Hydralazine and Organic Nitrates Restore Impaired Excitation-Contraction Coupling by Reducing Calcium Leak Associated with Nitroso-Redox Imbalance*†§

Raul A. Dulce1, Omer Yigner1, Daniel R. Gonzalez2, Garrett Goss1, Ning Feng3, Meizi Zheng4*, and Joshua M. Hare1‡

From the 1Interdisciplinary Stem Cell Institute (ISCI), University of Miami Miller School of Medicine, Miami, Florida 33136, the 2Department of Cardiology, Gulhane Military Medical Academy, Haydarpasa Hospital, 34668 Istanbul, Turkey, the 3Division of Cardiology, Johns Hopkins School of Medicine, Baltimore, Maryland 21287, the 4Departamento de Ciencias Basicas Biomedicas, Facultad de Ciencias de la Salud, Universidad de Talca, 3460000 Talca, Chile, and the 5**Center for Integrative Medicine, School of Medicine, University of Maryland, Baltimore, Maryland 21207

Background: Hydralazine and organic nitrates have clinical benefits for heart failure, but the underlying mechanism is controversial.

Results: Hydralazine reduced sarcoplasmic reticulum Ca2+ leak and improved Ca2+ cycling and contractility; nitroglycerin enhanced contractile efficiency; both were impaired by nitroso-redox imbalance.

Conclusion: These agents exert complementary effects on nitroso-redox imbalance.

Significance: New mechanistic insights for redox-targeted treatments of heart failure.

Although the combined use of hydralazine and isosorbide dinitrate confers important clinical benefits in patients with heart failure, the underlying mechanism of action is still controversial. We used two models of nitroso-redox imbalance, neuronal NO synthase-deficient (NOS1−/−) mice and spontaneously hypertensive heart failure rats, to test the hypothesis that hydralazine (HYD) alone or in combination with nitroglycerin (NTG) or isosorbide dinitrate restores Ca2+ cycling and contractile performance and controls superoxide production in isolated cardiomyocytes. The response to increased pacing frequency was depressed in NOS1−/− compared with wild type myocytes. Both sarcomere length shortening and intracellular Ca2+ transient ([Ca2+]i) responses in NOS1−/− cardiomyocytes were augmented by HYD in a dose-dependent manner. NTG alone did not affect myocyte shortening but reduced [Ca2+]i across the range of pacing frequencies and increased myofilament Ca2+ sensitivity thereby enhancing contractile efficiency. Similar results were seen in failing myocytes from the heart failure rat model. HYD alone or in combination with NTG reduced sarcoplasmic reticulum (SR) leak, improved SR Ca2+ reuptake, and restored SR Ca2+ content. HYD and NTG at low concentrations (1 μM), scavenged superoxide in isolated cardiomyocytes, whereas in cardiac homogenates, NTG inhibited xanthine oxidoreductase activity and scavenged NADPH oxidase-dependent superoxide more efficiently than HYD. Together, these results revealed that by reducing SR Ca2+ leak, HYD improves Ca2+ cycling and contractility impaired by nitroso-redox imbalance, and NTG enhanced contractile efficiency, restoring cardiac excitation-contraction coupling.

Heart failure (HF)3 is a common and increasingly prevalent cause of morbidity and mortality. Prior to the African-American heart failure trial, the only pharmacologic strategy that improved survival in HF was neurohormonal blockade therapy. This trial revealed increased survival among African-American patients with advanced HF treated with the combination of isosorbide dinitrate (ISDN) and hydralazine (HYD) (1–3). However, the precise mechanism(s) by which this regimen reduced mortality remains unclear.

HF is characterized by impaired excitation-contraction (E-C) coupling. For instance, increased contractile force upon increasing frequency of stimulation (force-frequency relationship, FFR) is compromised in failing hearts, giving rise to a blunted or negative FFR (4–6). Defects in calcium (Ca2+) handling may be responsible for this phenomenon (7, 8). It is increasingly appreciated that altered signaling in both Ca2+ cycling and contractility; nitroglycerin enhanced contractile efficiency, restoring cardiac excitation-contraction coupling.

The abbreviations used are: HF, heart failure; CM, cardiomyocyte; E-C, excitation-contraction; FFR, force-frequency response; HYD, hydralazine; ISDN, isosorbide dinitrate; NTG, nitroglycerin; NO/redox, nitroso-redox; NOS1−/−, neuronal nitric-oxide synthase deficient mice; NOX, NADPH oxidase; ONOO−, peroxynitrite; PLB, phospholamban; ROS, reactive oxygen species; RyR2, cardiac ryanodine receptor; SERCA2, SR Ca2+ -ATPase 2; SHHF, spontaneously hypertensive heart failure rats; SL, sarcomere length; SR, sarcoplasmic reticulum; WKY, Wistar Kyoto; XOR, xanthine oxidoreductase; DHE, dihydroethidium; ANOVA, analysis of variance.

* This work was supported, in whole or in part, by National Institutes of Health Grants HL-65453, SR01AG025017, and R01 HL094849 (to J. M. H.).
† This article contains supplemental Tables S1–S3.
‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Biomedical Research Building (BRR), 1501 NW 10th Ave., Room 916, Miami, FL 33136. Tel.: 305-243-5579; Fax: 305-243-5584; E-mail: JHare@med.miami.edu.
consequence of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak, an altered E-C coupling occurs by depleting the SR Ca\(^{2+}\) stores resulting in possible impaired contractile function of the heart.

Increased xanthine oxidoreductase (XOR)-, and possibly NADPH oxidase (NOX)-mediated reactive oxygen species (ROS) production also occurs in failing hearts (13–15). Importantly, disruption in signaling pathways due to oxidative stress can be intensified by NO deficiency, a situation of NO/redox disequilibrium (16, 17). One possible explanation of the clinical benefits of HYD-ISDN is restoration of the balance between formation of reactive oxygen and reactive nitrogen species (2).

We used two established models of cardiac NO/redox imbalance to test the hypothesis that HYD and organic nitrates restore NO bioavailability and reactivity, ameliorate NO/redox disequilibrium, and improves myocardial contractility as measured by FFR. Accordingly, we examined the effects of each drug, alone and in combination, on sarcomere length (SL) shortening, Ca\(^{2+}\) cycling, and SR Ca\(^{2+}\) leak in neuronal NO synthase-deficient (NOS1\(^{-/-}\)) mice and spontaneously hypertensive heart failure (SHHF) rats. ROS production by XOR and NOX were determined in vitro in NOS1\(^{-/-}\) hearts.

**EXPERIMENTAL PROCEDURES**

**Animal Models and Myocyte Isolation**—All protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Miami following the Guide for the Care and Use of Laboratory Animals (NIH Publication 85–234, revised 1996).

Cardiomyocytes (CMs) were isolated from C57BL/6J mice (WT, 3–5 months old, n = 25; Jackson Laboratories, Bar Harbor, ME) and transgenic mice with homozygous deletions for NOS1 (B6;129S4-Nos1\(^{J/J}\), 3–5 months old, n = 41) or male SHHF rats (22–24 months old, n = 4; Charles River Laboratories Inc., Wilmington, MA) and their normotensive controls, Wistar Kyoto rats (WKY, n = 5) hearts as described in detail (18). Briefly, hearts were harvested and retrograde perfused through the aorta in a modified Langendorf system with 3% bovine serum albumin (Sigma; Biochemical Corp.) and protease type XIV (Sigma).

**Protocols**—Cells were loaded with Fura-2. The SL and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{i}}\)) were measured simultaneously in CMs stimulated at 0.5, 1, 2, 3, and 4 Hz. Experiments were repeated after a 10-min incubation with HYD (0.1, 1, and 10 \(\mu\)M; Sigma), nitroglycerin (NTG, 0.1, 1 and 10 \(\mu\)M; American Regent Laboratories, Inc., Shirley, NY), 10 \(\mu\)M ISDN (Alexis Biochemicals, Enzo Life Sciences, Inc., PA), or NTG or ISDN. Experiments in SHHF rat CMs were carried out similarly, with the exception that the concentration of drugs was as follows: 10 \(\mu\)M HYD, 10 \(\mu\)M NTG, and their combination. All experiments were conducted at 37 °C.

**Contractility and Ca\(^{2+}\) Measuring**—SL shortening (%ΔSL) was recorded with an IonOptix iCCD camera and calculated as: (resting SL – peak SL) \(\times 100\)/resting SL. Ca\(^{2+}\), was measured using a dual excitation (340/380 nm) spectrofluorometer (IonOptix LLC, Milton, MA). The “in vivo” calibration was performed using solutions containing 10 \(\mu\)M ionomycin (Sigma) as described by Gryniewicz et al. (19) and the [Ca\(^{2+}\)]\(_{\text{i}}\), was calculated using the following equation,

\[
[Ca^{2+}] = K^* \times (Sf_2/Sb_2) \times (R - R_{\text{max}})/(R_{\text{max}} - R) \quad (\text{Eq. 1})
\]

where \(K^*\) (apparent dissociation constant for Fura-2) in adult myocytes was 224 \(nM\). \(R_{\text{min}}\) and \(R_{\text{max}}\) as well as the scaling factors (Sf, and Sb) were extracted from the calibration curves.

**SR Ca\(^{2+}\) Leak and SR Ca\(^{2+}\) Load Measurement**—SR Ca\(^{2+}\) leakage was assessed with tetracaine as described by Shannon et al. (20). Briefly, after pacing was stopped, a fast switch to a 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode solution (Na\(^+\) was replaced by an equimolar amount of Li\(^+\)) was performed. After 60 s, similar to Bassani et al. (21), a rapid switching to 0 Na\(^+\), 0 Ca\(^{2+}\) solution containing 20 mM caffeine to assess SR Ca\(^{2+}\) content was followed. Following recovery of the cell, the same pacing protocol was assessed. After stop pacing, a switch to 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode solution containing 1 mmol/liter of tetracaine (Sigma) was performed. The observed drop in the Fura-2 ratio compared with the non-tetracaine treated condition was considered the Ca\(^{2+}\) leak for a particular CM. After assessing the Ca\(^{2+}\) leak, tetracaine was washed out by superfusing fresh 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode solution and then a new caffeine challenge was applied to estimate the SR Ca\(^{2+}\) load. SR Ca\(^{2+}\) contents were calculated considering that SR represents 3.5% and cytosol 65% of the CM volume. The following equation from Shannon et al. (20) was used:

\[
[Ca^{2+}]_{\text{SR}} = [Ca^{2+}]_{\text{calf}} + (\beta_{\text{max-SR}} \times [Ca^{2+}]_{\text{calf}})/(K_{d-SR} + [Ca^{2+}]_{\text{SR}})
\]

[Ca\(^{2+}\)\(_{\text{SR}}\)]\(_{\text{SR}}\) is the SR Ca\(^{2+}\) content, [Ca\(^{2+}\)\(_{\text{calf}}\)]\(_{\text{calf}}\) is the SR Ca\(^{2+}\) released by caffeine, \(\beta_{\text{max-SR}}\), and \(K_{d-SR}\) are the usual Michaelis parameters for SR Ca\(^{2+}\) binding. SR leak-SR load pairs were grouped by comparable SR Ca\(^{2+}\) loads and expressed as a leak-load relationship.

**Detection of Superoxide by DHE Staining**—Fresh isolated mouse CMs were incubated at room temperature for 30 min with dihydroethidium (DHE, 3 \(\mu\)M; Molecular Probes). After washing with PBS, cells were fixed with 2% paraformaldehyde (room temperature) on a Zeiss LSM-710 confocal microscope. Nuclear fluorescence captured at 562 nm was quantified using the Image-Pro plus software (MediaCybernetics, Silver Spring, MD) and normalized by cytosolic fluorescence.

**NOX-dependent Superoxide Production**—NOX-dependent superoxide production was measured in heart homogenates from NOS1\(^{-/-}\) mice using lucigenin (5 \(\mu\)M)-enhanced chemiluminescence (β-NADPH 300 \(\mu\)M; room temperature) on a microplate luminometer (PerkinElmer, Sunnyvale, CA). Chemiluminescence readings were expressed as integrated light units. Experiments were performed in the presence of increasing concentrations of HYD and NTG (0.01, 0.1, and 1 \(\mu\)M).

**XOR Activity**—XOR activity was investigated by measuring uric acid (22) and superoxide production. Heart homogenates from NOS1\(^{-/-}\) mice were passed through a Sephadex G-25 column (GE Healthcare) and XOR-dependent superoxide production was measured using Amplex Red (Molecular Probes,
Hydralazine and Nitroglycerin Restore Impaired E-CC

Eugene, OR). The effluent was assessed at 295 nm for uric acid. Both assay plates were incubated at 37°C for 30 min in the presence or absence of allopurinol, and HYD and NTG at increasing concentrations.

Assessment of Ca²⁺ Myofilament Responsiveness—Myofilament responsiveness to Ca²⁺ was assessed using the steady-state relationship between SL and [Ca²⁺], in intact single CMs tetanized by high-frequency (10 Hz) stimulation after exposure to thapsigargin (0.2 μM for 15 min), as described previously (23). With this approach, the Ca²⁺, was reversibly clamped during the tetanic contracture for 20 s and then rapidly returned to resting levels upon cessation of electrical stimulation via the Na⁺/Ca²⁺ exchanger. The steady-state levels of Ca²⁺ achieved during tetanus exposure were regulated by subjecting CMs to Tyrode solutions containing increasing concentrations of Ca²⁺ (0.1, 1.0, 5.0, 10, and 20 mM CaCl₂).

Western Blot Immunoanalysis—Hearts from 12 NOS1⁻/⁻ mice were perfused with Krebs solution (30 min; n = 6), or Krebs plus 10 μM HYD (30 min; n = 4), or 10 μM ISDN (30 min; n = 2). Hearts were homogenized in cold RIPA buffer containing the protease inhibitor mixture and phosphatase inhibitors. Samples were electrophoresed using a NuPAGE 10% Bis-Tris gel (Invitrogen) and transferred to PVDF membranes (Bio-Rad Laboratories). Immunoblot analysis was performed with goat polyclonal antibody for SERCA2 (Santa Cruz Biotechnology, Inc.), mouse monoclonal antibody for phospholamban (PLB; Pierce, Thermo Scientific), rabbit polyclonal for phospho-PLB-Ser16 (Pierce, Thermo Scientific), PLB phospho Thr-17 (Badrilla, Leeds, UK), NCX1 (RDI, Flanders, NJ), and GAPDH (as loading control; Santa Cruz). Phosphorylated PLB were detected using a rabbit polyclonal antibody for phospho-PLB-Thr17 (Pierce, Thermo Scientific), rabbit polyclonal for phospho-PLB-Ser16 (Pierce, Thermo Scientific), PLB phospho Thr-17 (Badrilla, Leeds, UK), NCX1 (RDI, Flanders, NJ), and GAPDH (as loading control; Santa Cruz). Phosphorylated PLB were expressed as a ratio of Ser(P)-16/PLB or Thr(P)-17/PLB and SERCA2, NCX1, or total PLB expression compared with GAPDH.

Statistical Analysis—Data are reported as mean ± S.E. Statistical significance was determined by Student’s t test, one-way ANOVA, or two-way ANOVA followed by Student’s-Newman-Keuls or Bonferroni’s post hoc tests, as appropriate, using the GraphPad Prism version 4.02 (GraphPad Prism Software Corporation, San Diego, CA). The null hypothesis was rejected at p < 0.05.

RESULTS

Hydralazine and Organic Nitrates Improved Contractility in NOS1⁻/⁻ CMs—To determine the effect of the drugs on CM contractility, we measured SL shortening in cells from WT and NOS1⁻/⁻ mice paced at 0.5, 1, 2, 3, and 4 Hz (Fig. 1, A and B). At baseline, SL shortening was similar in NOS1⁻/⁻ and WT CMs (0.5 Hz; Table 1). However, as previously described (12), the increased SL shortening due to augmented pacing frequency was blunted in NOS1⁻/⁻ CMs compared with WT (4 Hz, Table 1, and Fig. 1, A and B, p < 0.0001 versus WT, two-way ANOVA).

HYD augmented the frequency-dependent SL shortening in NOS1⁻/⁻ CMs in a concentration-dependent manner (Figs. 1C and 2E, supplemental Table S1). In contrast, NTG alone did not affect the SL shortening in NOS1⁻/⁻ (Fig. 1C, supplemental Table S1). To test whether the drug combination would have a synergistic effect, NOS1⁻/⁻ myocytes were incubated with HYD plus NTG. Although this treatment restored the FFR in a dose-dependent manner, the response was not greater than hydralazine alone (Figs. 1C and 2E, Supplemental Table 1). In WT CMs, neither HYD nor NTG, alone or in combination, affected frequency-dependent SL shortening (data not shown).

We next assessed whether ISDN, the pharmacologically employed nitrate, had similar effects as NTG. Indeed, the combination of HYD and ISDN also induced an increased frequency-dependent SL shortening in NOS1⁻/⁻ compared with untreated NOS1⁻/⁻ CMs (Fig. 1C and supplemental Table S1; p = 0.004) at an equimolar concentration of 10 μM. Similar to NTG, 10 μM ISDN did not alter contractility in NOS1⁻/⁻ (Fig. 1C and supplemental Table S1).

Divergent Effects of Hydralazine and Nitroglycerin on Ca²⁺ Transient in NOS1⁻/⁻ CMs—Resting diastolic [Ca²⁺], values were slightly elevated in NOS1⁻/⁻ compared with WT CMs, over the range of studied frequencies (Table 1; p < 0.01) as previously described (12, 24). When the [Ca²⁺], transient (Δ[Ca²⁺]) was studied, [Ca²⁺], FFR was clearly depressed in NOS1⁻/⁻ CMs compared with WT, as previously described.
Hydralazine and Nitroglycerin Restore Impaired E-CC

TABLE 1
Baseline myocyte performance

|                    |          | WT       |          |          | NOS1<sup>−/−</sup> |
|--------------------|----------|----------|----------|----------|--------------------|
|                    | 0.5 Hz   | 4 Hz     | 0.5 Hz   | 4 Hz     |                    |
| Cells<sup>a</sup>  |          |          |          |          |                    |
| Sl. shortening (%)<sup>b</sup> |          |          |          |          |                    |
| Δ[Ca<sup>2+</sup>]<sub>i</sub> transient (nM) | 4.75 ± 0.30 | 9.30 ± 0.50 | 4.27 ± 0.30 | 5.90 ± 0.40<sup>*</sup> |
| Δ[Ca<sup>2+</sup>]<sub>i</sub> decay (τ s) | 0.198 ± 0.009 | 0.091 ± 0.004 | 0.231 ± 0.013<sup>†</sup> | 0.101 ± 0.006 |
| SR C<sup>2+</sup> load (μM) | 42.0 ± 7.4 | 70.6 ± 7.5 | 37.9 ± 7.2 | 52.4 ± 4.7<sup>‡</sup> |

<sup>a</sup> Five to 10 cells per heart were studied.

<sup>b</sup> Sl. shortening is calculated as: (resting SL – peak SL)/resting SL × 100.

<sup>†</sup> p < 0.001 versus WT (unpaired Student’s t test).

<sup>‡</sup> p < 0.05 versus WT (unpaired Student’s t test).

<sup>‡</sup> p < 0.01 versus WT (unpaired Student’s t test).

(Fig. 2, A and B, p < 0.0001 versus WT; two-way ANOVA) (18, 24, 25). HYD induced a Δ[Ca<sup>2+</sup>]<sub>i</sub> increase in a concentration-dependent manner (Fig. 2C) as well as significantly increased the Δ[Ca<sup>2+</sup>]<sub>i</sub>-FFR (Fig. 2D, supplemental Table S2, p < 0.05). Thus, the increase in contractility in response to HYD was mirrored by a concomitant increase in Δ[Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2E).

Although NTG did not affect contractility, it induced a concentration-dependent decrease in the Δ[Ca<sup>2+</sup>]<sub>i</sub> amplitude compared with control NOS1<sup>−/−</sup> myocytes most clearly seen at 4 Hz. This decrease was reproduced using 10 μM ISDN (Fig. 2C and supplemental Table S2). The combination of HYD and NTG (Fig. 2D) or ISDN (supplemental Table S2) on NOS1<sup>−/−</sup> CMs restored the increasing [Ca<sup>2+</sup>]<sub>i</sub>-FFR (frequency p = 0.0213 or p = 0.0041, respectively; two-way ANOVA) by reducing the Δ[Ca<sup>2+</sup>]<sub>i</sub> amplitude toward the levels in WT CMs (p = 0.042 or p = 0.0005 versus HYD alone, respectively). Thus, for any given improvement in cardiac contraction due to HYD, organic nitrates offset the increase in [Ca<sup>2+</sup>]<sub>i</sub>, thereby enhancing contractile efficiency (Fig. 2, D and E). Neither HYD nor NTG, alone or in combination (10 μM), altered the [Ca<sup>2+</sup>]<sub>i</sub>-FFR in WT CMs (data not shown).

We also examined the peak [Ca<sup>2+</sup>]<sub>i</sub>, decay time constant (τ), which was significantly higher in NOS1<sup>−/−</sup> than WT CMs (p < 0.0001, Table 1, Fig. 3, A and B). All treatments accelerated τ (particularly at 0.5 Hz, Fig. 3B and supplemental Table S3) in a dose-dependent manner (p < 0.0001 HYD alone or in combination with NTG versus NOS1<sup>−/−</sup> baseline; p = 0.001 NTG alone versus NOS1<sup>−/−</sup> baseline, two-way ANOVA). ISDN was equally effective in reducing τ, either alone or combined with 10 μM HYD (p < 0.001; supplemental Table S3). Reuptake of Ca<sup>2+</sup> assessed by decay of caffeine-induced Ca<sup>2+</sup> transients in a Na<sup>+</sup> and Ca<sup>2+</sup>-free solution, which avoids any Ca<sup>2+</sup> flux throughout the sarcolemma, was slower in NOS1<sup>−/−</sup> (K<sub>i</sub> = 0.1888/s; T<sub>50</sub> = 3.65 ± 0.12 s, fitted by an one-phase exponential equation) compared with WT cells (K = 0.2724/s; T<sub>50</sub> = 2.53 ± 0.08 s; p = 0.0005). Treatment with either 10 μM HYD or NTG increased the SR Ca<sup>2+</sup> reuptake rate in NOS1<sup>−/−</sup> (K = 0.240/s, T<sub>50</sub> = 2.88 ± 0.17 s, p = 0.0003 and K = 0.2392/s, T<sub>50</sub> = 2.88 ± 0.11 s, p < 0.0001, respectively). The combination exerted an additive effect (K = 0.2919/s, T<sub>50</sub> = 2.36 ± 0.06 s, p < 0.0001) suggesting that the improvement in the regular field-stimulated Δ[Ca<sup>2+</sup>]<sub>i</sub> decay was mediated by enhanced SERCA2 activity. This speculation was confirmed by assessing phosphorylation of PLB in cardiac homogenates from NOS1<sup>−/−</sup> hearts treated with either

![Figure 2](image-url)
10 μM HYD or ISDN. HYD increased phosphothreonine 17 (Thr(P)-17) for pentameric PLB (p = 0.0393); but not on total PLB (p = 0.0597) or monomeric PLB (Fig. 3, C and D). ISDN increased total Thr(P)-17 (p = 0.011; Fig. 3, E and F). Neither treatment significantly increased phosphoserine 16 (Ser(P)-16; Fig. 3, D–F).

Hydralazine Alone and in Combination with Nitroglycerin, Lowers SR Ca²⁺ Leakage Toward Normal—We next assessed SR Ca²⁺ leak and the SR Ca²⁺ leak-load relationship, using the technique of Shannon et al. (20) using tetracaine, a RyR2 blocker (Fig. 4A). SR Ca²⁺ leak was augmented in NOS1⁻/⁻ (R² = 0.5225 for WT and R² = 0.8827 for NOS1⁻/⁻, Fig. 4B; p = 0.0002). The decreased SR Ca²⁺ content and increased diastolic Ca²⁺ exhibited by NOS1⁻/⁻ compared with WT CMs are consistent with the elevated Ca²⁺ leak. HYD reduced the leak in a concentration-dependent manner in NOS1⁻/⁻ myocytes (Fig. 4C; p = 0.0012). Neither 1 nor 10 μM HYD significantly affected the SR Ca²⁺ leak in WT CMs (data not shown).

NTG did not significantly affect the SR Ca²⁺ leak in NOS1⁻/⁻ cells (Fig. 4D). In contrast, the combination of NTG plus HYD reduced the leak in a concentration-dependent manner as shown by the leak-load relationship (Fig. 4E; p = 0.0001). Fig. 4F shows the drug concentration-SR Ca²⁺ leak response relationship. The IC₅₀ for HYD was 0.0705 ± 0.0473 μM (at

**FIGURE 3.** Hydralazine and organic nitrates enhance Ca²⁺ influx to the SR in NOS1⁻/⁻ cardiomyocytes. A, Ca²⁺ decay time constant (tau, τ) versus frequency in NOS1⁻/⁻ (■) compared with WT (○) CMs. B, Tau values at 0.5 Hz in WT control or NOS1⁻/⁻ CMs treated with or without HYD, NTG, or a combination (HYD + NTG) at the indicated concentrations (μM) (n = 5–21 cells) (*, p < 0.05; **, p < 0.01; and ***, p < 0.001 versus NOS1⁻/⁻ control; two-way ANOVA). C, PLB phosphorylation at threonine 17 (Thr(P)-17) in baseline or 10 μM HYD-treated NOS1⁻/⁻ hearts (total and pentameric Thr(P)-17 PLB). D, representative Western blots for all studied proteins in the absence or presence of 10 μM HYD, E, PLB Thr(P)-17 in baseline or 10 μM ISDN-treated NOS1⁻/⁻ hearts (total and monomeric Thr(P)-17 PLB). Phosphorylated PLB at Ser-16 (Ser(P)-16, bottom) was also measured. F, representative Western blots. No significant changes were observed in Ser(P)-16, SERCA2, or NCX1 with either treatment, †, p < 0.05 versus NOS1⁻/⁻ control; Student’s t test.
Hydralazine and Nitroglycerin Restore Impaired E-CC

but found no significant difference compared with control, whereas 10 μM NTG significantly reduced the degree of the SR Ca\(^{2+}\) leak in WT (data not shown).

Cardiomyocyte SR Ca\(^{2+}\) Content—Because SR Ca\(^{2+}\) storage is the key determinant of FFR, we estimated the Ca\(^{2+}\) content by rapidly infusing caffeine after pacing the myocytes. Although increasing the pacing augmented the SR Ca\(^{2+}\) content in both NOS1\(^{-/-}\) and WT CMs (p < 0.05, Table 1), the increase was smaller in NOS1\(^{-/-}\) (38.2%) than WT (66.6%), which was consistent with previous studies (25). In the presence of HYD, NOS1\(^{-/-}\) CMs also exhibited a rise in SR Ca\(^{2+}\) content in response to increasing frequency (p < 0.05, data not shown). SR Ca\(^{2+}\) content evaluated at 4 Hz was increased by HYD in a concentration-dependent manner, reaching a load similar to WT CMs (p < 0.01) (Fig. 4H). We also determined that, similar to HYD alone, the combination of equal concentrations of NTG and HYD increased the SR Ca\(^{2+}\) load in a concentration-dependent manner in NOS1\(^{-/-}\) CMs (Fig. 4H). However, 10 μM HYD alone or combined with 10 μM NTG abolished the frequency-induced rise in SR Ca\(^{2+}\) content in WT CMs (p = 0.001 and p = 0.005, respectively; data not shown). NTG alone did not affect the SR Ca\(^{2+}\) load in NOS1\(^{-/-}\) (Fig. 4H) or WT myocytes (data not shown).

Detection of Superoxide by DHE Staining—To determine the molecular underpinnings for improved E-C coupling in NOS1\(^{-/-}\) myocytes in response to these drugs, we used the superoxide-sensitive dye DHE to assess the scavenging capacity of HYD and NTG alone, or in combination at 0.1, 1, or 10 μM (Fig. 5, A and B). As DHE is oxidized by superoxide and then translocated to the nucleus (Fig. 5B), the ratio between fluorescence in the nucleus and cytoplasm indexes superoxide production. Although the lowest drug concentrations did not affect superoxide detection, increasing concentrations of HYD as well as NTG scavenged superoxide (p < 0.05; Fig. 5A). In combination they had an additive effect (p < 0.01; Fig. 5A). Thus, at concentrations that reduced the SR Ca\(^{2+}\) leak, HYD alone or in combination with NTG exhibited an antioxidant effect.

NOX-dependent Superoxide Production—We also studied activation of specific oxidases, XOR and NOX, in mouse heart homogenates in the presence of either HYD or NTG. NOX activity of WT (n = 3) and NOS1\(^{-/-}\) (n = 4) hearts was similar (2.29 ± 0.15 versus 2.19 ± 0.13 integrated light units/μg of protein, respectively). HYD (0.1–1 mM, IC\(_{50}\) 0.42 ± 0.23 mM) decreased NOX activity (Fig. 5C; p < 0.01 for both concentrations versus control) in NOS1\(^{-/-}\) cardiac homogenates. NTG (>0.01 mM, IC\(_{50}\) 0.013 ± 0.06 mM) also inhibited NOX-dependent superoxide production (Fig. 5C; p < 0.05 for 0.01 mM, p < 0.01 for 0.1 and 1 mM versus control).

XOR Activity—NOS1\(^{-/-}\) hearts (n = 3) exhibited up-regulated XOR activity compared with WT (n = 3) using the Amplex Red detection of XOR-mediated ROS production assay (Fig. 5D; 2.1 ± 0.18 versus 1.63 ± 0.09 milliunits/μg of protein, respectively; p < 0.05), confirming previous findings in this mouse (25–27). Amplex Red XOR activity was inhibited by allopurinol (>0.01 mM, IC\(_{50}\) 0.19 ± 0.06 mM, Fig. 5D; n = 3) and HYD (IC\(_{50}\) of 0.7 ± 0.3 mM, Fig. 5E; n = 3) in NOS1\(^{-/-}\) cardiac homogenates. Interestingly, NTG (IC\(_{50}\) 1.0 ± 0.4 mM, Fig. 5E; n = 3) also inhibited XOR. Because the Amplex Red assay...
Hydralazine and Nitroglycerin Restore Impaired E-CC

measures superoxide levels, reduced activity could also reflect increased O$_2^-$ scavenging. To address this possibility, we measured uric acid conversion as a direct measurement of enzymatic activity. With this approach, NOS1$^{-/-}$ hearts ($n = 3$) again exhibited increased XOR activity compared with WT ($n = 3$) (Fig. 5D; 9.8 ± 0.46 versus 8.5 ± 0.21 milliunits/mg tissue, respectively, $p < 0.05$). Allopurinol (>0.1 mm) inhibited XOR enzyme activity in NOS1$^{-/-}$ mice (Fig. 5F, $n = 3$). Interestingly, HYD did not, but NTG did display XOR-inhibitory activity in NOS1$^{-/-}$ (Fig. 5F, $n = 3$).

**Organic Nitrates Increased Myofilament Responsiveness to Ca$^{2+}$ in NOS1$^{-/-}$ Myocytes**—Because our FFR data suggested that organic nitrates improve efficiency in myofilament responsiveness to Ca$^{2+}$, we assessed the mechanism by which NTG offsets the HYD-induced Δ[Ca$^{2+}$], increase toward normal, and directly tested the myofilament responsiveness in NOS1$^{-/-}$ as well as WT CMs in the presence of 10 μM NTG. Responsiveness of myofilaments to Ca$^{2+}$ (both sensitivity and maximal response) was lower in NOS1$^{-/-}$ compared with WT CMs (EC$_{50}$,NOS1$^{-/-}$ = 0.627 ± 0.0185 and EC$_{50}$,WT = 0.398 ± 0.018 μM, respectively; $p = 0.0009$, Fig. 6, A–D). In agreement with previous studies using different NO donors (23, 28), NTG reduced myofilament responsiveness to Ca$^{2+}$ in WT (not shown). Surprisingly, but consistent with our results, NTG increased responsiveness to Ca$^{2+}$ in NOS1$^{-/-}$ (Fig. 6, B and D), as shown by a leftward shift (EC$_{50}$,NOS1$^{-/-}$/NTG = 0.427 ± 0.0186 μM, $p = 0.0016$; Fig. 6C). Therefore, NTG restored the impaired myofilament sensitivity in NOS1$^{-/-}$ toward normal levels observed in WT CMs. Analysis of [Ca$^{2+}$]$_i$-SL shortening loops confirm that sensitivity to Ca$^{2+}$ is deficient in NOS1$^{-/-}$ CMs but treatment with 10 μM ISDN improves it toward WT (Fig. 6E).

**Frequency-stimulated Contractility and Ca$^{2+}$ Transients Are Improved by Hydralazine and Nitroglycerin in CMs from Heart Failure Rats**—To study the effects of HYD and NTG in a model of heart failure (dilated cardiomyopathy), we used SHHF rats. Usually, the SL shortening amplitude in rat CMs is flat or negative in response to increasing the pacing rate. We recently showed that SL shortening drops more abruptly in SHHF than WKY control CMs (13). Here, we also observed a significant decrease in contractility in SHHF compared with WKY CMs ($p = 0.0002$, Fig. 7, A and B). Treatment with 10 μM HYD significantly improved SL shortening in SHHF myocytes ($p = 0.0414$). Consistent with observations in NOS1$^{-/-}$, 10 μM NTG did not affect the SL shortening in SHHF myocytes ($p = 0.2553$). The combination of HYD and NTG showed a strong trend toward increased contractility in SHHF myocytes ($p = 0.0839$), which also exhibited an FFR pattern similar to HYD alone (Fig. 7A). Furthermore, there was a significant difference at 4 Hz compared with the SHHF control ($p = 0.0359$, Fig. 7B). Normalization of SL shortening as a percentage of 0.5 Hz pacing showed that HYD alone as well as in combination with NTG significantly improved FFR in failing CMs ($p = 0.035$ and $p = 0.0013$, respectively, data not shown).

The Δ[Ca$^{2+}$], increased in WKY CMs in response to pacing ($p = 0.0019$, Fig. 7C), compared with the flat pattern in SHHF CMs. In agreement with the results obtained in NOS1$^{-/-}$ mice, in failing CMs Δ[Ca$^{2+}$]$_i$ amplitude was augmented by 10 μM NTG.
**DISCUSSION**

Our major new finding is that HYD and NTG corrected the increased Ca$^{2+}$ leak from the SR, thus normalizing contractility-frequency responses by modulating Ca$^{2+}$ cycling in models of NO/redox imbalance. Although HYD improved contractile force in parallel with increasing Ca$^{2+}$ transients and total restoration of leak, NTG offset Ca$^{2+}$ cycling without impairing contractility, thereby improving contractile efficiency. Direct measure of myofilament sensitivity confirmed that NTG restores depressed myofilament sensitivity to Ca$^{2+}$ in NOS1$^{-/-}$ myocytes. Importantly, NTG inhibited two major sources of cellular ROS production, NOX and XOR, and also exhibited ROS scavenging capacity. HYD scavenged ROS but did not inhibit XOR. Although NOX activity appeared to be inhibited by HYD, this effect could be an epiphenomenon due to the superoxide scavenging properties of HYD, because NOX activity was only assessed by NADPH-induced superoxide production. Despite the lack of NOS1-derived NO and the fact that NOS3 is uncoupled under oxidative stress (29), peroxynitrite...
(ONOO\textsuperscript{−}) was measurable in NOS1\textsuperscript{−/−} homogenates and was reduced by both HYD and NTG, in a dose-dependent manner (data not shown).

**Model of NO-Redox Imbalance—NOS1\textsuperscript{−/−} mice** are widely used to investigate the involvement of NO in E-C coupling because CMs exhibit a particularly impaired performance due to deficient NO production and consequent NO/redox imbalance (12, 25). Sears et al. (30) showed that NOS1\textsuperscript{−/−} CMs have larger $\Delta$Ca\textsuperscript{2+}, which is consistent with larger $I_{Ca}$ and augmented SL shortening compared with control animals, at low frequencies. However, we (12, 18) and others (31) have shown that the contractile reserve is impaired in NOS1\textsuperscript{−/−} as the increase in $\Delta$Ca\textsuperscript{2+} and contractility in response to pacing are depressed. Consistently, we have found that the reduced SR Ca\textsuperscript{2+} load was associated with augmented SR Ca\textsuperscript{2+} leak. In contrast, Sears et al. (30) found that the SR Ca\textsuperscript{2+} load was higher in NOS1\textsuperscript{−/−}, when measured at 1 Hz. In our hands, SR Ca\textsuperscript{2+} loads at low frequencies were not different between strains, but during pacing with rates more physiologic for murine myocytes, were depressed versus WT (25). Similarly, Wang and colleagues (32) demonstrated that the SR Ca\textsuperscript{2+} leak is reduced in NOS1\textsuperscript{−/−} myocytes, suggesting that NOS1-derived NO induces RyR2 leak. However, in contrast to our experiments, this group studied the SR Ca\textsuperscript{2+} leak at room temperature (22°C), whereas, we assessed the leak at 37°C. This simple detail may be responsible for the differences. We recently demonstrated that SHHF rats, a representative heart failure model that exhibits oxidative stress as a hallmark (13), share certain specific features with NOS1\textsuperscript{−/−} mice. Both models exhibit increased superoxide production associated with elevated XOR activity (25) and blunted NOS1 activity, evidenced by hyponitrosylation of RyR2 (12). Moreover, increased SR Ca\textsuperscript{2+} leak, reduced SR Ca\textsuperscript{2+} load, and depressed Ca\textsuperscript{2+} and force-frequency responses are characteristics of these models (13, 18). Unlike SHHF rats, NOS1\textsuperscript{−/−} mice are not considered a representative model for heart failure. Rather, they develop age-related cardiac hypertrophy (a pre-heart failure stage) and exhibit accelerated mortality (33, 34). Additionally, after myocardial infarction, NOS1\textsuperscript{−/−} also undergo exaggerated remodelling and higher mortality than WT controls (27). Consequently, NOS1\textsuperscript{−/−} mice are an excellent model of NO/redox imbalance that share features with a model of heart failure, the SHHF rat.

**Therapeutic Use of Hydralazine and Organic Nitrates**—Recently, Cole et al. (2) reviewed the use of HYD-ISDN as a treatment for heart failure (1, 3) highlighting the fact that the mechanism for this regimen to reduce all-cause mortality remains to be established. We show for the first time that this regimen restores disrupted NO/redox equilibrium and in so doing restores E-C coupling derangements.

The effects of HYD and NTG resemble, in part, those of XOR inhibitors. Allopurinol improves myocyte contraction without changing $\Delta$Ca\textsuperscript{2+}, enhancing contractile efficiency in both NOS1\textsuperscript{−/−} mice and heart failure models (25, 35, 36). In large animal models, allopurinol restores mechano-energetic uncoupling (15). Interestingly, XOR inhibitors did not have clinical benefits in a broad heart failure population, although it may improve outcomes in patients with high uric acid levels (37) and may benefit patients with hypertension and ischemic heart disease (38–40).

**Mechanism of Action and Pharmacology of Hydralazine—**HYD is a peripheral vasodilator used for decades to treat essential hypertension and heart failure. The mechanism of its effect on the vasculature remains unknown. It is proposed to inhibit the endoplasmic reticulum Ca\textsuperscript{2+} release in vascular smooth muscle (41, 42), although the molecular mechanism by which HYD regulates the IP\textsubscript{3} receptor (a member of the superfamily of homotetrameric ligand-gated intracellular Ca\textsuperscript{2+} channels, which includes RyR2) has not been elucidated. Because HYD increases contractility and Ca\textsuperscript{2+} transients in myocardial fibers (43), it may positively affect the unregulated RyR2 activity in NOS1\textsuperscript{−/−}.

We showed that HYD enhanced depressed NOS1\textsuperscript{−/−} CM contractility and [Ca\textsuperscript{2+}], responses resulting from NO/redox imbalance. Importantly, HYD restored the increased SR Ca\textsuperscript{2+} leakage both alone or in combination with NTG. This feature suggests a regulatory action of HYD on RyR2 gating, possibly depressing Ca\textsuperscript{2+} release in diastole and enhancing gating in systole.

Accordingly, a more efficient handling of Ca\textsuperscript{2+} would underlie the recovery of the SR Ca\textsuperscript{2+} content observed in CMs treated with HYD or HYD plus NTG. Biochemically, HYD + NTG exhibited an additive superoxide scavenging capacity at concentrations as low as 1 μM, as shown by DHE staining. This effect may be correlated with the additive acceleration in SR Ca\textsuperscript{2+} reuptake in NOS1\textsuperscript{−/−} induced by combining HYD with NTG. Peroxynitrite links superoxide production to Ca\textsuperscript{2+} reuptake. Although ONOO\textsuperscript{−} generation depends on superoxide availability, PLB phosphorylation is decreased due to ONOO\textsuperscript{−}-dependent activation of phosphatases, impairing Ca\textsuperscript{2+} decay and relaxation in NOS1\textsuperscript{−/−} (31, 44, 45). Furthermore, HYD and NTG scavenged ONOO\textsuperscript{−} in NOS1\textsuperscript{−/−} heart homogenates (data not shown). Together these findings suggest a role for scavenging of ONOO\textsuperscript{−} on the recovery of the Ca\textsuperscript{2+} reuptake rate, a key process in E-C coupling in mouse CMs (44, 46). Considerable evidence supports the hypothesis that oxidative stress induces cardiac injury by oxidizing cellular constituents, including proteins critical for E-C coupling, and largely by diminishing NO bioactivity (9), thus playing a role in heart failure pathophysiology (47). Therefore, the effect of HYD, which reduced ONOO\textsuperscript{−} in NOS1\textsuperscript{−/−} hearts (data not shown), and scavenged XOR-mediated superoxide production may impact the redox regulation of RyR2 gating and the pharmacology of the response. Importantly, our results agree with Leiro et al. (48), who suggested antioxidant properties for HYD.

**Mechanism of Action and Pharmacology of Organic Nitrates—**Janero et al. (49) showed that NTG increases cardiac nitrosylation in vivo, and this effect was potentiated by xanthine oxidase inhibition with allopurinol, highlighting the interactions between NO and XOR-derived superoxide. Importantly, decreased NOS1 in cardiac SR contributes to depressed contractile reserve in response to pacing. It has been hypothesized that NTG may restore ion channel S-nitrosylation in NOS1\textsuperscript{−/−} myocytes (24). We showed that NTG and ISDN restored the normal amplitude of calcium transients toward WT levels in NOS1\textsuperscript{−/−} cells treated with HYD. This effect would be consis-
tent with a cGMP-dependent regulation of L-type Ca\(^{2+}\) channels and/or restoration of RyR2 S-nitrosylation (33, 50) and occurs without affecting cardiac contractility, which correlates with the observed improvement in myofilament sensitivity induced by NTG in NOS1\(^{-/-}\) CMs. This improvement may also be attributed to the restoration of the S-nitrosylation/nor-

mal redox state of redox-sensitive sites on sarcomeric proteins. Thus, this responsiveness is evident across the broad range of pacing frequencies tested, confirming that organic nitrates enhance contractile efficiency. It is reasonable to speculate that the lack of NOS1 and the resulting NO/redox imbalance enhance contractile efficiency. It is reasonable to speculate that the lack of NOS1 and the resulting NO/redox imbalance impaired by NO/ redox imbalance, thereby modulating E-C coupling toward a more efficient performance.

Inhibition of NOX and XOR by NO has been previously demonstrated (26, 51–53), supporting our results. In contrast to the low concentrations required to scavenge superoxide production in isolated myocytes, the effective concentrations of NTG to inhibit the substrate-induced activity of these enzymes in cardiac homogenates exceeded achievable blood levels of this drug (10 ng/ml, 44 nm) (54).

Despite the successful use of nitrates such as NTG or ISDN in the treatment of a variety of cardiovascular diseases, nitrate tolerance during long-term use in heart failure has been described. Co-treatment with HYD has been demonstrated to prevent this effect (55) and several authors have tried to explain the mechanism of this phenomenon (56–58).

The effects of organic nitrates on SR Ca\(^{2+}\) leak appear to be uncertain and depend strongly on several variants such as the concentration or nature of the NO donor. As shown in the supplemental material, NTG exerted differential effects along the range of concentrations from 0.1 to 100 \(\mu\)M. On the other hand, ISDN reduced the SR leak in NOS1\(^{-/-}\) at very low SR Ca\(^{2+}\) contents but did not affect it at increasing Ca\(^{2+}\) loads. We speculate that this differential response may correspond to the delicate equilibrium of the nitrosylation/denitrosylation cycle involved in the regulation of RyR2 activity (50).

Combination of HYD and NTG—The effect of NTG in NOS1\(^{-/-}\) hearts may appear puzzling. At lower concentrations (0.1 and 1 \(\mu\)M), NTG did not significantly affect SL shortening, Ca\(^{2+}\) transient, SR Ca\(^{2+}\) leak or Ca\(^{2+}\) decline. It reduced the Ca\(^{2+}\) frequency response with a concomitant acceleration of Ca\(^{2+}\) decline at 10 \(\mu\)M. However, despite 10 \(\mu\)M NTG improving Ca\(^{2+}\) reuptake, there was a trend toward increasing SR Ca\(^{2+}\) leak (or at least effecting no change on SR Ca\(^{2+}\) leak; Fig. 3E), which would counteract the improvement in Ca\(^{2+}\) influx by the SR, thereby abolishing the SR Ca\(^{2+}\) content recovery. This balancing may explain why there was no change in SR Ca\(^{2+}\) load, although this was not reflected on the Ca\(^{2+}\) amplitude, which was reduced. Because Ca\(^{2+}\) influx \((I_{Ca})\) was enhanced in NOS1\(^{-/-}\) (30), the reduced RyR2 gating in the presence of 10 \(\mu\)M NTG (or ISDN) may be caused by the down-regulation of the enhanced \(I_{Ca}\) in NOS1\(^{-/-}\) by exogenous NO (as previously shown) (24). Thus, whereas hydralazine alone reduced RyR2 leak, when combined with NTG (or ISDN), down-regulation of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (via inhibition of \(I_{Ca}\)) would oppose the effect of hydralazine, thereby leading to a reduced Ca\(^{2+}\) transient amplitude. Together with the reduced leak and increased reuptake under these conditions, the contractile reserve was recovered in NOS1\(^{-/-}\). Thus, the evidence of improved EC coupling in this model using the HYD-NTG treatment would be restoration of Ca\(^{2+}\) levels toward those exhibited by WT CMs and recovery of the increasing Ca\(^{2+}\) frequency response and myofilament responsiveness to Ca\(^{2+}\), where NTG played a key role.

Both HYD and NTG scavenged superoxide but were more potent in combination. However, we must consider that the chemical reactions of each molecule with superoxide yield different products. Although HYD can effectively scavenge the radical, the combination of NO and superoxide yields ONOO\(^{-}\), a reactive nitrogen species even more harmful than superoxide. In this sense, we speculate that ONOO\(^{-}\) may target RyR2 in a similar manner as superoxide. Therefore, the SR Ca\(^{2+}\) leak was still elevated in the presence of NTG. Fig. 4F showed that despite 10 \(\mu\)M NTG inducing a trend toward an increased leak in NOS1\(^{-/-}\), 100 \(\mu\)M NTG restored it to baseline. This observation suggests that higher concentrations of NTG not only scavenge superoxide but also the surplus would counteract the deficiency of NO in NOS1\(^{-/-}\) and is perhaps the mechanism of the combination of NTG and HYD.

Effects in SHHF Rats—Although this study is focused on NOS1\(^{-/-}\) myocytes as a model of NO/redox imbalance, we also tested the effect of these drugs alone or in combination on failing CMs from SHHF rats. The results obtained in SHHF CMs correlate with those in NOS1\(^{-/-}\) mice. HYD alone or in combination with NTG improved the Ca\(^{2+}\)-frequency response and FFR. NTG alone induced a trend to increase contractility at 4 Hz with no changes in \(\Delta\text{Ca}^{2+}\), suggesting an improvement in myofilament sensitivity. These results allow us to speculate that our approach may be extrapolated to heart failure in humans.

Conclusion—Our data show novel effects of HYD and NTG. In combination they quench superoxide in isolated cells, concomitantly with physiologic functional effects, including improved myocardial contractility and Ca\(^{2+}\) cycling. At higher in vitro concentrations, this combination scavenges superoxide and peroxynitrite production from two major ROS generating enzymatic systems, NOX and XOR. This improvement in myocyte performance, normalization of SR Ca\(^{2+}\) leak, regulation of RyR2 gating, and myofilament sensitivity by NTG may be associated in part with normalizing NO/redox equilibrium. Thus, these drugs in combination exert direct myocardial effects that provide a mechanistic basis for the favorable functional and structural responses in the treatment of congestive heart failure.

Acknowledgments—We thank Drs. Ivonne Schulman and Wayne Balkan for critical reading of the manuscript.

REFERENCES

1. Taylor, A. L., Ziesche, S., Yancy, C., Carson, P., D’Agostino, R., Jr., Ferdin-and, K., Taylor, M., Adams, K., Sabolinski, M., Worcel, M., and Cohn,
Hydralazine and Nitroglycerin Restore Impaired E-CC

J. N. (2004) Combination of isosorbide dinitrate and hydralazine in blacks with heart failure. *N. Engl. J. Med.* **351**, 2049–2057

2. Cole, R. T., Kalogeropoulos, A. P., Georgioupolou, V. V., Gheorghiade, M., Quyyumi, A., Yancy, C., and Butler, J. (2011) Hydralazine and isosorbide dinitrate in heart failure. Historical perspective, mechanisms, and future directions. *Circulation* **123**, 2414–2422

3. Taylor, A. L., Sabolinski, M. L., Tam, S. W., Ziesche, S., Ghali, J. K., Archambault, W. T., Worcel, M., and Cohn, J. N. (2012) Effect of fixed-dose combined isosorbide dinitrate/hydralazine in elderly patients in the African-American heart failure trial. *J. Card. Fail.* **18**, 600–606

4. Antoons, G., Vangeluwe, P., Volders, P. G., Bito, V., Holemans, P., Ceci, M., Wuytack, F., Caroni, P., Mubagawa, K., and Sidip, K. R. (2006) Increased phospholamban phosphorylation limits the force-frequency response in the MLP−/− mouse with heart failure. *J. Mol. Cell Cardiol.* **40**, 350–360

5. Lamberts, R. R., Hamdani, N., Soekhoe, T. W., Boontje, N. M., Zaremba, R., Walker, L. A., de Tombe, P. P., van der Velden, J., and Stienen, G. J. (2007) Frequency-dependent myofibril Ca2+ desensitization in failing rat myocardium. *J. Physiol.* **582**, 695–709

6. Rossman, E. I., Petre, R. E., Chaudhary, K. W., Piacentino, V., Janssen, A. D., Li, D., Berkowitz, D. E., and Hare, J. M. (2004) Neuronal nitric-oxide synthase negatively regulates myocardial oxygen consumption and increases myocardial oxygen consumption by nitric oxide derived from endothelial nitric oxide synthase. *Circ. Res.* **96**, 355–362

7. O’Rourke, B., Kass, D. A., Tomasselli, G. F., Kääb, S., Tunin, R., and Marbán, E. (1999) Mechanisms of altered excitation-contraction coupling in canine tachycardia-induced heart failure. I: experimental studies. *Circ. Res.* **84**, 562–570

8. Yano, M., Ikeda, Y., and Matsuzaki, M. (2005) Altered intracellular Ca2+ handling in heart failure. *J. Clin. Invest.* **115**, 556–564

9. Hare, J. M. (2004) Nitroso-redox balance in the cardiovascular system. *N. Engl. J. Med.* **351**, 2112–2114

10. Hare, J. M., and Stampler, J. S. (2005) NO/redox disequilibrium in the failing heart and cardiovascular system. *J. Clin. Invest.* **115**, 509–517

11. Terentyev, D., Gyo¨rke, I., Belevych, A. E., Terentyeva, R., Sridhar, A., Nishijima, Y., de Blanco, E. C., Khanna, S., Sen, C. K., Cardounel, A. J., N. Engl. J. Med. **334**, 1322–1329

12. Gonzalez, D. R., Beigi, F., Treuer, A. V., and Hare, J. M. (2007) Deficient hydralazine and nitroglycerin Restore Impaired E-CC

13. Rossman, E. I., Petre, R. E., Chaudhary, K. W., Piacentino, V., Janssen, A. D., Li, D., Berkowitz, D. E., and Hare, J. M. (2004) Neuronal nitric-oxide synthase negatively regulates myocardial oxygen consumption and increases myocardial oxygen consumption by nitric oxide derived from endothelial nitric oxide synthase. *Circ. Res.* **96**, 355–362

14. Saraiya, R. M., Minhas, K. M., Raju, S. V., Barouch, L. A., Pitz, E., Schuleri, K. H., Vila-Petroff, M. G., Younes, A., Egan, J., Kabuki, N. A., Neumann, C. A., Burnett, A. L., O’Rourke, B., Dizdaroglu, M., Marba´n, E., and Hare, J. M. (2004) S-Nitroso-redox equilibrium during caffeine contractures in cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 15944–15948

15. Khan, S. A., Lee, K., Minhas, K. M., Gonzalez, D. R., Raju, S. V., Tejani, A. D., Li, D., Gao, D., and Hare, J. M. (2005) Regulation of myocardial contractility and calcium cycling. *Circulation* **120**, 1455–1459

16. Senzaki, H., Kass, D. A., Marbán, E., and Hare, J. M. (2004) Role of ryanodine receptor on myocardial contractility. *J. Mol. Cell Cardiol.* **36**, 33–42

17. Yano, M., Ikeda, Y., and Matsuzaki, M. (2005) Altered intracellular Ca2+ signaling in failing heart. *J. Mol. Cell Cardiol.* **39**, 582–597

18. Amado, L. C., Saliaris, A. P., Raju, S. V., Lehrke, S., St John, M. E., Senzaki, H., Kass, D. A., Marbán, E., and Hare, J. M. (2004) Neuronal nitric-oxide synthase negatively regulates myocardial calcium handling. *Circulation* **112**, 3415–3422

19. Layland, J., Stith, M., and Shah, A. M. (2002) Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes. *J. Physiol.* **540**, 457–467

20. Chen, C. A., Wang, T. Y., Varadarajah, S., Reyes, L. A., Hemann, C., Talukder, M. A., Chen, Y. R., Druhan, L. J., and Zweier, J. L. (2010) S-Glutathio-nylation uncouples eNOS and regulates its cellular and vascular function. *Nature* **468**, 1115–1118

21. Sears, C. E., Bryant, S. M., Ashley, E. A., Dygate, C. A., Ravkovic, S., Wallis, H. L., Neubauer, S., Terrar, D. A., and Casadei, B. (2003) Cardiac neuronal nitric-oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ. Res.* **92**, e52–e59

22. Wang, H., Kohr, M. J., Tranyham, C. J., Wheeler, D. G., Janssen, P. M., and Ziolo, M. T. (2008) Neuronal nitric-oxide synthase signaling within cardiac myocytes targets phosphorylamban. *Am. J. Physiol. Cell Physiol.* **294**, C1566–C1575

23. Barouch, L. A., Cappola, T. P., Harrison, R. W., Skaf, M. W., Sokol, G. O., Koebeisi, Z. A., Marbán, E., and Hare, J. M. (2004) Role of ryanodine receptor on myocardial contractility. *J. Mol. Cell Cardiol.* **35**, 637–644

24. Amado, L. C., Saliaris, A. P., Raju, S. V., Lehrke, S., St John, M. E., Xie, J., Stewart, G., Pitton, T., Minhas, K. M., Brawn, J., and Hare, J. M. (2005) Xanthine oxidase inhibition ameliorates cardiovascular dysfunction in dogs with pacing-induced heart failure. *J. Mol. Cell Cardiol.* **39**, 531–536

25. Ekuland, U. E., Harrison, R. W., Shokek, O., Thakkar, R. N., Tunin, R. S., Senzaki, H., Kass, D. A., Marbán, E., and Hare, J. M. (1999) Intravenous allopurinol decreases myocardial oxygen consumption and increases me-
Hydralazine and Nitroglycerin Restore Impaired E-CC

J. Clin. Invest. 115, 500–508

Leiro, J. M., Alvarez, E., Arranz, J. A., Cano, E., and Orallo, F. (2004) Antioxidant activity and inhibitory effects of hydralazine on inducible NOS/COX-2 gene and protein expression in rat peritoneal macrophages. Int. Immunopharmacol. 4, 163–177

Janero, D. R., Bryan, N. S., Sajo, F., Dhawan, V., Schwalb, D. J., Warren, M. C., and Feelsch, M. (2004) Differential nitrosylation of blood and tissue constituents during glyceryl trinitrate biotransformation in vivo. Proc. Natl. Acad. Sci. U.S.A. 101, 16958–16963

Beigi, F., Gonzalez, D. R., Minhas, K. M., Sun, Q. A., Foster, M. W., Khan, S. A., Treuer, A. V., Dulce, R. A., Harrison, R. W., Saraiva, R. M., Premer, C., Schulman, I. H., Stamler, J. S., and Hare, J. M. (2012) Dynamic denitrosylation via S-nitrosogluthathione reductase regulates cardiovascular function. Proc. Natl. Acad. Sci. U.S.A. 109, 4314–4319

Harrison, C. B., Drummond, G. R., Sobey, C. G., and Selemidis, S. (2010) Evidence that nitric oxide inhibits vascular inflammation and superoxide production via a p47^{phox}-dependent mechanism in mice. Clin. Exp. Pharmacol. Physiol. 37, 429–434

Shin yashiki, M., Pan, C. J., Lopez, B. E., and Fukuto, J. M. (2004) Inhibition of the yeast metal reductase heme protein fre1 by nitric oxide (NO). A model for inhibition of NADPH oxidase by NO. Free Radic. Biol. Med. 37, 713–723

Cote, C. G., Yu, F. S., Zulueta, J. I., Vosatka, R. J., and Hassoun, P. M. (1996) Regulation of intracellular xanthine oxidase by endothelial-derived nitric oxide. Am. J. Physiol. 271, L869-L874

Thadani, U., and Whitsett, T. (1988) Relationship of pharmacokinetic and pharmacodynamic properties of the organic nitrates. Clin. Pharmacokinet. 15, 32–43

Gogia, H., Mehra, A., Parikh, S., Raman, M., Ajit-Uppal, J., Johnson, J. V., and Elkayam, U. (1995) Prevention of tolerance to hemodynamic effects of nitrates with concomitant use of hydralazine in patients with chronic heart failure. J. Am. Coll. Cardiol. 26, 1575–1580

Daiber, A., Mül sch, A., Hink, U., Mollnau, H., Warnholtz, A., Oelze, M., and Mü nzel, T. (2005) The oxidative stress concept of nitrate tolerance and the antioxidant properties of hydralazine. Ann. J. Cardiol. 96, 25i-36i

Mü nzel, T., Daiber, A., and Mül sch, A. (2005) Explaining the phenomenon of nitrate tolerance. Circ. Res. 97, 618–628

Bauer, J. A., and Fung, H. L. (1991) Concurrent hydralazine administration prevents nitroglycerin-induced hemodynamic tolerance in experimental heart failure. Circulation 84, 35–39

Mechanical efficiency in dogs with pacing-induced heart failure. Circ. Res. 85, 437–445

Hare, J. M., Mangal, B., Brown, J., Fisher, C., Jr., Freudenberger, R., Colucci, W. S., Mann, D. L., Liu, P., Givertz, M. M., and Schwarz, R. P. (2008) Impact of oxygenprin in patients with symptomatic heart failure. Results of the OPT-CHF study. J. Am. Coll. Cardiol. 51, 2301–2309

Bergamini, C., Cicoira, M., Rossi, A., and Vassanelli, C. (2009) Oxidative stress and hyperuricaemia. Pathophysiology, clinical relevance, and therapeutic implications in chronic heart failure. Eur. J. Heart Fail. 11, 444–452

Noman, A., Ang, D. S., Ogston, S., Lang, C. C., and Struthers, A. D. (2010) Effect of high-dose allopurinol on exercise in patients with chronic stable angina. A randomised, placebo controlled crossover trial. Lancet 375, 2161–2167

Ziolo, M. T. (2008) Targeting of phospholamban by peroxynitrite decreases S-nitrosoglutathione reductase activity and inhibitory effects of hydralazine on inducible COX-2 gene and protein expression in rat peritoneal macrophages. Int. Immunopharmacol. 4, 163–177

Janero, D. R., Bryan, N. S., Sajo, F., Dhawan, V., Schwalb, D. J., Warren, M. C., and Feelsch, M. (2004) Differential nitrosylation of blood and tissue constituents during glyceryl trinitrate biotransformation in vivo. Proc. Natl. Acad. Sci. U.S.A. 101, 16958–16963

Beigi, F., Gonzalez, D. R., Minhas, K. M., Sun, Q. A., Foster, M. W., Khan, S. A., Treuer, A. V., Dulce, R. A., Harrison, R. W., Saraiva, R. M., Premer, C., Schulman, I. H., Stamler, J. S., and Hare, J. M. (2012) Dynamic denitrosylation via S-nitrosogluthathione reductase regulates cardiovascular function. Proc. Natl. Acad. Sci. U.S.A. 109, 4314–4319

Harrison, C. B., Drummond, G. R., Sobey, C. G., and Selemidis, S. (2010) Evidence that nitric oxide inhibits vascular inflammation and superoxide production via a p47^{phox}-dependent mechanism in mice. Clin. Exp. Pharmacol. Physiol. 37, 429–434

Shin yashiki, M., Pan, C. J., Lopez, B. E., and Fukuto, J. M. (2004) Inhibition of the yeast metal reductase heme protein fre1 by nitric oxide (NO). A model for inhibition of NADPH oxidase by NO. Free Radic. Biol. Med. 37, 713–723

Cote, C. G., Yu, F. S., Zulueta, J. I., Vosatka, R. J., and Hassoun, P. M. (1996) Regulation of intracellular xanthine oxidase by endothelial-derived nitric oxide. Am. J. Physiol. 271, L869-L874

Thadani, U., and Whitsett, T. (1988) Relationship of pharmacokinetic and pharmacodynamic properties of the organic nitrates. Clin. Pharmacokinet. 15, 32–43

Gogia, H., Mehra, A., Parikh, S., Raman, M., Ajit-Uppal, J., Johnson, J. V., and Elkayam, U. (1995) Prevention of tolerance to hemodynamic effects of nitrates with concomitant use of hydralazine in patients with chronic heart failure. J. Am. Coll. Cardiol. 26, 1575–1580

Daiber, A., Mül sch, A., Hink, U., Mollnau, H., Warnholtz, A., Oelze, M., and Mü nzel, T. (2005) The oxidative stress concept of nitrate tolerance and the antioxidant properties of hydralazine. Ann. J. Cardiol. 96, 25i-36i

Mü nzel, T., Daiber, A., and Mül sch, A. (2005) Explaining the phenomenon of nitrate tolerance. Circ. Res. 97, 618–628

Bauer, J. A., and Fung, H. L. (1991) Concurrent hydralazine administration prevents nitroglycerin-induced hemodynamic tolerance in experimental heart failure. Circulation 84, 35–39