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RESEARCH ARTICLE | *Signaling and Stress Response*

Cardiac hyporesponsiveness in severe sepsis is associated with nitric oxide-dependent activation of G protein receptor kinase

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Dal-Secco D, DalBó S, Lautherbach NE, Gava FN, Celes MR, Benedet PO, Souza AH, Akinaga J, Lima V, Silva KP, Kiguti LR, Rossi MA, Kettelhut IC, Pupo AS, Cunha FQ, Assreuy J. Cardiac hyporesponsiveness in severe sepsis is associated with nitric oxide-dependent activation of G protein receptor kinase. *Am J Physiol Heart Circ Physiol* 313: H149–H163, 2017. First published May 19, 2017; doi:10.1152/ajpheart.00052.2016.—G protein-coupled receptor kinase isoform 2 (GRK2) has a critical role in physiological and pharmacological responses to endogenous and exogenous substances. Sepsis causes an important cardiovascular dysfunction in which nitric oxide (NO) has a relevant role. The present study aimed to assess the putative effect of inducible NO synthase (NOS2)-derived NO on the activity of GRK2 in the context of septic cardiac dysfunction. C57BL/6 mice were submitted to severe septic injury by cecal ligation and puncture (CLP). Heart function was assessed by isolated and perfused heart, echocardiography, and β-adrenergic receptor binding. GRK2 was determined by immunofluorescence and Western blot analysis in the heart and isolated cardiac myocytes. Sepsis increased NOS2 expression in the heart, increased plasma nitrite + nitrate levels, and reduced isoproterenol-induced isolated ventricle contraction, whole heart tension development, and β-adrenergic receptor density. Treatment with 1400W or with GRK2 inhibitor prevented CLP-induced cardiac hyporesponsiveness 12 and 24 h after CLP. Increased labeling of total and phosphorylated GRK2 was detected in hearts after CLP. With treatment of 1400W or in hearts taken from septic NOS2 knockout mice, the activation of GRK2 was reduced. 1400W or GRK2 inhibitor reduced mortality, improved echocardiographic cardiac parameters, and prevented organ damage. Therefore, during sepsis, NOS2-derived NO increases GRK2, which leads to a reduction in β-adrenergic receptor density, contributing to the heart dysfunction. Isolated cardiac myocyte data indicate that NO acts through the soluble guanylyl cyclase/cGMP/PKG pathway. GRK2 inhibition may be a potential therapeutic target in sepsis-induced cardiac dysfunction.

NEW & NOTEWORTHY The main novelty presented here is to show that septic shock induces cardiac hyporesponsiveness to isoproterenol by a mechanism dependent on nitric oxide and mediated by G protein-coupled receptor kinase isoform 2. Therefore, G protein-coupled receptor kinase isoform 2 inhibition may be a potential therapeutic target in sepsis-induced cardiac dysfunction.

β-adrenergic receptors; G protein receptor kinase; inducible nitric oxide synthase; nitric oxide; sepsis

SEPSIS, the main cause of death in critically ill patients, often leads to profound organ dysfunction (11, 19). While sepsis may be initiated by several inflammatory and infectious events (19), the pathogenesis of cell and organ injuries remains obscure. Tissue hypoperfusion consequent to cardiovascular collapse seems to play a critical role in these events (33, 38). The cardiovascular dysfunction observed in the latter phases of severe sepsis and septic shock (50) has long been considered a consequence of heart dysfunction, and it is associated with a pronounced decrease of peripheral vascular resistance (3, 30, 38). Myocardial depression affects both the left and right myocardial compartments (49), although the left myocardial dysfunction is more relevant for the cardiovascular collapse (9, 13).

Although there is evidence that in vitro depression of cardiac myocyte contraction can be induced by proinflammatory cytokines, nitric oxide (NO), and septic serum (22, 23), the mechanism underlying cardiac depression is not fully clarified. The myocardial-depressing substance was proposed to represent a mix of cytokines (9, 23) that triggers the production of reactive oxygen and/or nitrogen species that, in turn, mediate cardiac tissue injury (14, 29). The reduction of the β-adrenergic response observed in septic shock that contributes to cardiac collapse (28, 43) may be associated with the upregulation of inducible NO synthase (NOS2) expression and the consequent increase of NO in heart tissue (22, 28, 36). Taken together, these reports suggest that the flood of NO derived from NOS2 activity in the early phases of sepsis may in some way modify heart reactivity.

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G protein-coupled receptor kinases (GRKs) are protein kinases that recognize and phosphorylate activated G protein-coupled receptors (37). Overall, there is good evidence that GRK2 is important for regulating several cardiac receptor responses (20, 39). There are studies showing that the expression of GRK2 is upregulated in neutrophils from septic patients (2) or in mice with severe septic injury (1). The upregulation of GRK2 is associated with the internalization of chemokine (C-X-C motif) receptor 2 (CXCR2), a chemokine receptor, in septic neutrophils (1). Consequently, there is a reduction in neutrophil migration in vitro (2) and in vivo (1), which correlates with an outcome of sepsis in human and experimental settings (1, 2). Moreover, NOS2-derived NO mediates the increase in GRK2 levels in neutrophils by a mechanism dependent on soluble guanylyl cyclase (sGC)/cGMP/PKG (34).

Therefore, we hypothesized that heart failure observed in sepsis could result from an increased activity and/or expression of both total and phosphorylated GRK2. In the present report, we tested this hypothesis and investigated the involvement of NO on GRK2 levels. Moreover, we provide evidence that GRK2 inhibition can be a relevant therapeutic target for severe sepsis.

MATERIALS AND METHODS

Animals

Female adult C57BL/6 (wild type) or NOS2 knockout (KO) mice weighing 25 g were housed in cages in a temperature- and light-controlled room and fed ad libitum. Neonatal cardiomyocytes were harvested from Swiss mice obtained from Universidade de São Paulo animal facility. The Animal Use Ethics Committee of University Federal de Santa Catarina approved the protocol, which was according to Brazilian Government Guidelines for Animal Use in Research and to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Experimental Procedures

Sepsis induction. The model of cecum ligation and puncture (CLP) to induce sepsis was used throughout (40). Briefly, mice were anesthetized with ketamine and xylazine (100 mg/kg ketamine and 10 mg/kg xylazine ip), and a 1-cm midline incision was made on the anterior abdomen. The cecum was exposed and ligated below the ileocecal junction without causing bowel obstruction. A transfixing puncture was made using a 21-gauge needle to induce septic injury (SI). The punctured cecum was gently squeezed to extrude intestinal content through the punctures. The cecum was placed back into the abdominal cavity, and the peritoneal wall and skin incision were closed. All animals received 40 ml/kg sterile PBS subcutaneously immediately after the surgery. Sham-operated (sham) animals (control group) underwent identical laparotomy but without CLP. Animals were evaluated every 12 h up to 36 h after CLP surgery. The results are expressed as percentages of survival. Surviving animals were euthanized by anesthetic overdose (200 mg/kg ketamine and 20 mg/kg xylazine).

Primary cultures of neonatal cardiac myocytes. Neonatal murine (1 day old) hearts were excised, and the ventricles were minced and transferred to a sterile buffer. The tissue was then subjected to six to seven subsequent enzymatic digestions with collagenase, each performed at 37°C for 12 min. The solution obtained from each digest was then transferred to a tube containing 1 ml newborn calf serum and centrifuged. Each cell pellet was suspended in newborn calf serum, and dissociated cells were pooled. To separate myocytes from nonmyocytes, the cell suspension was layered onto discontinuous Percoll density gradients consisting of two phases. After being washed to remove all traces of Percoll, myocytes were cultured in DMEM with 5% FCS, 10% horse serum, and 1% penicillin-streptomycin for 48 h (21). The next day, all myocytes were cultured in glucose and serum-free media 30 min before experiment procedures.

Treatment protocols. IN VIVO TREATMENTS. Mice were treated with *N*-[3-(aminomethyl)-benzyl]acetamidine (1400W; 1 mg/kg) subcutaneously 30 min before and 6 and 12 h after CLP or sham surgery. Animals were treated with GRK2 inhibitor (0.2 mg/kg) subcutaneously 2, 6, and 12 h after CLP or sham surgery. For survival experiments, animals received an additional treatment with GRK2 inhibitor 24 h after sepsis induction. Groups treated with 1400W received increasing doses (0.3, 1.0, and 3.0 mg/kg) 30 min before and 6 and 12 h after CLP surgery. 1400W was diluted in PBS buffer, and GRK2 inhibitor was diluted in PBS-DMSO (1%). Control animals (sham or SI) received PBS or PBS-DMSO (0.2 ml sc). After 12 or 24 h of sepsis induction, echocardiography was performed, and the heart or plasma was harvested for assays.

IN VITRO TREATMENTS. Cardiac myocytes were pretreated for 1 h with 1400W (100 μM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; sGC inhibitor, 30 μM), or KT-5823 (PKG inhibitor, 10 μM). LPS (10 μg/ml) plus interferon (IFN)-γ (500 ng/ml) were then added in all experimental groups except in the control group (cardiac myocytes plus medium). Treatment with 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine (BAY 41-2272; sGC activator, 30 μM) was performed in the absence of LPS + IFN-γ. After 24 h, cells were properly prepared for immunofluorescence or Western blot assays.

Nitrite + nitrate assay. Nitrite + nitrate (NO_x) was measured in plasma harvested 12 h after surgery. As previously described (16), zinc sulfate-deproteinized plasma samples were subjected to quantitative conversion of nitrate to nitrite. Nitrate was converted to nitrite using *Escherichia coli* nitrate reductase for 3 h at 37°C (45). Standard curves of nitrite and nitrate (0–150 μM) were run simultaneously. Values are expressed as micromoles of NO_x.

Plasma aspartate transaminase and alanine transaminase levels. Aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured in plasma harvested 24 h after surgery using commercially available clinical assay kits (Bio-Clin, Belo Horizonte, MG, Brazil). The results were calculated by linear regression and are expressed as units per milliliter (45).

Myocardial functional experiments. Myocardial contractile activity was evaluated in ventricular cones according to previously described procedures (42) with some modifications. The ventricular cones were harvested 12 and 24 h after sepsis induction or sham operation from mice treated with PBS or 1400W (0.2 ml/animal or 1 mg/kg 1400W at 30 min before and 6 and 12 h after surgery). Mice were deeply anesthetized with ketamine (100 mg/kg ip) and xylazine (10 mg/kg ip) and decapitated, the chest was opened, and the heart was rapidly removed and placed in Krebs-Henseleit modified solution maintained at 37°C and pH 7.4 continuously bubbled with 5% CO_2 and O_2. The left and right atria were then excised. Ventricular cones were mounted transversally between two platinum electrodes under a resting tension of 2.0 g in oxygenated Krebs-Henseleit solution. Stimulation was done using a PowerLab/415 system ML415 (AD Instruments, Castle Hill, NSW, Australia) at a frequency of 2 Hz, duration of 4 ms, and supramaximal voltage (threshold + 50%, 3 V). Twenty minutes after the ventricular cone was positioned in the transducer for stabilization, concentration-response curves to isoproterenol (10^{-8}–10^{-12} mol/l) were performed. Only preparations with stable basal contractile activity were included in this study. At the end of the experiment, ventricular cones were weighed and the data are presented as muscle tension per milligram of tissue wet weight. The potency of isoproterenol was determined as pD_2 values (–log of agonist concentration inducing 50% of the maximal response). In addition to ventricular cones, whole and perfused hearts by Langendorff preparation (25) were used to study heart function. Sham or septic animals were treated subcutaneously

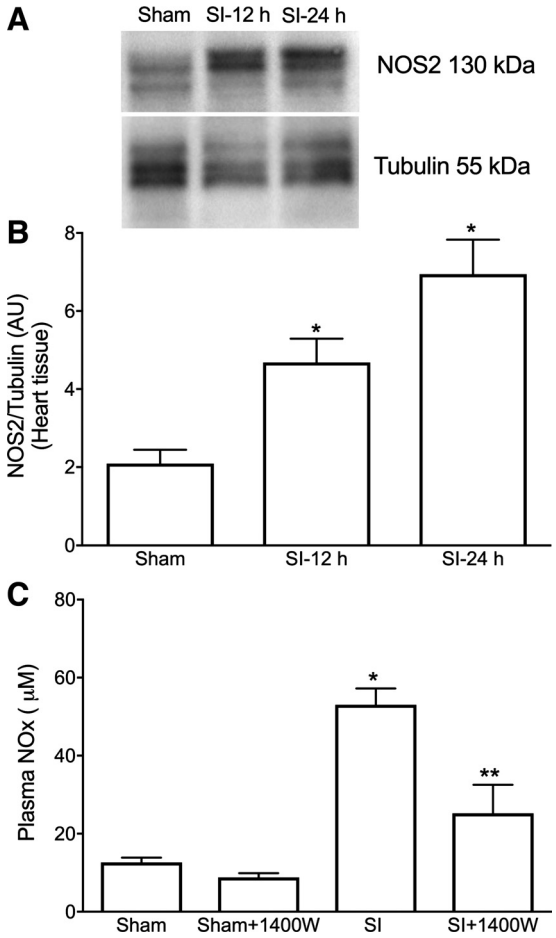


Fig. 1. Inducible nitric oxide (NO) synthase (NOS2) expression on cardiac tissue and plasma nitrite + nitrate (NO_x) levels of mice subjected to septic injury (SI). *A*: 12 or 24 h after sepsis induction, heart tissues were harvested and analyzed for NOS2 protein content by Western blot analysis. The protein content was normalized to tubulin. *B*: data represent means ± SE; $n = 6$ for each group. AU, arbitrary units. *$P < 0.05$ vs. the sham-operated (sham) group. *C*: mice were treated with PBS (0.2 ml sc; SI) or 1400W (1.0 mg/kg sc) and submitted to cecal ligation and puncture (CLP) or sham surgery as described in MATERIALS AND METHODS. Plasma was collected 12 h after sepsis induction, and NO_x levels were assayed as described in MATERIALS AND METHODS. Results are expressed as plasma NO_x (in μM). Values are means ± SE; $n = 6$ for each group. *$P < 0.05$ vs. the control (sham) group; **$P < 0.05$ vs. the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

with PBS or PBS-DMSO (1%, 0.2 ml/animal), 1400W (1 mg/kg, 30 min before and 6 and 12 h after surgery), or GRK2 inhibitor (0.2 mg/kg, 2, 6, and 12 h after surgery). Mice were deeply anesthetized with tribromoethanol (250 mg/kg ip), heparinized (200 IU ip), and decapitated, and the heart was rapidly excised and mounted immediately on the apparatus. The thorax was opened, and the heart was carefully dissected, held with forceps by the opened aortic root, and fixed on a cannula on the perfusion apparatus. The cannula was attached to the constant perfusion flow (2.0 ml/min) of a reservoir containing oxygenated Krebs-Henseleit solution at 37°C and pH 7.4. After 30 min of stabilization (isometric tension of 1.0 g), concentration-response curves to isoproterenol ($10^{-8}-10^{-12}$ mol/l) were performed. The data are presented as systolic tension (in g) and heart rate (in beats/min).

Echocardiography. In vivo cardiac function of sham or septic animals treated subcutaneously with PBS or PBS-DMSO (1%, 0.2 ml/animal), 1400W (1 mg/kg, 30 min before and 6 and 12 h after surgery), or GRK2 inhibitor (0.2 mg/kg, 2, 6 and 12 h after surgery) was evaluated by echocardiography (24). Mice were anesthetized with 1.5% isofluorane (Isoforine Isoflurane) in an inhalation chamber. Body temperature was monitored, and cardiac parameters were obtained through VEVO2100 machine using a 30-MHz transducer. Cardiac output and end-systolic volume were calculated in the long axis by bidimensional mode.

[^{3}H]CGP-12177 binding. Hearts from sham, septic mice, and septic mice treated with 1400W or GRK2 inhibitor as described above were harvested 12 and 24 h after sepsis induction. A binding protocol was performed as previously described (31). The heart was excised and immediately immersed in Krebs-Henseleit solution at 4°C. The tissue was chopped into small segments of 2 × 2 mm and incubated in 1 ml modified Krebs-Henseleit solution with different concentrations of the hydrophilic radioligand [^{3}H]CGP-12177 (12–1250 pM, GE Healthcare Bioscience, Pittsburgh, PA) for 12 h at 4°C, which, under these conditions, selectively labels cell surface β-adrenergic receptors (31). Free radioligand was removed by a successive wash on immersion in tubes containing 2 ml modified Krebs-Henseleit and blotted on a piece of filter paper. Each piece was left in 0.5 ml NaOH (0.3 M) at 37°C until complete solubilization. An aliquot was used for protein quantification by the Bradford (6) method (Bio-Rad, Hercules, CA), and the other was used to quantify radioactivity in a liquid

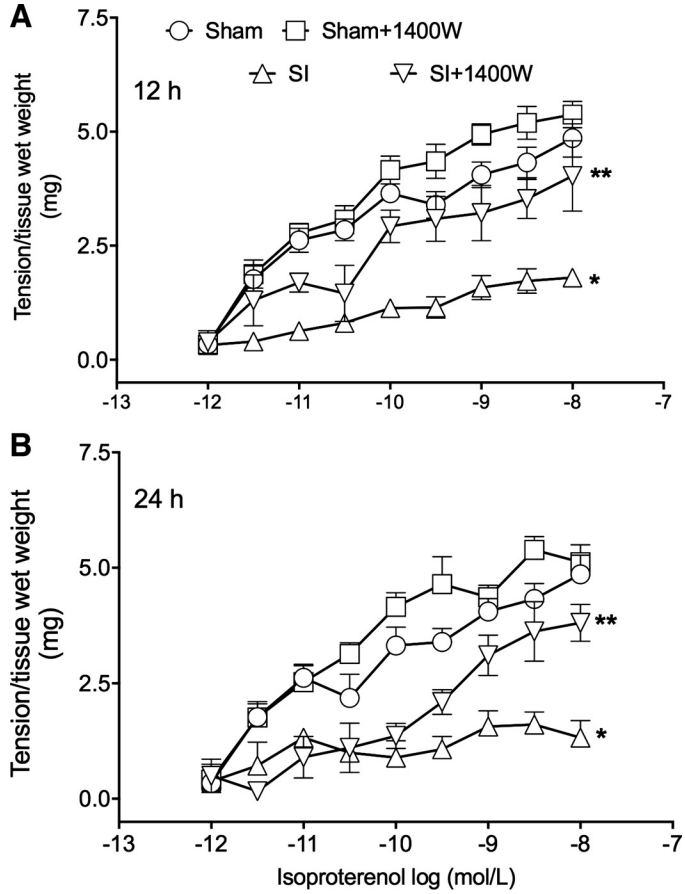


Fig. 2. Effect of 1400W on the responsiveness to isoproterenol of ventricle cones harvested from mice subjected to SI. Mice were treated with PBS or 1400W as described and submitted to CLP or sham surgery. Ventricular cones were collected and mounted (as detailed in MATERIALS AND METHODS) 12 h (*A*) and 24 h (*B*) after CLP surgery. After an equilibration period (20 min), concentration-response curves to isoproterenol ($10^{-8}-10^{-12}$ mol/l) were performed. The control group represents sham animals. Data are means ± SE; $n = 8$ for each group. *$P < 0.05$ vs. the control (sham) group; **$P < 0.05$ vs. the SI group (one-way ANOVA test).

Table 1. *pD_2 values obtained from the concentration-response curve to isoproterenol 12 and 24 h after septic injury*

Experimental Groups	pD_2 Values, $10^{-8}-10^{-12}$ mol/l	
	12 h	24 h
Sham	10.36 ± 0.24	10.36 ± 0.24
Septic injury	9.95 ± 0.26*	8.51 ± 0.33*
Septic injury + 1400W	10.50 ± 0.33†	9.51 ± 0.20†

Data are means ± SE; $n = 8-12$ animals/group. *$P < 0.05$, 24 h vs. the sham (control) group; †$P < 0.05$ vs. the septic injury group (by one-way ANOVA test).

scintillation counter (1900TR, Packard, Perkin-Elmer). Nonspecific binding was defined as the binding of the radioligand in the presence of 10 μM propranolol. Data were analyzed by nonlinear regression using GraphPad Prism software (version 5.0, San Diego, CA) and are presented as maximal radioligand binding capacity (B_{max}; in fmol/mg protein) and the radioligand binding equilibrium dissociation constant (K_d).

Immunofluorescence assay for GRK2 or phosphorylated GRK2. For GRK2 or phosphorylated (p)GRK2 measurements, heart tissues from sham or septic mice treated with PBS or 1400W were harvested 12 and 24 h after sepsis induction. Cardiac myocytes treated with 1400W, ODQ, and KT-5823 and stimulated with LPS + IFN-γ or BAY 41-2272 were harvested 24 h after stimuli. Levels of GRK2 or pGRK2 were determined by immunofluorescence (7, 10). Frozen cardiac tissue sections (5 μm) or cardiac myocytes were fixed with paraformaldehyde (4%) for 10 min in a wet chamber at room temperature. Slides were incubated with PBS containing 1% BSA for 1 h and then incubated with rabbit anti-mouse GRK2 monoclonal antibody (1:200) or rabbit anti-mouse pGRK2 (Ser^{29}) monoclonal antibody (1:200) overnight and then with Alexa fluor 594 (goat anti-rabbit, 1:400). Slides were mounted using DAPI and sealed with enamel. The results of the qualitative analysis are expressed as fluorescence intensity of stained heart tissue (magnification of ×40) or cardiomyocytes (magnification of ×40) in a confocal fluorescent microscopic using Leica Qwin software (Leica Imaging Systems, Cambridge, UK) in conjunction with a Leica microscope, video camera, and online computer (7, 10). The relative quantification of immunostaining from cardiac tissue was achieved through densitometry analysis using the National Institutes of Health ImageJ 1.36b imaging software (Bethesda, MD). We accurately captured one image of each heart tissue per section (3 images/group). All images were acquired by the same microscope settings. In brief, a range of red colored pixels was visually selected using threshold color plugins from the ImageJ imaging software. A threshold value for the optical density that better discriminated staining from the background was obtained, and the settings of this threshold were recorded using the Plugins macro. All images were analyzed by the recorded macro to dispose of any subjectivity. The results are expressed as optical density, and the graphic data represent the average values obtained by analyzing the images of cardiac tissue at indicated groups (5).

Western blot analysis for NOS2, GRK2, and pGRK2. For NOS2 levels, heart tissue from sham or septic mice was harvested 12 and 24 h after surgery and the content of NOS2 was determined by Western blot analysis (45). For GRK2 and pGRK2 levels, heart tissue from sham mice, septic mice (wild type) treated with PBS or 1400W, or sham or septic (NOS2 KO) mice, or isolated cardiac myocytes stimulated with LPS + IFN-γ were harvested 24 h after the indicated treatments, and the content of GRK2 or pGRK2 was determined by Western blot analysis. Tissues or cells were disrupted and homogenized in ice-cold RIPA assay buffer containing protease and phosphatase inhibitors [100 mM Tris·HCl (pH 7.4), 2 mM EDTA, 2 μg aprotinin, 0.1 mM PMSF, 200 mM NaF, and 2 mM sodium orthovanadate, Cell Signaling Technology, Danvers, MA]. The homogenate was then centrifuged at 1,000 *g* for 15 min at 4°C. The pellet was discarded, and the supernatant was aliquoted in Laemmli buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromophenol blue) and boiled for 5 min. Protein (20 μg) was then separated on 8% SDS-PAGE and analyzed by Western blot analysis using primary antibodies against NOS2 (1:1,000), GRK2 and pGRK2 (1:1,000), or tubulin (1:2,000) overnight at 4°C. After a wash with Tris-buffered saline-Tween 20 buffer (2 mM Tris, 1 M NaCl, and 0.2% Tween 20, pH 7.4), membranes were incubated at room temperature with the secondary antibody (goat anti-rabbit for NOS2, GRK2, and pGRK2 or goat anti-mouse for tubulin, 1:5,000) for 2 h. Semiquantitative analysis was performed by Image Laboratory Software 4.1 according to the manufacturer's instructions (Bio-Rad). The results are expressed as NOS2/tubulin or GRK2/tubulin in arbitrary units. Tubulin served as the loading protein control.

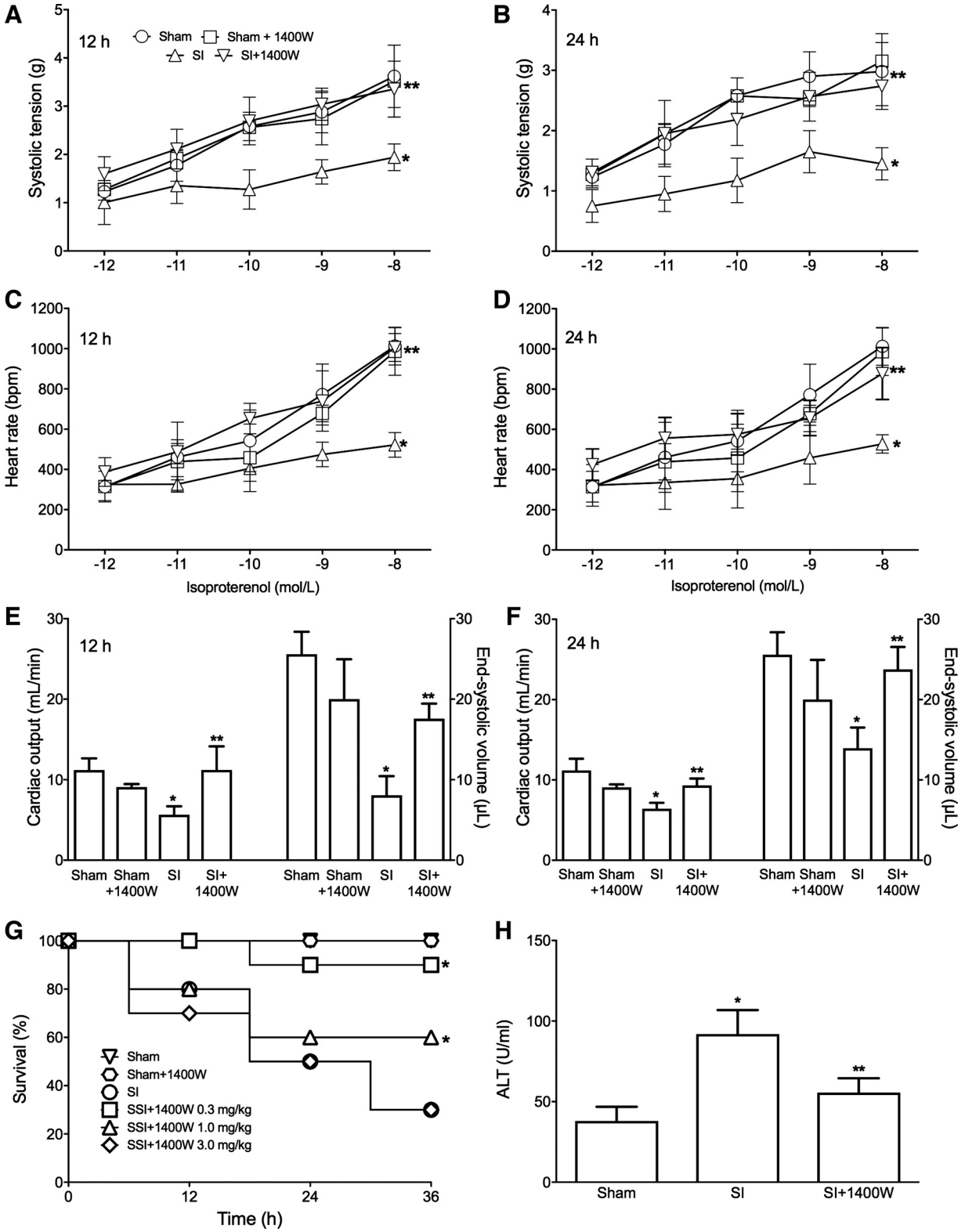
S-nitrosylation in cardiac tissue. The biotin switch method to detect *S*-nitrosylation was performed according to previously described procedures (8). Twenty-four hours after surgery, mice were euthanized by a lethal dose of ketamine and xylazine. Hearts were perfused with wash buffer (250 mM HEPES, 0.1 mM EDTA, and 0.01 mM neocuproine), embedded in Tissue-Tek medium, and immediately frozen in liquid nitrogen. Sections of 10 μm were obtained with a cryostat. Sections were washed three times with wash buffer and permeabilized with wash buffer containing 1% Triton X-100 for 15 min. Sections were incubated with 20 mM *N*-ethyl maleimide to block SH groups for 30 min at 4°C and washed three times with wash buffer containing 1% Triton X-100 and incubated with 1 mM sodium ascorbate and 0.05 mM *N*-(3-malemidylpropionyl) biocytin for 1 h at room temperature. Sections were incubated overnight at 4°C with rabbit anti-mouse polyclonal antibody GRK2 (1:200 dilution, Santa Cruz Biotechnology) or rabbit anti-mouse pGRK2 (Ser^{29}) polyclonal antibody (1:200 dilution, Abcam). Sections were then washed three times with PBS containing 1% Triton X-100 and incubated with Alexa fluor 568 goat anti-rabbit (1:400 dilution) and avidin-linked FITC (1:400 dilution) (Invitrogen Life Technologies, Carlsbad, CA) for 1 h at room temperature and washed three times with PBS containing 1% Triton X-100. Sections were mounted in Gel Mount aqueous mounting medium (Sigma-Aldrich, St. Louis, MO). Confocal fluorescence microscopy was performed in a Leica DMI6000B microscope, and images were acquired with LAS AF Lite (Leica Microsystems) software.

Fig. 3. Effect of 1400W on cardiac parameters, on survival rate, and on marker of hepatic injury in mice subjected to septic injury (SI). Mice were treated with PBS or 1400W as described and submitted to CLP or sham surgery. Hearts were collected and mounted in Langendorff setup 12 h (*A* and *C*) or 24 h (*B* and *D*) after CLP surgery. After an equilibration period (30 min), concentration-response curves to isoproterenol ($10^{-8}-10^{-12}$ mol/l) were performed to evaluate systolic tension (*A* and *B*) and heart rate (*C* and *D*). Control group represents sham animals. For echocardiography, animals were treated as described and anesthetized with isoflurane to evaluate cardiac output and end-systolic volume at 12 h (*E*) or 24 h (*F*) after CLP or sham surgery. Data are means ± SE; $n = 8$ for each group. *$P < 0.05$ vs. the control (sham) group; **$P < 0.05$ vs. the SI group (one-way ANOVA test). In survival experiments (*G*), animals were treated with PBS or 1400W (0.3–3.0 mg/kg sc) and observed every 12 h until 36 h after surgery. The results are expressed as percentages of survival for all indicated groups; $n = 10$ for each group. *$P < 0.05$ compared with the SI group (Mantel-Cox log-rank test). *H*: plasma was collected 24 h after sepsis induction. Results are expressed as alanine transaminase (ALT) levels (in U/ml); $n = 6$ for each group. *$P < 0.05$ compared with the control (sham) group; **$P < 0.05$ compared with the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

Bacterial counts. Bacterial counts were determined as previously described (17). In brief, samples of blood and lung tissue from sham, septic, or GRK2 inhibitor-treated septic mice were harvested, plated on Muller-Hinton agar dishes (Difco Laboratories), and incubated for 24 h at 37°C.

Statistical Analysis

Data are reported as means ± SE of values obtained from 4−10 animals/group according to experimental group. Comparisons were made using ANOVA followed by a Bonferroni's test for unpaired



values. The survival rate was evaluated by the Mantel-Cox log-rank test. P values of ≤ 0.05 were considered significant.

Compounds, Antibodies, and Reagents

The following compounds were used in this study: isoproterenol hydrochloride (selective β-adrenergic receptor agonist), propranolol (nonselective β-adrenergic receptor antagonist), 1400W (a selective NOS2 inhibitor) (15), *E. coli* LPS (serotype 0111:B4), *E. coli* nitrate reductase and Griess reagent, *N*-ethyl maleimide, neocuproine, L-ascorbic acid, and mercury chloride (all obtained from Sigma-Aldrich). Methyl([5-nitro-2-furyl]vinyl)-2-furoate (GRK2 inhibitor) (46) and KT-5823 (PKG inhibitor) were obtained from Calbiochem (La Jolla, CA). ODQ (sGC inhibitor) and BAY 41-2272 (sGC activator) were obtained from Cayman Chemical. The selective β-adrenergic receptor radioligand [^{3}H]CGP-12177 was obtained from GE Healthcare Bioscience (Pittsburgh, PA). IFN-γ was from R&D Systems (Minneapolis, MN). Drugs were diluted in sterile Dulbecco's PBS [containing (in mM) 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, and 8.1 $NaHPO_4$, pH 7.4] or in Krebs-Henseleit modified solution [containing (in mM) 118.4 NaCl, 4.7 KCl, 1.9 $CaCl_2$, 1.2 KH_2PO_4. 1.2 $MgSO_4 \cdot 7H_2O$, 25 $NaHCO_3$, and 11.6 glucose, pH 7.4]. Bradford reagent was obtained from Bio-Rad, and liquid scintillation cocktail was from Perkin-Elmer. Collagenase type 2 (CLS2) was obtained from Worthington; forane isoflurane was obtained from Isoforine from Cristália (Itapira, SP, Brazil). *N*-(3-malemidylpropionyl) biocytin was obtained from Molecular Probes (Eugene, OR), and avidin-linked FITC was from Invitrogen Life Technologies. Antibodies were obtained as follows: rabbit anti-mouse GRK2 monoclonal antibody, mouse anti-mouse tubulin, goat anti-rabbit IgG-horseradish peroxidase, and goat anti-mouse (Santa Cruz Biotechnology); rabbit anti-mouse pGRK2 (Ser^{29}) monoclonal antibody (Abcam); rabbit anti-mouse NOS2 (Sigma); and Alexa fluor 568 or 594 goat anti-rabbit (Invitrogen).

RESULTS

Plasma NO_x Levels and NOS2 Expression Are Increased During Severe Sepsis

NOS2 expression was augmented in heart tissue 12 and 24 h after sepsis induction (Fig. 1, *A* and *B*). NO_x levels increased in plasma from septic animals 12 h after SI (Fig. 1*C*). Pretreatment of septic mice with 1400W substantially reduced NO_x levels (Fig. 1*C*). NO_x levels in plasma from sham animals were not changed by 1400W treatment.

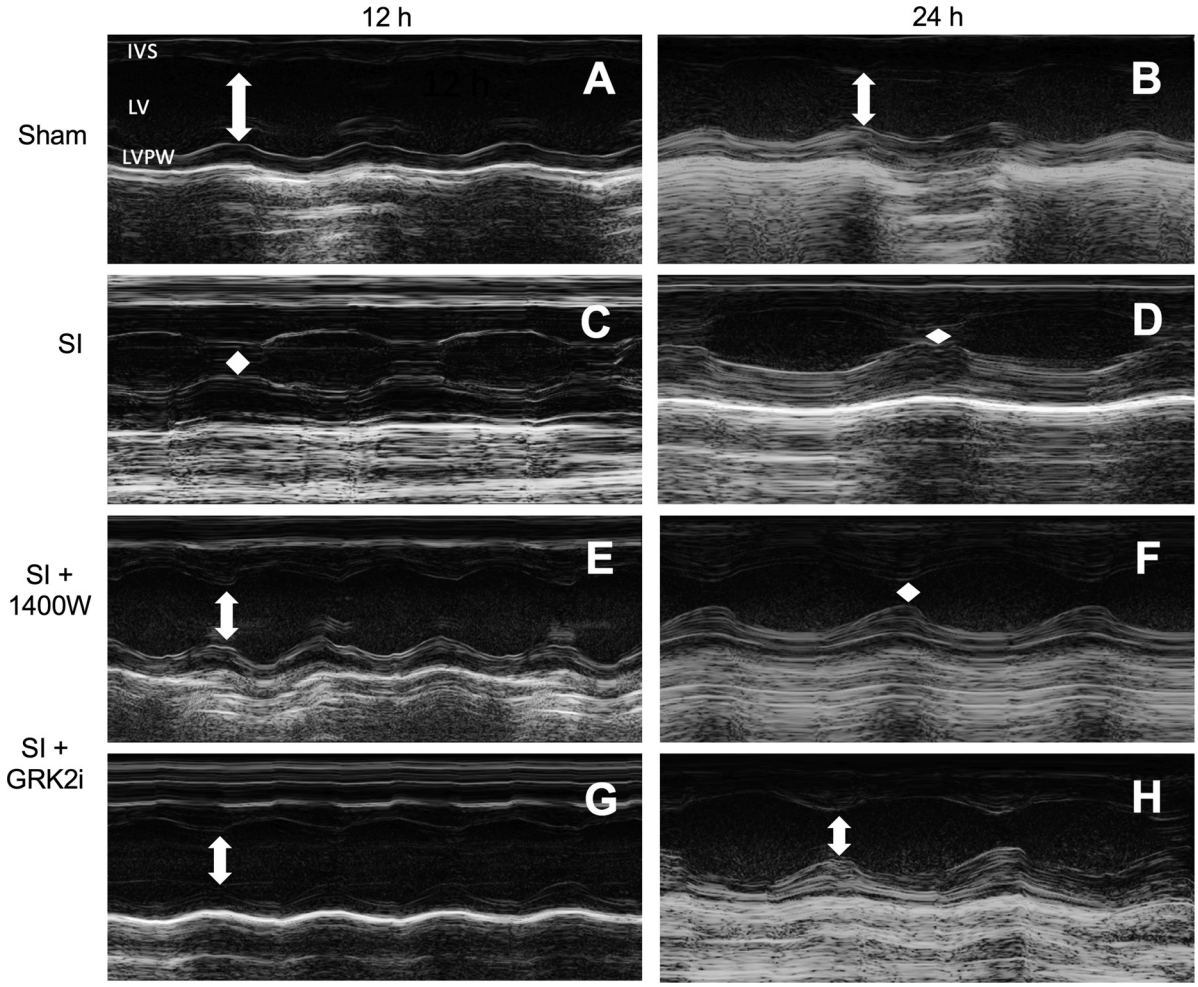


Fig. 4. Effect of 1400W and GRK2 inhibitor (GRK2i) in in vivo cardiac function in mice subjected to SI. Echocardiographic images (left ventricle, M mode) were obtained in mice treated with PBS-DMSO (1%; 0.2 ml sc), 1400W (1.0 mg/kg sc), or GRK2i (0.2 mg/kg) as described and submitted to CLP or sham surgery (control group). Animals were anesthetized with isoflurane and prepared to echocardiography 12 h (*A*, *C*, *E*, and *G*) or 24 h (*B*, *D*, *F*, and *H*) after CLP surgery. White arrows indicate the left ventricular cavity (LVC) in systole. IVS, interventricular septum; LVPW, left ventricular posterior wall.

NO Induces a Loss in Ventricular Cone Responsiveness to Isoproterenol During Severe Sepsis

There was a substantial reduction (~70%) in the maximal ventricular cone tension in response to isoproterenol 12 and 24 h after SI compared with sham mice (Fig. 2, *A* and *B*). Treatment with 1400W restored the isoproterenol response (Fig. 2). Pretreatment with 1400W did not affect the isoproterenol response in sham animals. Quantitative data and pD_2 parameter are shown in Table 1, evidencing that the potency of isoproterenol was reduced in septic animals. Treatment with 1400W restored the potency of isoproterenol to control levels.

NOS2 Inhibition Improves Cardiac Parameters In Vitro and In Vivo, Mortality, and Liver Injury During Severe Sepsis

There was a significant decrease (~50%) in systolic tension and heart rate in response to isoproterenol in isolated hearts at 12 (Fig. 3, *A* and C) and 24 h (Fig. 3, *B* and *D*) after SI. Treatment with 1400W prevented the loss in the isoproterenol response (Fig. 3, *A–D*) but did not affect the isoproterenol response in sham animals. Septic mice presented a significant reduction in cardiac output (~50%) and end-systolic volume (~60%) at 12 h (Figs. 3*E* and 4*C*) and 24 h (Figs. 3*F* and 4*D*) after SI. Echocardiography from sham animals presented normal anatomy and systolic function (Fig. 4, *A* and *B*), whereas septic mice presented increases in the interventricular septum and left ventricle posterior wall, with an important reduction of left ventricular cavity during systole and diastole (Fig. 4, *C* and *D*). These alterations were observed at 12 h but were more intense at 24 h after SI, contributing to the reduction of cardiac output (Fig. 3*E*) due to the impaired ventricular filling (Fig. 3*F*). Treatment with 1400W prevented the reduction of cardiac output and end-systolic volume (Figs. 3, *E* and *F*, and 4, *E* and *F*). Moreover, treatment of septic mice with 1400W protected animals against SI-induced mortality (~60% survival; Fig. 3*G*). Importantly, the protective effect was lost at higher 1400W doses (3 mg/kg). Liver damage was evidenced by higher ALT levels 24 h after septic injury (Fig. 3*H*), and treatment with 1400W prevented the hepatic injury.

Loss of β-Adrenergic Receptors in Heart Tissue During Severe Sepsis

The saturation analysis of the specific binding of the selective β-adrenergic receptor radioligand [^{3}H]CGP-12177 to heart tissue segments yielded a B_{max} of 27.6 ± 2.8 fmol/mg protein and K_d of 202 ± 68 pM. There was a significant decrease in the B_{max} of [^{3}H]CGP-12177 in heart tissue segments 12 h (~40% reduction) and 24 h (~70% reduction) after SI compared with sham mice (Fig. 5). The K_d values of [^{3}H]CGP-12177 were 212 ± 42 and 174 ± 54 pM in heart tissues 12 and 24 h after SI, respectively. Treatment of mice with 1400W or GRK2 inhibitor restored B_{max} in heart tissue segments to values close to the control group (Fig. 5). Pretreatment with 1400W or GRK2 inhibitor did not affect the B_{max} of [^{3}H]CGP-12177 in heart tissue segments from sham animals.

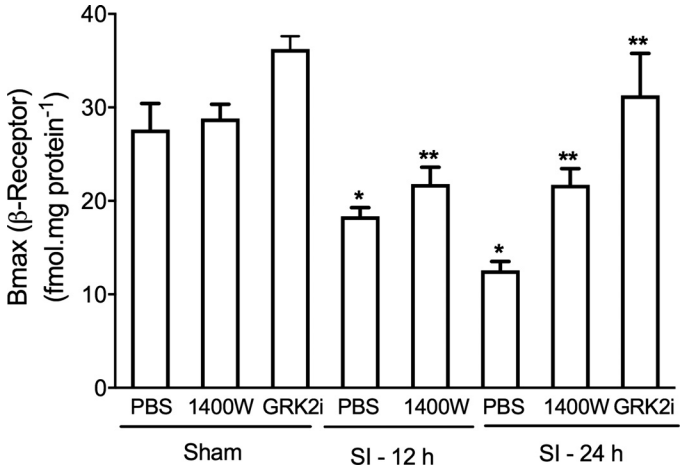


Fig. 5. Effect of 1400W or GRK2i on β-adrenergic receptor binding in ventricular tissue of mice subjected to SI. Mice were treated with PBS, 1400W (1 mg/kg sc), or GRK2i (0.2 mg/kg sc) and submitted to CLP or sham surgery. Binding of [^{3}H]CGP-12177 was performed in ventricular segments from sham mice, septic mice, and septic mice treated with 1400W. After 12 and 24 h, binding to β-adrenergic receptors was evaluated. A similar protocol was used for GRK2i except that the assay was performed 24 h after CLP surgery. Data are means ± SE; $n = 6$ for each group). *$P < 0.05$ vs. the control (sham) group; **$P < 0.05$ vs. the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

NO Increases GRK2 and pGRK2 Labeling in Heart Tissue During Severe Sepsis

GRK2 labeling intensity increased in heart tissue from septic mice 12 h (~30%; Fig. 6*B*) and 24 h (~70%; Fig. 6*D*) after CLP surgery. Pretreatment with 1400W substantially reduced sepsis-induced GRK2 intensity to control levels (Fig. 6*A*) 12 and 24 h after SI (Fig. 6, *C* and *E*). Moreover, a significant increase in pGRK2 staining was observed in heart tissue 12 1 (Fig. 6*G*) and 24 h (Fig. 6*I*) after CLP. Pretreatment with 1400W reduced pGRK2 staining 12 and 24 h after SI induction (Fig. 6, *H* and *J*). Pretreatment with 1400W did not affect GRK2 or pGRK2 in hearts from sham mice (data not shown). Quantitative analysis of optical density confirmed that treatment with 1400W reduces GRK2 (Fig. 6*K*) and pGRK2 (Fig. 6*L*) staining 12 and 24 h after SI induction.

NOS2 Is Essential for GRK2 Activation During Severe Sepsis

Western blot analysis confirmed the increased GRK2 or pGRK2 protein content in cardiac tissues during severe sepsis and that 1400W reduced it to control levels (Fig. 7). Moreover, GRK2 or pGRK2 protein content was diminished in cardiac tissue of NOS2 KO mice during severe sepsis (Fig. 7).

GRK2 Inhibitor Ameliorates Cardiac Parameters In Vitro and In Vivo, Mortality, and Liver Injury During Severe Sepsis

The ~50% decrease in systolic tension and heart rate in response to isoproterenol in isolated hearts from septic mice at 12 h (Fig. 8, *A* and *C*) and 24 h (Fig. 8, *B* and *D*) after SI was prevented by treatment of animals with GRK2 inhibitor (Fig. 8, *A–D*). GRK2 inhibition did not affect the isoproterenol response in sham animals. Septic mice presented a significant

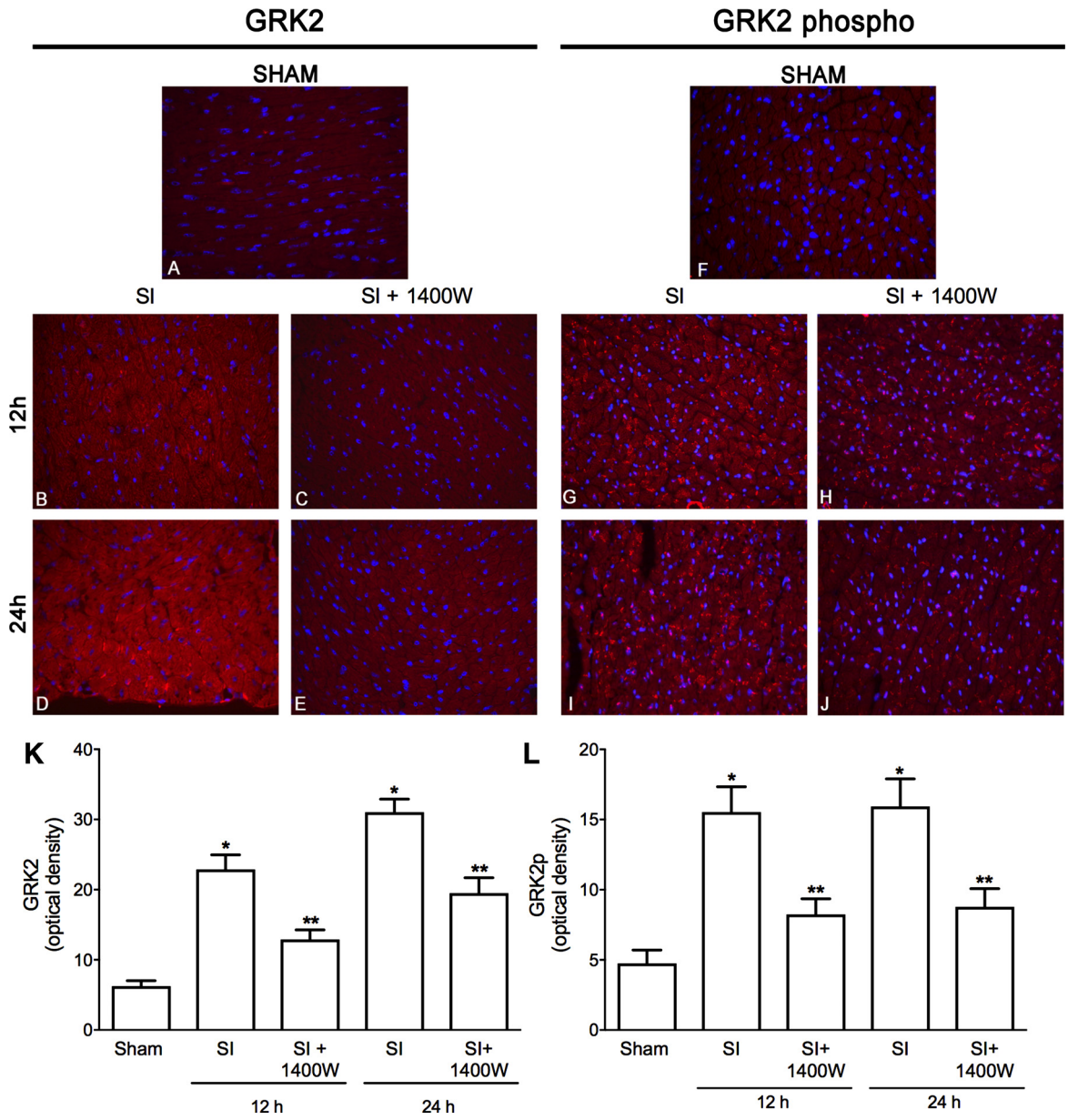


Fig. 6. Effect of 1400W on GRK2 and phosphorylated (p)GRK2 levels in the hearts of mice subjected to SI. Mice were treated with PBS or 1400W and submitted to CLP or sham surgery. GRK2 levels were evaluated 12 h and 24 h after SI induction in hearts from sham mice (control group; *A*), septic mice (SI; *B*: 12 h and *D*: 24 h), or septic mice treated with 1400W (*C*: 12 h and *E*: 24 h). pGRK2 levels were evaluated 12 and 24 h after SI induction in hearts from sham mice (control group; *F*), PBS-treated septic mice (SI; *G*: 12 h and *I*: 24 h), or 1400W-treated septic mice (*H*: 12 h and *J*: 24 h). Cell nuclei are colored blue, and red staining represents GRK2 (*A–E* and *K*) or pGRK2 (*F–J* and *L*). *A–L*: overlay of nuclei and GRK2 or pGRK2 expression. Shown are representative images of 3 animals/group taken from 4 fields/section at a magnification of ×40. The quantitative analysis was made using ImageJ analysis and represents the optical density of immunostaining average of GRK2 (*K*) and pGRK2 (*L*). Values means ± SE; $n = 3$ for each group. *$P < 0.05$ vs. the sham group; **$P < 0.05$ vs. the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

reduction in cardiac output (~50%) and end-systolic volume (~60%) at 12 h (Fig. 8*E*) and 24 h (Fig. 8*F*) after SI. Treatment with GRK2 inhibitor prevented the reduction of cardiac output and end-systolic volume (Fig. 8, *E* and *F*). GRK2 inhibitor improved cardiac output and end-systolic volume (Fig. 4, *G* and *H*). Moreover, treatment of septic mice with GRK2 inhibitor protected animals against SI-induced mortality (~65% survival; Fig. 8*G*). Liver damage was evidenced by higher plasma AST and ALT levels 24 h after septic injury (Fig. 8*H*), and treatment with GRK2 inhibitor prevented the hepatic injury. GRK2 inhibitor did not affect bacteremia and lung bacterial load of septic mice (data not shown).

Experiments on LPS + IFN-γ-Stimulated Neonatal Isolated Cardiac Myocytes

GRK2 labeling intensity increased in myocytes incubated with LPS + IFN-γ (~60%; Fig. 9*B*) as well as in myocytes incubated with BAY 41-2272 only (~75%; Fig. 9*D*) 24 h after stimuli. Pretreatment with 1400W, ODQ, or KT-5823 substan-

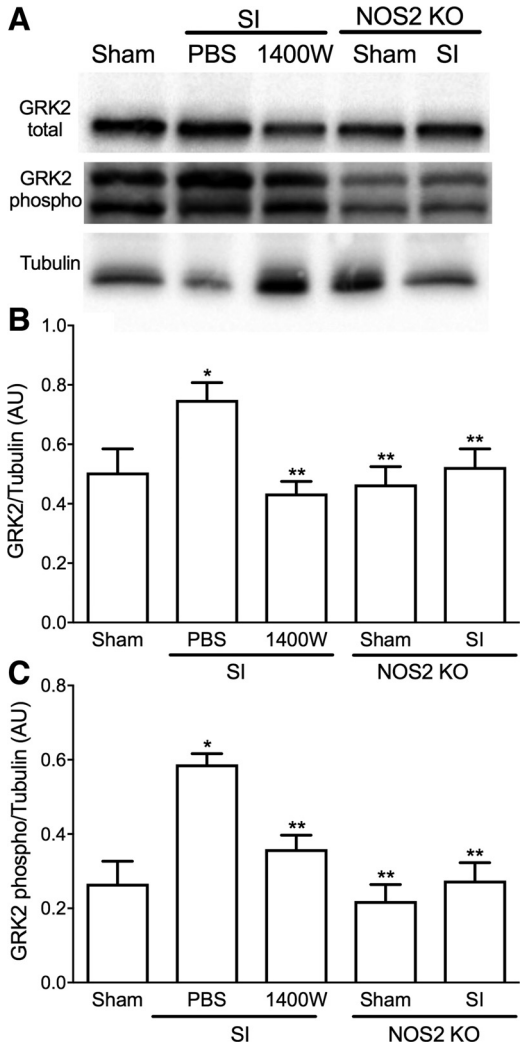


Fig. 7. *A–C*: effect of 1400W and NOS2 genetic deletion on the content and activation of GRK2 in the heart of mice subjected to SI. Mice [wild-type and NOS2 knockout (KO)] were treated with PBS or 1400W and submitted to CLP or sham surgery. GRK2 (*B*) and pGRK2 (*C*) levels were evaluated 24 h after SI induction. Values are means ± SE; $n = 6$ for each group. *$P < 0.05$ vs. the sham group; **$P < 0.05$ vs. the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

tially reduced LPS + IFN-γ-induced GRK2 intensity to control levels after 24 h (Fig. 9, *C*, *E*, and *F*, respectively). Western blot experiments revealed a significant increase in GRK2 (Fig. 10, *A* and *B*) and pGRK2 (Fig. 10, *A* and *C*) levels in cardiac myocytes 24 h after LPS + IFN-γ. Pretreatment with 1400W, ODQ, or KT-5823 reduced GRK2 and pGRK2 levels 24 h after the stimulus induction (Fig. 10).

Involvement of Nitrosylation in GRK2 Activation in the Heart During Severe Sepsis

S-nitrosylation, GRK2, or pGRK2 labeling intensity was increased in heart tissue from septic mice 24 h after CLP surgery. Image merging suggested that both *S*-nitrosylation and GRK2 contents are increased during sepsis and that GRK2 seems to be nitrosylated. However, the images suggest that upon activation pGRK2 does not seem to correlate with nitrosylation anymore (Fig. 11). Proper controls in the absence of ascorbate or presence of mercury chloride were performed (not shown).

DISCUSSION

The main findings of the present report are *1*) sepsis increases the expression of NOS2 in the heart and increases plasma NO_x levels; *2*) cardiac β-receptor density and the functional responsiveness to isoproterenol decrease in sepsis; *3*) sepsis induces loss in cardiac output and end-systolic volume; *4*) the content of active GRK2 is increased in heart tissue and in isolated cardiac myocytes; *5*) blockage of NOS2 activity and the ensuing reduction on NO levels restores β-adrenergic receptor density and the heart functional response, reduces GRK2 activity, increases survival, and prevents liver lesion; *6*) direct inhibition of GRK2 activity restores heart functional response and β-adrenergic receptor density, increases survival, and prevents hepatic injury; and *7*) the soluble GC/cGMP/PKG pathway seems to be the effectors of NO action on GRK2 in cardiac myocytes.

It has been well demonstrated that sepsis induces the expression of several proinflammatory proteins, i.e., NOS2 and its product, with NO being the most prominent one (18). Our data confirm those findings showing that levels of NOS2 protein increase sharply and remain high during 24 h of sepsis. That the enzyme is fully active was demonstrated by the substantial increase in NO_x levels and its reduction when animals were treated with 1400W.

Notwithstanding the limitations of murine models when translating into human studies, several findings obtained in murine models have counterparts in the human condition (1, 2, 4, 35). Levy et al. (26) demonstrated that the reduction of cardiac performance did not correlate with reductions in the energy supply since myocardial glucose uptake and glycogen deposits were even increased. On the other hand, high NOS2 expression and the consequent high amounts of NO produced have been described in different models of sepsis and in human septic shock (48, 53). For instance, treatment of patients with a NOS inhibitor also reestablished the cardiovascular responsiveness to the vasoconstrictors (35), like what is shown here. Moreover, NOS2-deficient mice preserved their cardiac function after endotoxin challenge (48) and nonspecific NOS inhibition restored cardiac output and stroke volume after LPS injection (48).

The molecular mechanisms by which NO mediates the reduction of heart function during severe sepsis have not been fully clarified. It is well accepted that severe sepsis induces myocardial contractility abnormalities, which contribute to the aggravation of this condition (3, 9, 22, 23, 44, 49, 50). Several mechanisms have been described to be involved in septic heart failure, including dysfunction of the intrinsic contraction apparatus such as loss of intercalated disk structural integrity (7) and reduction of β-adrenergic response (4, 28, 43).

Having established that NOS2 is expressed and functional, we went to study what would be its contribution to the loss in the response of heart toward β-adrenergic stimulation using a murine model of sepsis. We found a substantial reduction of the density of β-adrenergic receptors in the membrane of the heart cells, which was prevented by the inhibition of NOS2 with 1400W. Since the simultaneous availability of NO and superoxide may lead to the formation of peroxynitrite (32), we

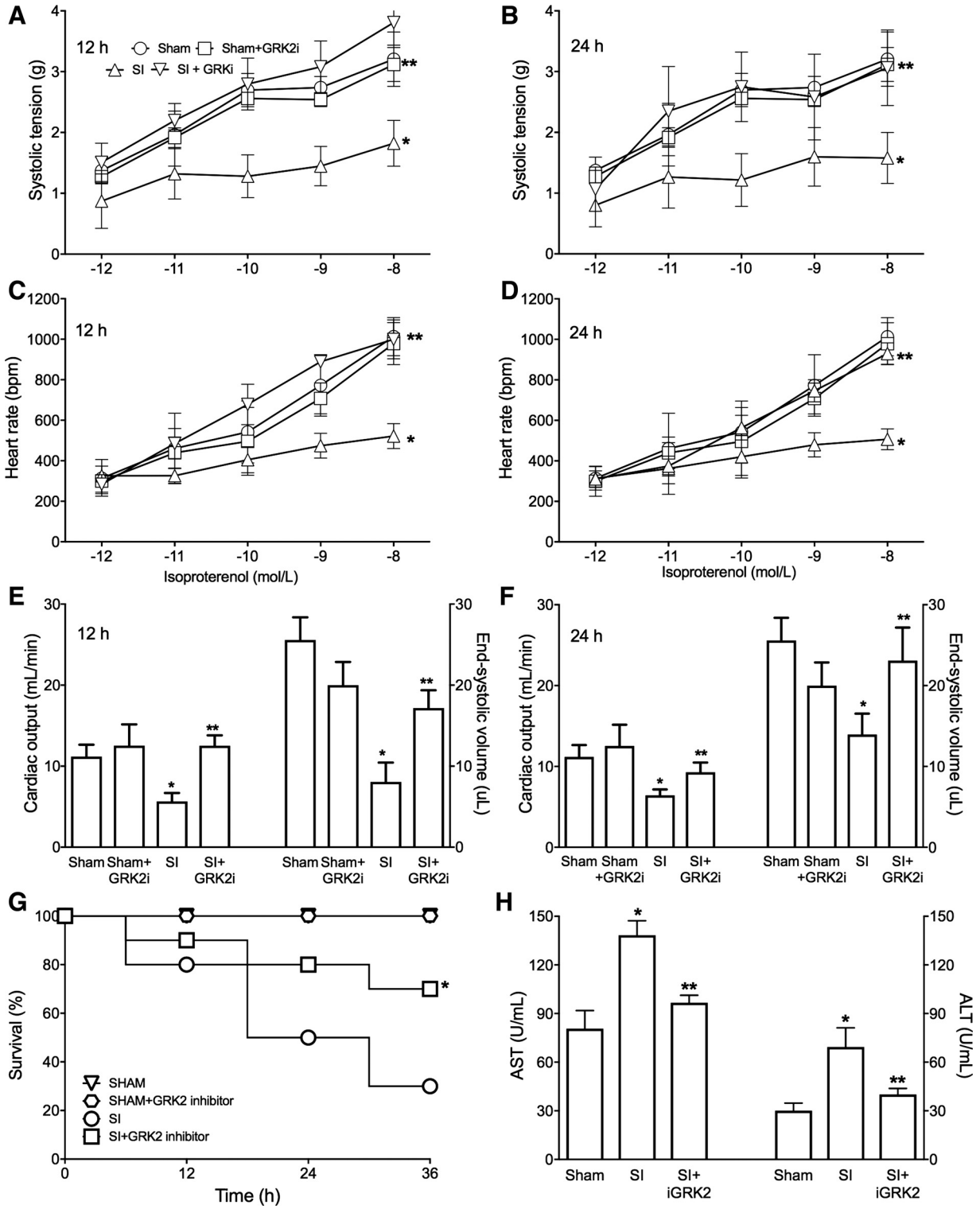


Fig. 8. Effect of GRK2i on cardiac responsiveness to isoproterenol in hearts, on in vivo cardiac function, in the survival rate, and on markers of hepatic injury in mice subjected to SI. Mice were treated with PBS or 1400W as described and submitted to CLP or sham surgery. Hearts were collected and mounted in a Langendorff setup 12 h (*A* and *C*) or 24 h (*B* and *D*) after CLP surgery. After an equilibration period (30 min), concentration-response curves to isoproterenol ($10^{-8}-10^{-12}$ mol/l) were performed to evaluate systolic tension (*A* and *B*) and heart rate (*C* and *D*). Control group represents sham animals. For echocardiography, animals were treated as described and anesthetized with isoflurane to evaluate cardiac output and end-systolic volume at 12 h (*E*) or 24 h (*F*) after CLP or sham surgery. Data are means ± SE; $n = 8$ for each group. *$P < 0.05$ vs. the control (sham) group; **$P < 0.05$ vs. the SI group (one-way ANOVA test). In survival experiments (*G*), animals were treated with PBS or 1400W (0.3–3.0 mg/kg sc) and observed every 12 h until 36 h after surgery. Results are expressed as percentages of survival for all indicated groups; $n = 10$ for each group. *$P < 0.05$ compared with the SI group (Mantel-Cox log-rank test). *H*: plasma was collected 24 h after sepsis induction. Results are expressed as ALT and aspartate transaminase (AST) levels (in U/ml); $n = 6$ for each group. *$P < 0.05$ compared with the control (sham) group); **$P < 0.05$ compared with the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

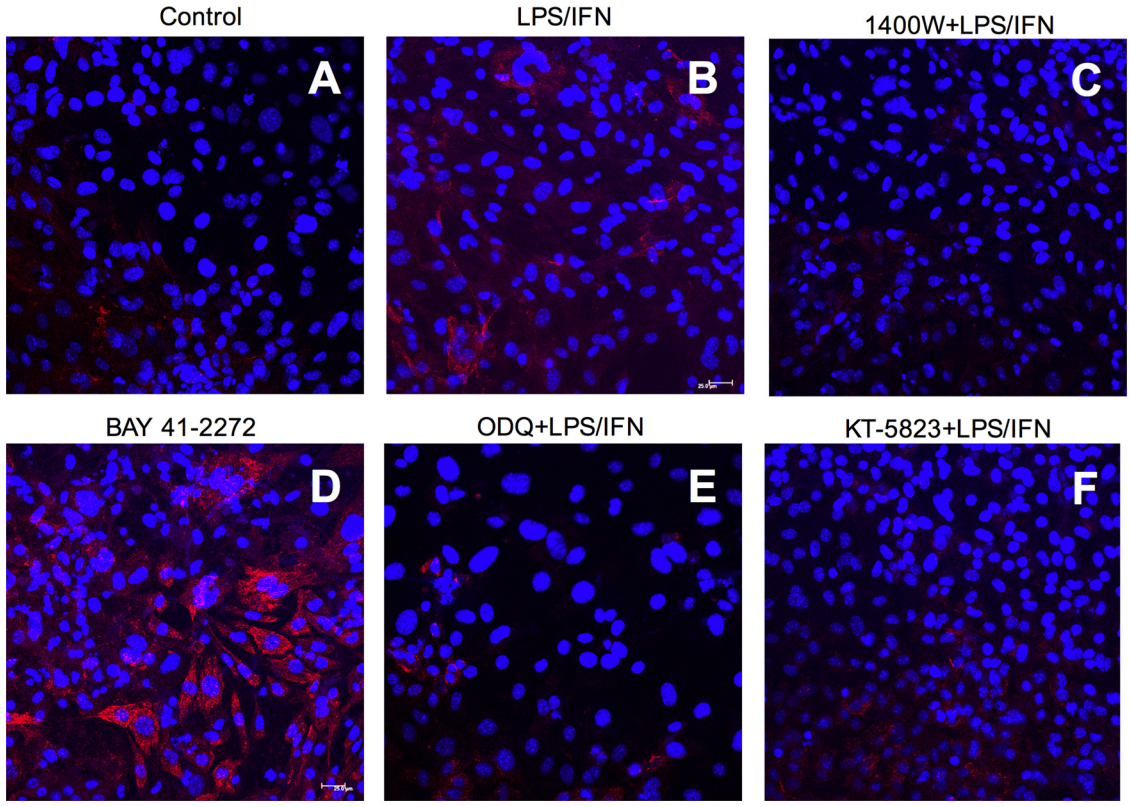


Fig. 9. Effect of several conditions on GRK2 levels in isolated neonatal cardiomyocytes. Myocytes were obtained as described in MATERIALS AND METHODS and treated with DMEM (control group; *A*), LPS + interferon (IFN)-γ (*B*), 1400W (100 μM; *C*), ODQ (30 μM; *E*), and KT-5823 (10 μM; *F*) 1 h before LPS + IFN-γ addition to the indicated experimental groups. BAY 41-2272 (30 μM; *D*) was added to the cells without activation with LPS + IFN-γ. After 24 h, cardiomyocytes were examined under the confocal microscope. Nuclei are blue and red indicates GRK2. For the sake of better visualization, the brightness of all was enhanced by 65% at the same time. White bar = 25 μm. Magnification: ×40.

cannot exclude the involvement of this species in the reduction of heart β-adrenergic receptor density. In any event, our results show that NOS2-derived NO induces cardiac hyporesponsiveness to β-adrenergic stimulus by reducing the number of adrenergic binding sites and affecting both systolic tension and heart rate. Moreover, it was also demonstrated that sepsis induces a substantial impairment of cardiac function, notably cardiac output and a reduction of end-systolic volume. These alterations were promptly avoided in animals in which NOS2 activity was reduced by 1400W. The improvement of the cardiovascular performance associates with the increase of the survival rate of the septic mice. Of note, the increase of the survival rate was not observed with a high dose of 1400W. The simplest explanation is that the progressive inhibition of NOS2 switches from a protective effect to a deleterious effect. In fact, besides the deleterious effect on the cardiovascular system described above, NO derived from NOS2 also has antimicrobial activity (34).

We were also interested in further exploring the mechanism involved in NO-induced reduction in the β-adrenergic response in the heart. An important feature of G protein-coupled receptors, including adrenergic receptors, is their rapid internalization from the plasma membrane into the endosomal cell compartments and the consequent desensitization to adrenergic stimuli (37). These events appear to be responsible for the reduction of agonist effects, including heart contraction induced by β-agonists (12, 51). Receptor activation induces an increase in the activity and the expression of GRKs, a family of ubiquitous cytosolic enzymes that phosphorylates G protein-coupled receptor to signal receptor desensitization and internalization (37). GRK2 is a member of GRKs expressed in several cell types, including leukocytes, cardiomyocytes, and smooth muscle cells (37, 39). We have described that GRK2 is increased in neutrophils from patients and mice during sepsis (1, 2, 34). The increase of GRK2 is associated with the reduction of CXCR2 and of chemotaxis toward agonists to this receptor (1). Considering these findings, we investigated the hypothesis that the reduction of heart β-adrenergic receptor density would involve GRK2. Inhibition of NOS2 activity by 1400W treatment prevented both the reduction in receptor density and enhancement of GRK2 levels and activity, clearly indicating that the three events are linked. Moreover, genetic deletion of NOS2 also prevented the increase in GRK2 level and activity in cardiac tissue from septic mice. Interestingly, both the increase in GRK2 levels and decrease in β-adrenergic receptor density in the heart were observed during the hypodynamic phase of sepsis, suggesting that this mechanism may contribute to the hypodynamic state observed in the late phases of sepsis.

Previously, it has been demonstrated that an induction of NOS2 is involved in the upregulation of GRK2 expression in septic neutrophils by a mechanism dependent on Toll-like receptor 2/4 activation, being the NO signaling mechanism linked to GRK2 induction dependent on the sGC/cGMP/PKG

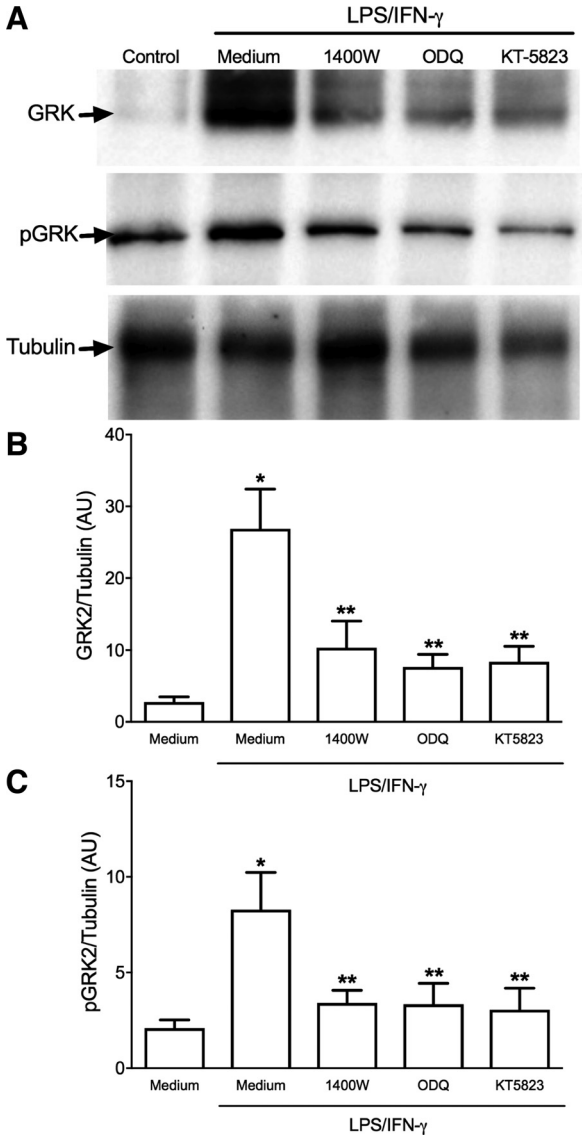


Fig. 10. *A–C*: involvement of the NO/cGMP/PKG pathway on GRK2 and pGRK2 levels in isolated cardiomyocytes from neonatal mice. Cardiomyocytes were treated with DMEM (control group), 1400W (100 μM), ODQ (30 μM), or KT-5823 (10 μM) 1 h before LPS + IFN-γ or DMEM addition to indicated experimental groups. After 24 h, cardiomyocytes were collected and analyzed for GRK2 or pGRK2 levels by Western blot analysis. Quantification is shown in *B* (GRK2) and *C* (pGRK2) as means ± SE; $n = 5$ for each group. *$P < 0.05$ vs. the sham group; **$P < 0.05$ vs. the SI group (one-way ANOVA followed by Bonferroni's multiple-comparison test).

pathway (34). In the same way, we demonstrated that inhibition of the sGC/PKG pathway in isolated cardiac myocytes stimulated with LPS + IFN-γ prevented alterations in GRK2 and pGRK2 levels, strongly suggesting that this pathway may be the effector of NO-induced GRK2 activation and β-adrenergic receptor decrease and the ensuing cardiac failure of sepsis. Rudiger and Singer (41) reviewed some mechanisms involved in the myocardial depression observed during sepsis, including the NO/cGMP/PKG pathway inducing inhibition of PKA and consequently reduction of cardiac contractility and relaxation, heart rate, and metabolism (41). In accordance with our results, it was recently demonstrated that atorvastatin pretreatment could improve left ventricular systolic and diastolic functions in vitro through the preservation of GRK2-mediated desensitization/downregulation of β_1-adrenergic receptors in a mouse model of sepsis (47). We also have checked whether *S*-nitrosylation plays a role in GRK2 activation. Although preliminary, the data obtained with confocal microscopy suggest that heart GRK2 seems to be nitrosylated in the basal condition and during sepsis. However, the activated form (pGRK2) does not seem to be correlated with *S*-nitrosylation. Our results showing that NO activates GRK2 is in contrast with other reports showing that NO inhibits GRK2 (for instance, see Refs. 27 and 52). Although we do not have a clear reason for these divergent results, it may be that sepsis modifies the mechanisms relating GRK2 activation/inhibition or that different levels of NO produced are determinant for the activation or inhibition of GRK2 activity.

The most important finding of the present report is related to the demonstration that direct inhibition of GRK2 had such a profound impact in the preservation of heart responsiveness and functionality. Central to that observation is the finding that GRK2 inhibition improved sepsis survival by >50%. At least part of this improvement can be ascribed to a reduction in organ damage, as judged by the prevention in liver damage during severe sepsis. The findings shown here provide substantial evidence that NOS2 and GRK2 inhibition lead to essentially the same effects, demonstrating the crucial roles of NO and GRK2 in the cardiac dysfunction of sepsis. Even with the necessary caution of translating mouse studies to the human clinical setting, the results suggest that GRK2 may be an important player in several aspects of sepsis and that interference with it may be a new option to change the course of this condition.

Taken together, our data suggest that severe sepsis induces cardiac hyporesponsiveness to isoproterenol by a mechanism dependent on NO and mediated by an increase in GRK2 and pGRK2 levels. Moreover, GRK2 inhibition restored cardiac function, augmented the survival rate, and prevented organ damage of septic mice, suggesting that GRK2 inhibition may be a new potential therapeutic to sepsis-induced cardiac dysfunction.

In summary, the present study provides an important insights on the mechanisms of septic shock-induced heart failure. During sepsis, NOS2-derivated NO mediates increases in GRK2 levels in cardiac myocytes, contributing to β-adrenergic receptor internalization and consequent heart hyporesponsiveness to a β-agonist and cardiac dysfunction. Our findings therefore not only reveal the crucial role of NO and GRK2 in septic cardiac dysfunction but also unravel a potential target for new therapies aimed to treat this dysfunction.

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GRANTS

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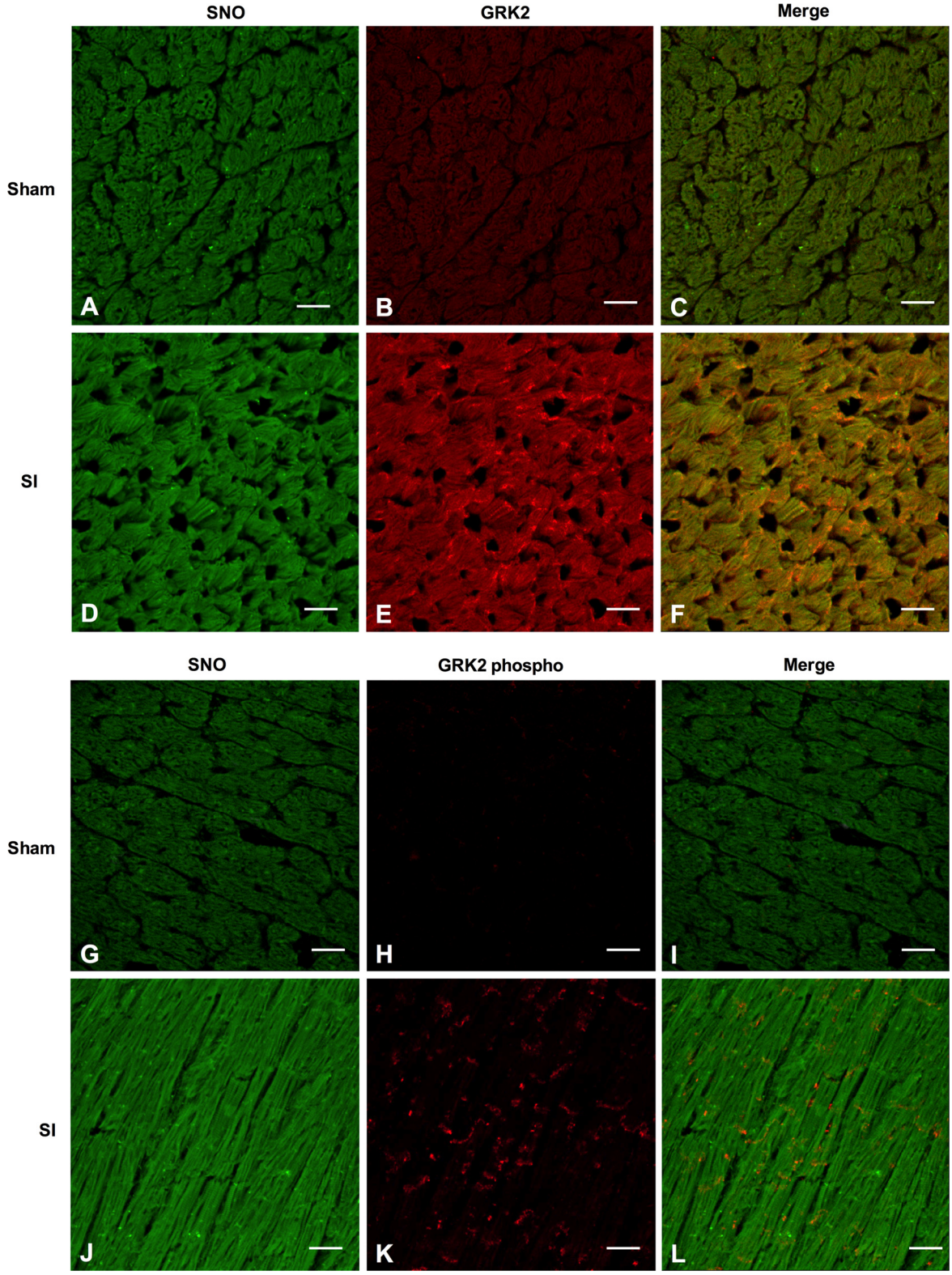

Fig. 11. Detection of *S*-nitrosylated proteins, GRK2, and pGRK2 in the hearts of mice subjected to SI. Twenty-four hours after CLP surgery, hearts were harvested, frozen, sliced (10 μm thick), and prepared for a biotin switch assay. The labeling for nitrosylated proteins is shown in *A*, *D*, *G*, and *J*, for GRK2 in *B* and *E*, and for pGRK2 in *H* and *K*. *C*, *F*, *I*, and *L*: overlay. Images were acquired at an optical magnification of ×20 with a ×5 digital zoom. White bar = 20 μm. For the sake of better visualization, brightness was increased by 40% and contrast was decreased by 40% in *A*–*L*.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

D.D.-S., F.Q.C., and J. Assreuy conceived and designed the research; D.D.-S., S.D., N.E.S.L., F.N.G., M.R.N.C., P.O.B., A.H.S., J. Akinaga, V.L., K.P.S., L.R.A.K., and A.S.P. performed experiments; D.D.-S., S.D., N.E.S.L., F.N.G., M.R.N.C., P.O.B., A.H.S., J. Akinaga, V.L., K.P.S., L.R.A.K., M.A.R., I.d.C.K., A.S.P., F.Q.C., and J. Assreuy analyzed data; D.D.-S., S.D., F.N.G., M.R.N.C., P.O.B., A.H.S., J. Akinaga, V.L., K.P.S., L.R.A.K., M.A.R., I.d.C.K., A.S.P., F.Q.C., and J. Assreuy interpreted results of experiments; D.D.-S. and A.S.P. prepared figures; D.D.-S., F.Q.C., and J. Assreuy drafted manuscript; D.D.-S., S.D., N.E.S.L., F.N.G., M.R.N.C., P.O.B., A.H.S., J. Akinaga, V.L., K.P.S., L.R.A.K., I.d.C.K., A.S.P., F.Q.C., and J. Assreuy edited and revised manuscript; D.D.-S., S.D., N.E.S.L., F.N.G., M.R.N.C., P.O.B., A.H.S., J. Akinaga, V.L., K.P.S., L.R.A.K., I.C.K., A.S.P., F.Q.C., and J. Assreuy approved final version of manuscript.

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