarcomere length, which is an index of Ca\(^{2+}\) sensitivity (42, 58). We also confirmed that endotoxin challenge by itself increased cardiomyocyte Ca\(^{2+}\) sensitivity in both genotypes, as observed previously in WT cardiomyocytes in the same mouse model (24). Others have reported that sepsis reduces Ca\(^{2+}\) sensitivity of the cardiac myofilament (34), but the apparent discrepancy with our current results might be explained by the fact that in this previous study intracellular Ca\(^{2+}\) transients were not depressed by LPS administration, possibly because a significantly lower dose of LPS was used. Therefore, it is conceivable that myofilament Ca\(^{2+}\) sensitivity is increased in our sepsis model to compensate for the significant decrease in systolic Ca\(^{2+}\) release. The LPS-induced decrease in cardiomyocyte Ca\(^{2+}\) sensitivity in the study by Layland et al. (34) was correlated with an increase in TnI phosphorylation (34). While we also observed a slight increase in TnI phosphorylation levels after LPS challenge, we did not find a difference between genotypes, indicating that other mechanisms are responsible for the improved Ca\(^{2+}\) sensitivity in GSNOR-CMTg cardiomyocytes. Therefore, we hypothesize that S-nitrosylation of one or more myofilament proteins during sepsis blunts the increase in Ca\(^{2+}\) sensitivity in WT cardiomyocytes, which is alleviated by GSNOR-dependent denitrosylation in GSNOR-CMTg cardiomyocytes. Previous studies reporting a reduction of myofilament Ca\(^{2+}\) sensitivity by protein S-nitrosylation provide support for this hypothesis (14, 50).

We also found that GSNOR-CMTg cardiomyocytes have slightly lower diastolic Ca\(^{2+}\) levels than WT cells both at baseline and after LPS administration during 1- and 2-Hz pacing. A possible explanation for this observation is that GSNOR overexpression might decrease S-nitrosylation levels of the ryanodine receptor, leading to decreased Ca\(^{2+}\) release from this Ca\(^{2+}\) channel. This hypothesis is in line with previous reports showing that the cardiac ryanodine receptor can be activated by GSNO (53) and inhibited by GSNOR activity (4). The lower diastolic Ca\(^{2+}\) level that is observed in WT cells after LPS administration at 4 Hz on the other hand might be due to LPS-induced inhibition of L-type Ca\(^{2+}\) channel function (59). Further studies to elucidate the effects of GSNOR overexpression on cardiomyocyte Ca\(^{2+}\) handling are warranted.

It has been proposed that caspase activation in cardiomyocytes during sepsis contributes to myocardial depression (31), while increased expression of the antiapoptotic factor Bcl2 has been shown to protect against septic cardiomyopathy (32). Furthermore, several different studies have highlighted proapoptotic (21) as well as antiapoptotic (35, 40) effects of protein S-nitrosylation. We however did not discover any impact of GSNOR overexpression on cardiac apoptotic pathways at 6 h after LPS injection. Similarly, cardiac tissue inflammation parameters were increased to the same extent in WT and GSNOR-CMTg mice challenged with LPS. Taken together with the observation that the protective effects of GSNOR overexpression persist in isolated cardiomyocytes, our results suggest that the improved cardiac phenotype of GSNOR-CMTg mice in our acute sepsis model is unrelated to anti-inflammatory or antiapoptotic effects of GSNOR. This finding is consistent with previous studies that were unable to detect significant cardiomyocyte apoptosis at early time points during
experimental sepsis (41). Nevertheless, it is conceivable that cardiac GSNOR overexpression might have an effect on cardiomyocyte apoptosis occurring at later time points during sepsis.

Since overexpression of a denitrosylase might be expected to cause compensatory changes in NO-producing enzymes, we examined the expression levels of the three NOS isoforms in cardiac tissue samples of WT and GSNOR-CMTg mice after both vehicle and LPS challenge. We found that increased cardiac GSNOR activity does not interfere with NOS2 induction after LPS. In addition, cardiac NOS1 protein levels and NOS3 gene expression were decreased to a similar extent in both genotypes during sepsis, in agreement with previous observations in septic WT cardiomyocytes (11, 24). These results suggest that GSNOR overexpression does not affect LPS-induced changes in NOS levels.

Importantly, we demonstrated a lower total level of protein S-nitrosylation after LPS in GSNOR-CMTg hearts compared with WT hearts. This observation supports our hypothesis that GSNOR protects against sepsis-induced myocardial dysfunction by decreasing cardiomyocyte protein S-nitrosylation. These results are also in agreement with the increased tissue protein S-nitrosylation that was observed in GSNOR-deficient mice after LPS challenge (37) and confirm a role for GSNOR in the regulation of sepsis-induced protein S-nitrosylation. The decrease in protein S-nitrosylation in GSNOR-CMTg samples observed in Fig. 9 is much smaller than the robust difference presented in Fig. 2, most likely because 1) the levels of protein S-nitrosylation in vivo are much lower than those obtained after in vitro administration of a high dose of GSNO, and 2) other parameters such as cofactor availability and the activity of the thioredoxin-TrxR system also influence total protein denitrosylation activity in vivo.

Of note, we only observed a slight tendency toward increased total protein S-nitrosylation after LPS challenge in WT hearts, which did not reach significance. This is in agreement with a previous study that was unable to demonstrate significant differences in protein S-nitrosylation of LPS-challenged rat hearts using a similar biotin switch-based detection method (8). However, the biotin switch technique is sufficiently sensitive to detect highly abundant and/or strongly S-nitrosylated proteins. Therefore, it is conceivable that LPS administration leads to relatively small increases in the S-nitrosylation level of specific proteins in the heart, below the detection limit of the current standard methods. To overcome these limitations, more sensitive methods remain to be developed to accurately measure the quantitative level of S-nitrosylation of different key regulators of cardiac function, based on novel approaches such as the recently described Cys-reactive tandem mass tag labeling procedure (28).

In conclusion, our results indicate that cardiac protein S-nitrosylation is responsible for the detrimental effects of NO in the myocardium during sepsis, and that augmentation of denitrosylation capacity in cardiomyocytes can reverse the sepsis-induced myocardial dysfunction in vivo. Our observations may lead to the development of safer, more targeted therapeutic approaches aimed at improving cardiac function in critically ill septic patients.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


