Broad Suppression of NADPH Oxidase Activity Exacerbates Ischemia/Reperfusion Injury Through Inadvertent Downregulation of Hypoxia-inducible Factor-1α and Uptregulation of Peroxisome Proliferator–activated Receptor-α

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Rationale: NADPH oxidase (Nox) 2 and Nox4 are major components of the Nox family which purposefully produce reactive oxidative species, namely O$_2^-$ and H$_2$O$_2$, in the heart. The isoform-specific contribution of Nox2 and Nox4 to ischemia/reperfusion (I/R) injury is poorly understood.

Objective: We investigated the role of Nox2 and Nox4 in mediating oxidative stress and myocardial injury during I/R using loss-of-function mouse models.

Methods and Results: Systemic (s) Nox2 knockout (KO), sNox4 KO, and cardiac-specific (c) Nox4 KO mice were subjected to I/R (30 minutes/24 hours, respectively). Both myocardial infarct size/area at risk and O$_2^-$ production were lower in sNox2 KO, sNox4 KO, and cNox4 KO than in wild-type mice. Unexpectedly, however, the myocardial infarct size/area at risk was greater, despite less O$_2^-$ production, in sNox2 KO+cNox4 KO (double-KO) mice and transgenic mice (Tg) with cardiac-specific expression of dominant-negative Nox, which suppresses both Nox2 and Nox4, than in wild-type or single KO mice. Hypoxia-inducible factor-1α was downregulated whereas peroxisome proliferator–activated receptor-α was upregulated in Tg-dominant-negative Nox mice. A cross with mice deficient in prolyl hydroxylase 2, which hydroxylates hypoxia-inducible factor-1α, rescued the I/R injury and prevented upregulation of peroxisome proliferator–activated receptor-α in Tg-dominant–negative Nox mice. A cross with peroxisome proliferator–activated receptor-α KO mice also attenuated the injury in Tg-dominant–negative Nox mice.

Conclusions: Both Nox2 and Nox4 contribute to the increase in reactive oxidative species and injury by I/R. However, low levels of reactive oxidative species produced by either Nox2 or Nox4 regulate hypoxia-inducible factor-1α and peroxisome proliferator–activated receptor-α, thereby protecting the heart against I/R, suggesting that Noxs also act as a physiological sensor for myocardial adaptation. (Circ Res. 2013;112:1135-1149.)

Key Words: cell survival ■ free radicals ■ ischemia/reperfusion ■ lipid metabolism ■ oxidative stress ■ reactive oxygen species

Res oxy species (ROS), such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), play an important role in regulating the cell growth and death of cardiac myocytes.1

The family of NADPH oxidases (Noxs) represents the only known enzyme system whose sole biological function is to purposefully produce O$_2^-$ or H$_2$O$_2$ by transferring electrons from NADPH to molecular oxygen. Nox2 and Nox4 are the major Nox isoforms in the heart.1 Nox2 is involved in cardiac remodeling after myocardial infarction,4 whereas Nox4 is a critical mediator of mitochondrial oxidative stress and mitochondrial dysfunction during heart failure.3

Nox2 and Nox4 are localized at distinct subcellular locations and seem to have distinct roles in the heart.3 Although it has been speculated that Nox isoforms play an important role in mediating increases in oxidative stress during ischemia/reperfusion (I/R), the role of Nox4 in mediating I/R injury has not been evaluated with genetically altered mouse models. The role of Nox2 in mediating I/R injury has been investigated...
Nonstandard Abbreviations and Acronyms

AAR area at risk
Ad adenovirus
DN dominant-negative
HIF hypoxia-inducible factor
LV leftventricle
Nox NADPH oxidase
NTg nontransgenic
PHD prollyhydroxylase
PPAR peroxisome proliferator—activated receptor
ROS reactive oxygen species
Tg transgenic

indirectly with p47phox knockout (KO) mice,6 but has only been examined directly with Nox2 KO mice in the context of preconditioning.7 Direct examination of the roles of Nox isoforms in mediating I/R injury would provide important clues for the development of an effective intervention to inhibit myocardial I/R injury.

Hypoxia-inducible factors (HIFs) are master regulators of hypoxia-regulated gene expression8 that mediate adaptive responses to low oxygen (O2) levels and oxidative stress. HIF-1 transcriptionally activates genes associated with angiogenesis, energy metabolism, nutrient transport, cell cycle, and cell migration.8 Activation of glycolytic genes by HIF-1 is considered critical for metabolic adaptation to hypoxia through increased conversion of glucose to pyruvate and, subsequently, lactate. A hypoxia-induced metabolic switch shunts glucose metabolites from mitochondria to glycolysis to maintain ATP production and prevent toxic ROS production.9 Therefore, HIFs play a protective role in I/R injury through regulation of the cardiac metabolism.10 Although it has been reported that ROS activate HIF-1α,11,12 the role of Nox in regulating the expression of HIF-1 during I/R remains to be elucidated.

Thus, the major goal of this study was to elucidate the functional roles of Nox2 and Nox4 during I/R in the heart. To this end, we investigated the effects of I/R in the context of Nox2 and Nox4 loss-of-function mouse models. Our results suggest that both Nox2 and Nox4 play critical roles in mediating ROS production and myocardial injury in response to I/R. Interestingly, a low level of ROS produced by either Nox2 or Nox4 is required for the heart to activate adaptive mechanisms, including regulation of HIF-1α and peroxisome proliferator–activated receptor α (PPARα). Thus, Nox2 and Nox4 both have pathological and adaptive roles in the heart during myocardial I/R.

Methods

An expanded Methods section is available in the Online Data Supplement.

Genetically Altered Mouse Models

TG-Nox4 and TG-dominant–negative Nox (DN-Nox) mice were generated with the use of α-myosin heavy-chain promoter13 on a C57BL/6J background. The baseline cardiac phenotypes of TG-Nox4 and TG-DN-Nox mice have been described.3 Nox4 flox/flox mice were generated as described previously.3 Cardiac-specific Nox4 KO mice were generated by crossing Nox4 flox/flox mice having a C57BL/6J background with α-myosin heavy-chain–Cre transgenic mice (alpha myosin heavy chain–Cre recombinase, courtesy of Dr M. Schneider, Imperial College, London, United Kingdom). Systemic Nox4 KO mice were generated by crossing Nox4 flox/flox mice with a C57BL/6J background with cytomegalovirus promoter–driven Cre mice purchased from Jackson Laboratory. Systemic Nox2 KO mice were also purchased from Jackson Laboratory. Cardiac-specific prolly hydroxylase (PHD) 2 KO mice were generated by crossing PHD2 flox/flox mice with a C57BL/6J background with alpha myosin heavy chain–Cre recombinase mice. We generated a genetic cross between Tg-DN-Nox and cardiac-specific PHD2−/− mice.11 We also generated a genetic cross between Tg-DN-Nox and PPARα−/− mice. We used only male mice in these experiments. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Statistical Analysis

Data are expressed as means±SEM. The between-group comparisons of means were performed by 1-way ANOVA, followed by t tests. The Bonferroni’s correction was done for multiple comparisons of means. P<0.05 was considered to be statistically significant.

Results

Individual Downregulation of Nox2 or Nox4 Attenuates I/R Injury in the Heart

We investigated the involvement of the different Nox isoforms in I/R injury in the heart. We first characterized O2− production in the heart after I/R, using superoxide dismutase (SOD)–inhibitable lucigenin chemiluminescence. Wild-type (WT) mice were subjected to 30 minutes of ischemia and 24 hours of reperfusion. O2− production from the heart homogenates at baseline, 6, and 24 hours after reperfusion was significantly higher than that at baseline (Figure 1A). To investigate the role that Nox4 in cardiomyocytes plays in mediating I/R injury, we applied I/R to cardiac-specific Nox4 KO (cNox4 KO) and WT mice. Nox4 expression was increased by I/R in WT mice, but was decreased in cNox4 KO mice at baseline and after I/R (Figure 1B). Downregulation of Nox4 led to a significant decrease in the infarct size/area at risk (AAR) after I/R, as evaluated with triphenyltetrazolium chloride and Alcian blue staining, compared with that in WT mice (Figure 1C). We also examined the role of Nox2 in the heart during I/R injury by subjecting systemic Nox2 KO mice to I/R. I/R increased Nox2 expression in WT mice, an effect that was abolished in Nox2 KO mice (Figure 1D). Knockdown of Nox2 also led to a significant decrease in the infarct size after I/R compared with that in WT mice (Figure 1E). Apoptotic cell death in the AAR, as evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling staining, was significantly decreased in both cNox4 KO and Nox2 KO mice compared with that in WT mice (Figure 1F). Consistent with terminal deoxynucleotidyl transferase dUTP nick end labeling staining, cleaved caspase-3 was decreased in both cNox4 KO and Nox2 KO mice compared with that in WT mice (Figure 1G). These results suggest that individual downregulation of either Nox2 or Nox4 reduces I/R injury, accompanied by decreases in apoptosis in the myocardium.

To investigate the mechanism by which apoptosis is attenuated in cNox4 KO and Nox2 KO mice, we evaluated mitochondrial function in the heart. Mitochondria were challenged with calcium overload and the rate of mitochondrial swelling was evaluated by light scattering, where decreases in the absorbance reflect passive swelling of the mitochondrial matrix. I/R-induced decreases in absorbance were significantly inhibited in cNox4 KO and Nox2 KO mice compared with...
that in WT mice (Figure 1H), suggesting that both Nox4 and Nox2 contribute to I/R-induced mitochondrial permeability transition pore opening.

The expression of cleaved caspase-12, a mediator of endoplasmic reticulum stress-induced apoptosis, in the ischemic area was significantly increased by I/R in WT mice. This increase was reduced in both cNox4 KO and Nox2 KO mice (Figure 1I). These data indicate that both Nox4 and Nox2 are involved in endoplasmic reticulum stress during I/R injury.

Because Nox4 is expressed not only in myocytes but also in nonmyocytes, to investigate the role of Nox4 in all cell types in the heart during I/R, we generated Nox4 systemic KO (sNox4 KO) mice by crossing Nox4 flox/flox mice with cytomegalovirus-Cre mice on a C57 background. The expression of Nox4 in the left ventricle (LV) was completely abolished in sNox4 KO mice (Online Figure IA). sNox4 KO mice showed a decrease in I/R injury compared with WT mice (Online Figure IB). The extent of I/R injury in sNox4 KO mice was similar to that in cNox4 KO mice (Online Figure IB) and systemic Nox2 KO mice (not shown). We also evaluated the O$_2^-$ production in the ischemic area. The extent of O$_2^-$ production in sNox4 KO was similar to that in cNox4 KO mice (Online Figure IC).

To elucidate the role of inflammatory responses in mediating I/R injury, the extent of cell infiltration was evaluated with hematoxylin-eosin staining. Although I/R increased the total cell density in the myocardium in WT, cNox4 KO, and Nox2 KO mice, there was no significant difference among the 3 groups (Online Figure IIA and IIB).

**Overexpression of DN-Nox Causes Broad Suppression of Nox Activity in the Heart**

To further investigate the role of Nox in the heart, we next examined transgenic mice with cardiac-specific overexpression of DN-Nox.$^1$ Although we initially named these mice Tg-DN-Nox4 mice, we have recently redesignated them as Tg-DN-Nox because we have discovered that DN-Nox inhibits other Nox isoforms besides Nox4 as well. For example, when cardiomyocytes were transduced with adenovirus (Ad)-Nox2 and Ad-DN-Nox, DN-Nox efficiently inhibited Nox2-induced O$_2^-$ production, evaluated as the SOD-inhibitable component of lucigenin chemiluminescence, in cardiomyocytes (Figure 2A). In addition, in sNox4 KO mice, O$_2^-$ production in the LV, as evaluated with dihydroethidium staining, was significantly decreased after injection of Ad-DN-Nox (Figure 2B). Moreover, O$_2^-$ production, evaluated as the SOD-inhibitable component of lucigenin chemiluminescence, in the mitochondrial fraction, where Nox4 is mainly expressed, and in the microsomal fraction, where Nox2 is mainly expressed, was suppressed in myocytes transduced with Ad-DN-Nox (Figure 2C and 2D). These results indicate that DN-Nox inhibits both Nox4 and Nox2 in cardiomyocytes.

To elucidate the mechanism of suppression of Nox isoforms by DN-Nox, we evaluated the interaction between DN-Nox and p22$^{phox}$ in myocytes. p22$^{phox}$ was effectively immunoprecipitated with anti-human influenza hemagglutinin antibody from DN-Nox-human influenza hemagglutinin-expressing cardiomyocytes. Importantly, the p22$^{phox}$ level in the supernatant after immunoprecipitation with anti-human influenza hemagglutinin agarose was markedly decreased in myocytes transduced with Ad-DN-Nox-human influenza hemagglutinin (Figure 2E), suggesting that DN-Nox effectively sequestrates p22$^{phox}$. In addition, we evaluated the NADP/NADPH ratio in the heart. The NADP/NADPH ratio of the heart homogenates was in the order WT>cNox4 KO, Nox2 KO>Tg-DN-Nox (Figure 2F), suggesting that the consumption of NADPH is reduced markedly in myocytes in the presence of DN-Nox. Taken together, the data suggest that DN-Nox broadly suppresses Nox isoforms by sequestrating p22$^{phox}$ from endogenous Noxs.

**Tg-DN-Nox and Double-Knockout of Nox2 and Nox4 Exhibit Exacerbated I/R Injury in the Heart**

To further understand the role of ROS produced by Nox isoforms, we conducted I/R experiments in transgenic mice with cardiac-specific overexpression of Nox4 (Tg-Nox4) and Tg-DN-Nox mice. Interestingly, the infarct size/AAR in Tg-Nox4 was not significantly different than in nontransgenic (NTg) mice (Online Figure IIIA). Apoptosis in the ischemic area, as evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling staining, was also similar in Tg-Nox4 and WT mice (Online Figure IIIB), despite an increase in O$_2^-$ production (Online Figure IIIC). However, unexpectedly, Tg-DN-Nox exhibited a significant increase in the infarct size/AAR (Figure 3A), which was accompanied by increases in apoptosis, as evaluated by the number of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive myocytes and the level of cleaved caspase-3 in the AAR after I/R (Figure 3B and 3C). Tg-DN-Nox also exhibited an increase in necrosis compared with NTg mice as evaluated with hairpin 2 staining (Online Figure IV).

We also evaluated cardiac function in Tg-DN-Nox mice using the Langendorff system. Although there was no difference in left ventricular pressure or dP/dt$_{max}$, indexes of cardiac systolic function, or in dP/dt$_{min}$, an index of cardiac diastolic function, between NTg and Tg-DN-Nox at baseline, Tg-DN-Nox showed a dramatic decrease in these parameters after I/R in glucose-rich substrates (Figure 3D, Online Figure V). LV end-diastolic pressure was significantly elevated in Tg-DN-Nox mouse hearts compared with NTg hearts after I/R (Figure 3D). Although heart rate decreased rapidly in Tg-DN-Nox mice after reperfusion, it recovered within 30 minutes (Online Figure V). Taken together, these data show that suppression of multiple Nox isoforms with DN-Nox exacerbated I/R injury and suppressed the recovery of LV function after reperfusion.

Because Nox2 and Nox4 are the major Noxs in the heart, the Tg-DN-Nox data raised the possibility that combined downregulation of Nox2 and Nox4 may exacerbate I/R injury. To address these issues, we generated a double-KO (DKO) of Nox2 and Nox4 by crossing cNox4 KO mice with systemic Nox2 KO mice. We confirmed that the expression levels of both Nox2 and Nox4 in the LV were decreased in DKO mice compared with that in WT mice (Online Figure VIA). There was no compensatory upregulation of Nox1 or Nox3 in DKO mice. The expression levels of other sources of ROS, such as nitric oxide synthase and xanthine oxidase, and the volume of mitochondria, as determined by the level of mitochondrial complex IV (COX IV), in Tg-DN-Nox and DKO mice were not significantly different from those in WT mice (Online Figure VIA). The expression levels of the antioxidants SOD1, Trx1, and catalase were also not altered in Tg-DN-Nox or DKO mice. Only SOD2, an enzyme that dismutates O$_2^-$ in
mitochondria, was decreased in Tg-DN-Nox and DKO mice compared with that in WT mice (Online Figure VIIB), possibly because of the decreased production of O$_2^\cdot$-$^{-}$. Interestingly, the antioxidant capacity was significantly increased in Tg-DN-Nox and DKO mice (Online Figure VIC). In addition, the NADP/NADPH ratio was significantly lower in DKO mouse hearts than in control hearts (Online Figure VID), consistent with the results obtained in Tg-DN-Nox mouse hearts. These data indicate that Tg-DN-Nox and DKO mice exhibit a similar phenotype regarding the redox state in the heart. Echocardiographically determined fractional shortening, LV chamber size, and wall thickness in DKO mice were all similar to those in WT and Tg-DN-Nox mice at baseline (Online Figure VII). Importantly, DKO mice exhibited a significantly greater infarct size/AAR (Figure 3E) and a greater increase in cardiomyocyte apoptosis in response to I/R (Figure 3F) than WT mice.

**Tg-DN-Nox and DKO Mice Exhibit Marked Downregulation of ROS in the Heart**

To elucidate the mechanism of exacerbation of I/R injury in Tg-DN-Nox and DKO mice, we evaluated ROS in the heart. Myocardial O$_2^\cdot$-$^{-}$ production at baseline, as evaluated with dihydroethidium staining, was lower in Tg-DN-Nox and DKO mice than in WT, cNox4 KO, and Nox2 KO mice (Online Figure VIA). In addition, O$_2^\cdot$-$^{-}$ production was significantly lower in Tg-DN-Nox and DKO than in WT, cNox4 KO, and Nox2 KO mice after I/R (Figure 4A and 4B). Consistent with these results, the O$_2^\cdot$-$^{-}$ and H$_2$O$_2$-producing activity of cardiac homogenates after I/R, evaluated with lucigenin chemiluminescence assays and Amplex Red assays, was in the order WT>cNox4 KO, Nox2 KO>Tg-DN-Nox, DKO (Figure 4C, Online Figure VIAIA). cNox4 KO and Tg-DN-Nox showed an equivalent decrease in O$_2^\cdot$-$^{-}$-producing activity in the mitochondrial fraction after I/R (Online Figure VIIIC). These data indicate that DN-Nox inhibits not only Nox4 but also other Nox isoforms.

We also evaluated H$_2$O$_2$ production and malondialdehyde+4-hydroxyalkenals content in LV blocks from NTg and Tg-DN-Nox mice at baseline, 30 minutes, and 24 hours after reperfusion, using the Amplex Red assay and lipid peroxidation-586 assay, respectively. H$_2$O$_2$ production was significantly lower in Tg-DN-Nox than in NTg mice at all time points (Figure 4D). Malondialdehyde+4-hydroxyalkenals content was also lower in Tg-DN-Nox than in NTg mice after I/R (Figure 4E). Taken together, these results suggest that both Nox2 and Nox4 contribute to oxidative stress in the heart at baseline and in response to I/R, whereas I/R-induced increases in oxidative stress were markedly suppressed in Tg-DN-Nox and DKO hearts after I/R.

**Marked Downregulation of Oxidative Stress Increases Cardiomyocyte Death in Response to Hypoxia/Reoxygenation In Vitro**

To analyze the role of ROS in mediating the cell survival response to I/R at the cellular level, cultured neonatal rat ventricular myocytes were subjected to hypoxia and reoxygenation. O$_2^\cdot$-$^{-}$ production was evaluated after 12 hours of hypoxia followed by 24 hours of reoxygenation. Pretreatment with either Ad-sh-Nox4 or Ad-sh-Nox2 decreased O$_2^\cdot$-$^{-}$ production in myocytes (Figure 5A). Pretreatment with Ad-DN-Nox or combined Ad-sh-Nox4 and Ad-sh-Nox2 markedly decreased O$_2^\cdot$-$^{-}$ production in myocytes even further (Figure 5A). Cell survival, as evaluated by Cell Titer-Blue assays, was significantly increased in myocytes pretreated with Ad-sh-Nox4 or Ad-sh-Nox2 compared with that in those pretreated with control Ad (Figure 5B and 5C). In contrast, pretreatment with Ad-DN-Nox or Ad-sh-Nox4 plus Ad-sh-Nox2 decreased cell survival in myocytes compared with pretreatment with control adenovirus (Figure 5D and 5E). To further elucidate the mechanism of cell survival, we evaluated mitochondrial permeability transition pore opening, an indicator of mitochondrial membrane potential, with 5,5$,6,6$-tetrachloro-1,1$,3,3$-tetraethylbenzimidazolylcarbocyanineiodide staining. Although pretreatment with Ad-sh-Nox2 or Ad-sh-Nox4 prevented hypoxia/reoxygenation-induced depolarization of the mitochondrial membrane potential, pretreatment with Ad-DN-Nox or Ad-sh-Nox4 plus Ad-sh-Nox2 failed to do so (Online Figure IXA). The mRNA level of PPAR gamma coactivator-1 alpha and the protein level of mitochondrial transcription factor were preserved by downregulation of Nox4 or Nox2. However, combined downregulation of the Nox isoforms showed further reduction of those levels (Online Figure IXB and IXC). These results indicate that, whereas mild downregulation of oxidative stress is protective, marked downregulation of oxidative stress increases cardiomyocyte death in response to hypoxia/reoxygenation in vitro through stimulation of mitochondrial permeability transition pore opening and suppression of mitochondrial biogenesis.

**Normalization of HIF-1alpha Expression Rescues the Increased I/R Injury in Tg-DN-Nox Mice**

The Langendorff experiments and in vitro experiments indicate that the altered cell metabolism may be responsible for the enhancement of I/R injury in the presence of marked suppression of ROS. HIF-1alpha plays an important role in regulating cell metabolism during I/R. Thus, to elucidate the role of Noxs in mediating the adaptive response to I/R injury, we investigated the role of Noxs in mediating HIF-1alpha expression. Time-course experiments showed that I/R increases the HIF-1alpha protein level in NTg hearts 1 hour and 6 hours after reperfusion (Figure 6A). HIF-1alpha was downregulated at baseline in Tg-DN-Nox (Figure 6B and 6C). HIF-1alpha remained downregulated in Tg-DN-Nox after reperfusion, whereas it was upregulated after I/R in Tg-Nox4 (Figure 6B and 6C). The expression level of HIF-1alpha was also significantly decreased in DKO mice hearts after I/R, whereas it was preserved in cNox4 KO and Nox2 KO mice hearts (Figure 6D). These results are consistent with the notion that Nox-derived ROS play an important role in inducing HIF-1alpha expression at baseline and during I/R in the heart.

To investigate the role of HIF-1alpha downregulation in mediating the exaggerated myocardial injury in Tg-DN-Nox after I/R, we crossed Tg-DN-Nox with PHD2 knockout mice, in which PHD2, an enzyme known to induce hydroxylation and downregulation of HIF-1alpha, is deleted in...
a cardiomyocyte-specific manner, and generated PHD2 heterozygous knockout (PHD2+/−) mice and Tg-DN-Nox/PHD2+/− mice. We then conducted I/R experiments in WT, PHD2+/−, Tg-DN-Nox, and Tg-DN-Nox/PHD2+/− mice. Whereas PHD2+/− mice did not exhibit a significant difference in I/R injury compared with WT mice, the increase in I/R injury observed in Tg-DN-Nox mice was significantly alleviated in Tg-DN-Nox/PHD2+/− mice (Figure 6E). The expression level of cleaved caspase-3 was also significantly lower in Tg-DN-Nox/PHD2+/− mice than in Tg-DN-Nox mice (Figure 6F).

HIF-1α expression was downregulated in Tg-DN-Nox hearts after I/R, but was normalized in Tg-DN-Nox4/PHD2+/− hearts (Figure 6G).

HIF-1α regulates glycolysis, fatty acid oxidation (FAO), and angiogenesis in the heart. Although glucose transporter-4 and hexokinase, key regulators of glycolysis, were downregulated in the Tg-DN-Nox mouse heart, expression of these molecules was normalized in the Tg-DN-Nox/PHD2+/− mouse heart (Figure 6G). However, PPARα, a key regulator of FAO, was not only upregulated in the Tg-DN-Nox mouse heart, but was...
also normalized in the Tg-DN-Nox/PHD2−/− mouse heart (Figure 6G). The cardiac ATP content 6 hours after reperfusion was preserved after downregulation of either Nox4 or Nox2. However, broad suppression of Nox isoforms by Tg-DN-Nox or combined downregulation of Nox2 and Nox4 by DKO induced significant reduction of the ATP content 6 hours after reperfusion. Importantly, the decrease in the ATP content observed in Tg-DN-Nox mice was significantly reversed in Tg-DN-Nox/PHD2+/− mice. These data indicate that both ROS and HIF-1α are involved in ATP production after I/R. To determine whether angiogenesis is affected in Tg-DN-Nox mice, we evaluated capillary density in the LV in NTg and Tg-DN-Nox mice at baseline. There was no significant difference in capillary density between groups (Online Figure XI). Taken together, these data suggest that normalization of HIF-1α expression rescues the increased I/R injury observed in Tg-DN-Nox mice, and is accompanied by a normalization of genes involved in glycolysis and FAO.

**Broad Suppression of Nox Activity Upregulates PPARα and Induces Triglyceride Deposition in the Heart**

Inadvertent activation of PPARα may be detrimental during I/R; I/R decreased endogenous PPARα expression after 1 h of reperfusion. However, both Tg-DN-Nox and DKO mice exhibited greater PPARα expression after 1 h of reperfusion than control and single KO mice did (Figure 7B, Online Figure XIIA). However, PPARα was further downregulated after I/R in Tg-Nox4 mice compared with that in NTg (Online Figure XIIB).

Knockdown of either Nox4 or Nox2 in myocytes using a short hairpin adenovirus did not change the expression level of either PPARα or medium-chain acyl-coenzyme A dehydrogenase (MCAD), a protein known to be regulated by PPARα, compared with control. However, overexpression of DN-Nox or combined downregulation of Nox2 and Nox4 upregulated both PPARα and MCAD (Figure 7C). In addition, the activity of a PPARα-luciferase reporter gene was increased in myocytes transduced with Ad-DN-Nox or both Ad-sh-Nox4 and Ad-sh-Nox2 (Figure 7D).

Although the expression of PPARα was downregulated in response to hypoxia/reoxygenation in control myocytes, it was not downregulated after hypoxia/reoxygenation in myocytes treated with Ad-DN-Nox or Ad-sh-Nox4 plus Ad-sh-Nox2 (Online Figure XIIIC), suggesting that suppression of both Nox2 and Nox4 prevents hypoxia/reoxygenation-induced downregulation of PPARα in a cell autonomous manner. Tg-DN-Nox and DKO mice showed equivalent

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**Figure 2.** DN-NADPH oxidase (Nox) inhibits Nox isoforms. A, Cardiomyocytes were transduced with either Ad-LacZ or Ad-Nox2 in the presence or absence of Ad-DN-Nox, and the cellular level of O2− was evaluated with superoxide dismutase (SOD)-inhibitable lucigenin chemiluminescence. *P<0.05.

B, O2− production in the left ventricle (LV) in systemic Nox4 KO mice, as evaluated with dihydroethidium (DHE) staining, was significantly decreased after injection of Ad-DN-Nox adenovirus. *P<0.05.

C and D, O2−-producing activity of mitochondrial and microsomal fractions from cardiomyocytes transduced with the indicated adenoviruses was evaluated with lucigenin chemiluminescence assays. *P<0.05 vs LacZ.

E, Lysates from myocytes transduced with Ad-LacZ or Ad-DN-Enox-human influenza hemagglutinin (HA) were used for immunoprecipitation with HA antibody. Immunoblots for p22phox, Nox4, and HA were performed. Immunoblot of remaining lysate after incubation with HA-agarose beads is also shown. F, NADP/NADPH ratio was measured in the indicated mouse hearts. *P<0.05 vs WT, #P<0.05 vs cNox4 KO, ##P<0.05 vs Nox2 KO. N.S. indicates not significant; and WT, wild type.
Figure 3. DN-NADPH oxidase (Nox) and double-knockout (DKO) of Nox2 and Nox4 exacerbate ischemia/reperfusion (I/R) injury.

A, Nontransgenic (NTg) and Tg-DN-Nox mice were subjected to ischemia (30 minutes) and reperfusion (24 hours). Infarct size was evaluated by triphenyltetrazolium chloride (TTC) and Alcian blue staining. *P<0.05. Bar, 1 mm.

B, Apoptotic cells in the ischemic area were evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining 24 hours after reperfusion. The number of TUNEL-positive myocytes was increased in the left ventricles (LVs) of Tg-DN-Nox mice compared with those of NTg mice. *P<0.05.

C, Expression levels of cleaved caspase-3 and tubulin in the indicated mice were evaluated by immunoblotting. *P<0.05.

D, Cardiac function in NTg and Tg-DN-Nox mice was evaluated using the isolated perfused heart (Langendorff) system. *P<0.05 vs NTg at same time point.

E, Wild-type (WT) and DKO mice were subjected to ischemia (30 minutes) and reperfusion (24 hours). Infarct size was evaluated by TTC and Alcian blue staining. *P<0.05. Bar, 1 mm. Apoptotic cells in the ischemic area were evaluated by TUNEL staining 24 hours after reperfusion. The number of TUNEL-positive myocytes was increased in the LVs of DKO mice compared with those of WT mice. *P<0.05, Bar, 10 μm. LVEDP indicates LV end-diastolic pressure.
decreases in HIF-1α and increases in PPARα and MCAD protein expression (Online Figure XIII).

The circulating level of triglyceride (TG) increases during I/R. To test whether upregulation of PPARα during I/R leads to increased accumulation of TG in the myocardium, we measured the TG content in the heart. Cardiac TG content was increased in Tg-DN-Nox and DKO mice compared with that in WT, cNox4, and Nox2 KO mice after I/R (Figure 7E). These results suggest that inadvertent upregulation of PPARα caused by combined suppression/downregulation of Noxs induces lipotoxicity in response to I/R.

**Downregulation of PPARα Attenuates the Increased I/R Injury in Tg-DN-Nox Mice**

To investigate the role of PPARα in mediating the enhancement of I/R injury observed in Tg-DN-Nox mouse hearts, we crossed Tg-DN-Nox with PPARα knockout mice and generated Tg-DN-Nox/PPARα−/− mice. We then
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conducted I/R experiments in WT, PPARα−/−, Tg-DN-Nox, and Tg-DN-Nox/PPARα−/− mice. Protein levels of PPARα and MCAD were upregulated in the Tg-DN-Nox mouse heart after I/R, but the upregulation was abolished in the Tg-DN-Nox/PPARα−/− mouse heart (Figure 8A). Consistent with the protein levels, the mRNA levels of target genes of PPARα, such as PDK4, AcoxI, CD36, carnitine palmitoyltransferase I, and MCAD, were upregulated in Tg-DN-Nox but were normalized in Tg-DN-Nox/PPARα−/− mice after I/R (Online Figure XIV A). Interestingly, PPARα−/− mice showed an increase in the LV HIF-1α protein level compared with WT mice (Online Figure XIVB). Thus, HIF-1α and PPARα negatively regulate one another.

Whereas PPARα−/− mice did not exhibit a significant difference in the extent of I/R injury compared with WT mice, Tg-DN-Nox, and Tg-DN-Nox/PPARα−/− mice. Protein levels of PPARα and MCAD were upregulated in the Tg-DN-Nox mouse heart after I/R, but the upregulation was abolished in the Tg-DN-Nox/PPARα−/− mouse heart (Figure 8A). Consistent with the protein levels, the mRNA levels of target genes of PPARα, such as PDK4, AcoxI, CD36, carnitine palmitoyltransferase I, and MCAD, were upregulated in Tg-DN-Nox but were normalized in Tg-DN-Nox/PPARα−/− mice after I/R (Online Figure XIV A). Interestingly, PPARα−/− mice showed an increase in the LV HIF-1α protein level compared with WT mice (Online Figure XIVB). Thus, HIF-1α and PPARα negatively regulate one another.

Figure 5. Combined downregulation of NADPH oxidase (Nox)2 and Nox4 exacerbates cell death caused by hypoxia/reoxygenation. A, O2−-producing activity of myocytes treated with the indicated adenoviruses was evaluated with lucigenin chemiluminescence assays after 12 hours of hypoxia and 24 hours of reoxygenation. *P<0.05. B–E, Cell survival in myocytes treated with the indicated adenoviruses was evaluated by Cell Titer-Blue assay at baseline and 1, 6, and 24 hours after reoxygenation. *P<0.05 vs sh-Scramble or sh-LacZ at same time point. N.S. indicates not significant.

Our results suggest that both Nox2 and Nox4 are major sources of ROS production in the heart in response to I/R, and that oxidative stress induced by Nox2 and Nox4 plays an important role in mediating myocardial I/R injury. However, low levels of oxidative stress produced by either endogenous Nox2 or Nox4 mediate essential adaptive mechanisms for myocardial survival against I/R through activation of HIF-1α and consequent suppression of the PPARα pathway.

Although O2− is rapidly dismutated to H2O2, which is diffusible and membrane permeable, as Nox2 and Nox4 are localized in distinct subcellular spaces,2 they may have distinct local targets promoting I/R injury. However, thus far, we have found that both Nox4 and Nox2 affect common cellular responses, namely, mitochondrial dysfunction and endoplasmic reticulum stress. Thus, unique targets of Nox2 and Nox4 in the context of I/R injury remain to be identified. Interestingly, downregulation of a single Nox is sufficient to achieve a >50% reduction of ROS. Furthermore, upregulation of Nox4 alone failed to enhance I/R injury in Tg-Nox4 mice compared with that in control mice. Thus, ROS produced by Nox2 and Nox4 in distinct subcellular localizations may cooperatively enhance further ROS production by both isoforms, which in turn promotes myocardial injury. In addition, although the reduction in superoxide production at the level of 25% to 50% is beneficial to I/R injury, >75% is detrimental in WT. These
data indicate a possibility that there is a threshold effect for ROS in terms of I/R injury.

Perhaps one of the most surprising findings in this work is the fact that a significant enhancement of infarct size/AAR after I/R occurs when both Nox2 and Nox4 are downregulated or inhibited. Importantly, ROS production in Tg-DN-Nox and DKO mice was markedly downregulated in the ischemic area, to an extent significantly greater than in either Nox2 KO or cNox4 KO mice. This indicates that a certain level of ROS is important for cell survival in response to I/R. Purposeful production of ROS in mitochondria could mediate upregulation of cell-protective mechanisms, such as preconditioning effects.18 ROS generated by Nox may act as a second messenger to alert cells and activate adaptive mechanisms.18 The protective mechanism activated by the low level of ROS seems quite powerful, because, in the absence...
of such a mechanism, the myocardial injury caused by I/R is even greater than usual, despite the fact that the cell-death mechanism activated by excessive ROS is no longer active under these conditions (Figure 8F).

Previous reports have suggested that Nox isoforms play some beneficial roles in the heart. For example, upregulation of angiogenic growth factors in response to I/R is inhibited in p47<sup>phox</sup>−/− mouse hearts. Stretch-induced production of ROS (X-ROS) from Nox2 increases Ca<sup>2+</sup> sensitivity by stimulating ryanodine receptors in healthy cardiomyocytes. Stimulation of angiogenesis in pressure-overload heart is attenuated in systemic Nox4−/−mice, which is accompanied by downregulation of HIF-1α.20 It should be noted, however, that cNox4−/− mice are protected from pressure overload. Thus, the protective action of Nox4 may originate from nonmyocytes during pressure overload. The molecular mechanism mediating the protective actions of Nox isoforms and how they are regulated by cellular levels of ROS seem to be dependent on stimulus and cell type.

As discussed below, as PHD2, one of the critical targets of ROS mediating the hypoxic adaptation during I/R, is localized primarily in cytosol, ROS produced by either Nox2 or Nox4 may be sufficient to inactivate this enzyme, whereas the absence of both Noxs seems to prevent this inactivation, an essential step for stabilizing HIF-1α. Consistent with the in vivo data, whereas cell death in cultured cardiomyocytes was attenuated by single downregulation of Nox2 or Nox4 after hypoxia/reoxygenation, it was exacerbated by expression of DN-Nox or combined downregulation of Nox4 and Nox2. Thus, ROS produced by either Nox2 or Nox4 acts as a second messenger to promote cell survival under stress in a cell-autonomous fashion.

HIF-1α is activated by low oxygen conditions during ischemia or by increased oxidative stress during I/R, and activation of HIF-1α reduces reperfusion injury in the heart. Although HIF-1α protects the heart through multiple mechanisms, one important mechanism particularly relevant for protecting the heart against acute I/R may be its effect on cardiac metabolism. Activation of endogenous...
HIF-1α stimulates the glycolytic pathway and inhibits the trichloroacetic acid cycle and FAO, thereby preserving ATP while reducing O2 consumption during I/R. The preservation of ATP production induced by stimulation of the glycolytic mechanism is protective against ischemia and facilitates restoration of the high-energy phosphate content after reperfusion. Although it has been speculated that Nox may be involved in the stabilization of HIF-1α, a definitive link between Nox and HIF-1α during I/R has not yet been established in vivo. We show here that combined inhibition/downregulation of Nox2 and Nox4 decreases HIF-1α at baseline and in response to I/R in the heart, suggesting that...
endogenous Noxs play an important role in HIF-1α regulation in response to I/R.

Hydroxylation of prolyl residues in HIF-1α promotes proteolytic degradation of HIF-1α by the ubiquitin-proteasome pathway. ROS increase the stability and transcription of HIF-1α through suppression of PHD. \(^1\)\(^-\)\(^2\) In response to ROS, PHD is inactivated and proline hydroxylation of HIF-1α is reduced, which in turn reduces ubiquitin-proteasomal degradation of HIF-1α. PHD1 knockout mice exhibit attenuation of myocardial I/R injury. \(^3\) However, PHD2 was found to be the most active isofrom in a number of cell lines. PHD2 has a preference for HIF-1α, whereas PHD1 and PHD3 primarily hydroxylate HIF-2α. \(^3\) Here, downregulation of PHD2 prevented the increase in myocardial infarct size after I/R in Tg-DN-Nox mice, suggesting that Noxs play an important role in regulating the activation of HIF-1α through inactivation of PHD2 during I/R.

Although overexpression of Nox4 was sufficient to enhance I/R-induced upregulation of HIF-1α, it did not significantly reduce the infarct size/AAR. Excessive production of ROS by overexpressed Nox4 may counteract the beneficial effect of HIF-1α. Alternatively, the beneficial effect of HIF-1α on I/R injury may have already been saturated at the level of HIF-1α upregulation seen in Ntg mice, which would be consistent with the fact that downregulation of PHD2 alone did not reduce the infarct size, despite upregulation of HIF-1α.

HIF-1α not only upregulates proteins involved in glycolysis but also downregulates those involved in FAO, including PPARα. \(^3\)\(^2\) Upregulation of HIF-1α, such as by I/R and downregulation of PHD2, rapidly increased the expression of PPARα, whereas downregulation of HIF-1α increased the PPARα level after hypoxia/reoxygenation, suggesting that HIF-1α negatively regulates PPARα in cardiomyocytes. Consistent with its effect on HIF-1α, combined inhibition/downregulation of Nox4 and Nox2 significantly upregulated PPARα in the heart at baseline and in response to I/R. Although a previous report suggested that ROS negatively regulate PPARα expression in response to repetitive ischemia, \(^3\) underlying mechanisms remain to be elucidated. Our results clearly show that endogenous Noxs negatively regulate PPARα expression through HIF-1α in the heart.

The effect of PPARα activation on I/R injury is controversial. \(^1\)\(^4\)\(^-\)\(^6\)\(^3\) In our study, an increase in PPARα expression was correlated with the extent of TG accumulation in the heart after reperfusion in Tg-DN-Nox and DKO mice. Downregulation of PPARα in this condition induced a significant attenuation of infarct size, apoptosis, and deposition of TG, indicating the causative involvement of endogenous PPARα in mediating the detrimental phenotype caused by suppression of both Nox2 and Nox4.

The upregulation of PPARα observed in Tg-DN-Nox mice was accompanied by upregulation of PPARα target genes, including CD36. Because myocardial ischemia rapidly increases the plasma free fatty acid concentration, \(^4\) these data suggest that upregulation of PPARα caused by the lack of Nox-derived ROS could stimulate free fatty acid uptake, which in turn induces TG deposition and consequent lipotoxicity. Stimulation of FAO may also delay the recovery of intracellular pH during reperfusion of ischemic hearts because of the increased production of H⁺ by glycolysis uncoupled from glucose oxidation. \(^3\) Inhibition of Noxs affected HIF-1α/PPARα signaling even at baseline but its effect seems to manifest only in post-I/R hearts, suggesting that the events associated with I/R, such as increases in free fatty acid concentration and acidosis, contribute to the phenotype in Tg-DN-Nox4 and DKO mice.

Interestingly, downregulation of PPARα increased the expression of HIF-1α in the heart, suggesting that PPARα and HIF-1α negatively regulate one another’s functions in the heart. Thus, the salutary effect seen in Tg-DN-Nox/PPARα−/− mice might be caused in part by an increase in HIF-1α and activation of glycolysis. Inhibition of glycolysis delays recovery of ATP during I/R in the Langendorff heart model. \(^2\) In fact, Tg-DN-Nox/PPARα−/− mice showed an improved recovery in ATP content 6 hours after reperfusion compared with Tg-DN-Nox mice, suggesting that endogenous Noxs play an important role in mediating the suppression of PPARα and downstream metabolic effects that are essential for myocyte survival during I/R.

There is an issue to be acknowledged as a limitation of this study. In terms of the pathophysiological equivalence of DN and KO Nox4 mice, cross-breeding of Nox4flox/flox-alpha myosin heavy chain-Cre recombinase-Nox2−/−-PHD2flox/flox (or PPARα−/−) mice was technically challenging and we could not generate them. However, additional lines of evidence suggest that DN-Nox and combined downregulation of Nox2/Nox4 exhibit identical effects on cardiomyocytes (Online Figures IX, X, XI and XIV).

In conclusion, both Nox2 and Nox4 play an essential role in mediating oxidative stress and myocardial injury after I/R. Importantly, however, low levels of ROS produced by either Nox2 or Nox4 are required to activate essential adaptive mechanisms to protect the heart against I/R, including preventing inactivation of HIF-1α and inhibition of PPARα. Unfortunately, these protective mechanisms are easily overcome by cell-death promoting mechanisms activated by the excessive ROS produced by coordinated activation of Nox2 and Nox4 during I/R (Online Figure XVII). Our study suggests that although either Nox2 or Nox4 can be targeted for the treatment of I/R, any such intervention should be carried out with extreme caution so as not to impair the physiological function of the Noxs.

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**Disclosures**

None.
References


**Novelty and Significance**

**What Is Known?**
- Oxidative stress plays an important role in mediating ischemia/reperfusion (I/R) injury in the heart.
- NADPH oxidases (Noxs) produce $\text{O}_2^-$ and $\text{H}_2\text{O}_2$, thereby contributing to oxidative stress during I/R in the heart.
- Myocardial I/R injury is suppressed in mice with genetic deletion of $\text{p}47^{\text{phox}}$, a cytosolic factor positively regulating NADPH oxidase (Nox) 2.
- Hypoxia-inducible factors play a protective role in I/R injury through regulation of the cardiac metabolism.

**What New Information Does This Article Contribute?**
- Both Nox2 and Nox4 contribute to increases in oxidative stress during I/R injury in the heart.
- Isoform-specific genetic deletion of either Nox2 or Nox4 significantly attenuates I/R injury in the heart.
- Combined deletion of Nox2 and Nox4 or overexpression of dominant-negative Nox, which suppresses multiple isoforms of Nox, markedly reduces oxidative stress during I/R, but exacerbates I/R injury.
- Physiological levels of reactive oxygen species (ROS) produced by either Nox2 or Nox4 play an essential role in activating hypoxia-inducible factor-1α, which in turn plays an essential role in mediating protection against I/R in part through downregulation of peroxisome proliferator–activated receptor-α.

Oxidative stress plays an important role in mediating myocardial injury during I/R. The molecular source of ROS during I/R and their role in mediating myocardial injury remain unclear. Using genetically altered mouse models, we found that both Nox2 and Nox4, major isoforms of the NADPH oxidase in the heart, contribute to oxidative stress during I/R, and suppression of either of them significantly reduces I/R injury by inhibiting excessive production of ROS. However, combined downregulation of both Nox2 and Nox4 paradoxically exacerbated I/R injury, although oxidative stress by I/R was markedly suppressed. These findings suggest that during I/R, low levels of ROS production from either Nox2 or Nox4 are required for activation of adaptive mechanisms essential for cardioprotection, which is mediated by the activation of hypoxia-inducible factor-1α and downregulation of peroxisome proliferator–activated receptor-α. Thus, ROS production by Nox seems to have dichotomous functions during I/R: excessive production of ROS by Nox2 and Nox4 is detrimental whereas minimum production of ROS by either Nox2 or Nox4 is essential for adaptation. Hence, broad Nox suppression during I/R could be detrimental for the heart.
Broad Suppression of NADPH Oxidase Activity Exacerbates Ischemia/Reperfusion Injury Through Inadvertent Downregulation of Hypoxia-inducible Factor-1α and Upregulation of Peroxisome Proliferator–activated Receptor-α

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Primary Culture of Neonatal Rat Ventricular Myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl:(WI) BR-Wistar rats (Harlan). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described.¹

Ischemia/Reperfusion in Vivo

Mice were housed in a temperature-controlled environment with 12 h light/dark cycles where they received food and water ad libitum. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with 65% oxygen. The animals were kept warm with heat lamps. Rectal temperature was monitored and maintained between 36°C and 37°C. The chest was opened by a horizontal incision at the third intercostal space. I/R was achieved by ligating the anterior descending branch of the left coronary artery with an 8-0 Prolene suture, with silicon tubing (1 mm outer diameter) placed on top of the left anterior descending coronary artery, 2 mm below the border between the left atrium and left ventricle (LV). Ischemia was confirmed by ECG change (ST elevation). After 30 min of occlusion, the silicon tubing was removed to achieve reperfusion, and the rib space and overlying muscles were closed. When recovered from anesthesia, the mice were extubated and returned to their cages. They were housed in a climate-controlled environment. Twenty-four hours after reperfusion, the animals were reanesthetized and intubated, and the chest was opened. After the heart was arrested at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. The left anterior descending coronary artery was occluded with the same suture, which had been left at the site of
the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm-thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe Photoshop (Adobe Systems Inc), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct size/AAR were expressed as percentages.2

**Langendorff-Perfused Mouse Heart Model of Global I/R**

Mice were anesthetized with pentobarbital (65 mg/kg IP) and treated intraperitoneally with 50 U of heparin. The heart was quickly removed and catheterized with a 22-gauge needle. The hearts were mounted on a Langendorff-type isolated heart perfusion system and subjected to retrograde coronary artery reperfusion with 37°C oxygenated Krebs-Henseleit bicarbonate buffer (mmol/L: NaCl 120, glucose 17, NaHCO₃ 25, KCl 5.9, MgCl₂ 1.2, CaCl₂ 2.5, EDTA 0.5), pH 7.4, at a constant pressure of 80 mm Hg. A balloon filled with water was introduced into the LV through the mitral valve orifice and connected to a pressure transducer via a plastic tube primed with water. LV pressures and LV dP/dt were recorded with a strip chart recorder (Astro-Med Inc). The LV end-diastolic pressure was set at 4 to 10 mm Hg at the beginning of perfusion by adjusting the volume of the balloon in the LV, and the volume was kept constant throughout the experiment. After a 30 min equilibration period, the heart was subjected to 30 min of global ischemia (at 37°C) followed by 60 min of reperfusion.³

**Evaluation of Apoptosis in Tissue Sections**
DNA fragmentation was detected in situ with the use of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), as described. Nuclear density was determined by manual counting of DAPI-stained nuclei in 6 fields for each animal with the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section with the same power objective.3

**Capillary Density**

Capillary density was evaluated as previously described.4 Heart samples were embedded in OCT compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Frozen sections (5 μm in thickness) were stained for alkaline phosphatase using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, and then counterstained with eosin. The capillary density was calculated as capillaries/1000 μm².

**Immunoblot Analysis**

Heart homogenates and cardiac myocyte lysates were prepared in a RIPA lysis buffer containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% deoxycholic acid, 10 mmol/L Na₄P₂O₇, 5 mmol/L EDTA, 0.1 mmol/L Na₃VO₄, 1 mmol/L NaF, 0.5 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Samples were subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride microporous membranes (Bio Rad) and probed with primary antibodies. The antibodies used in this study include anti-Nox4, raised as previously described ⁵, and anti-Nox1, anti-Nox2, anti-Nox3, and anti-nitric oxide synthase antibodies (Abcam), anti-HIF-1α and anti-COX IV antibodies (Cell Signaling Technology), anti-PDK4
antibody (BD Biosciences), anti-PPARα and anti-MCAD antibodies (Cayman), anti-GLUT4 and Hexokinase antibodies (Santa Cruz Biotechnology Inc), anti-actinin and anti-tubulin antibodies (Sigma), and xanthine oxidase antibody (Novus).

**Adenoviruses**

Adenovirus vectors harboring Nox4 (Ad-Nox4), DN-Nox (Ad-DN-Nox), shRNA-Nox4 (Ad-sh-Nox4), or shRNA-Nox2 (Ad-sh-Nox2) were generated using the AdMax system (Microbix) as described. In brief, the recombinant adenoviruses were generated in HEK293 cells by co-transfection with a cosmid (pBHGloxΔE1,3Cre) containing the adenovirus type 5 genome (devoid of E1 and E3) and pDC316, a shuttle vector, containing a gene of interest. Adenovirus vectors harboring LacZ (Ad-LacZ) and Scramble shRNA (Ad-sh-Scr) were used as controls. Adenovirus vector harboring shRNA-HIF-1α (Ad-sh-HIF-1α) was kindly gifted by Dr. M. Abdellatif (University of Medicine and Dentistry of New Jersey).

**Viability of the Cells**

Viability of the cells was measured by Cell Titer Blue (CTB) assays (Promega). In brief, cardiac myocytes (1 X10^5 per 100 μl) were seeded onto 96-well dishes. The cells were preincubated with the indicated adenovirus for 48 h. Viable cell numbers were measured before hypoxia and after 12 h of hypoxia (<0.3% O₂ and 5% CO₂, 37°C) followed by 1, 6, and 24 h of reoxygenation (21% O₂ and 5% CO₂, 37°C) under conditions of serum deprivation. The CTB assays were performed according to the supplier's protocol.

**Luciferase Assay**

Transfection of plasmids into myocytes was performed with Fugene 6 (Roche).
Luciferase activity was measured with a luciferase assay system (Promega). The method of in vivo reporter gene assays has been described.7

**Fractionation**

To isolate crude nuclear fractions, we used procedures previously described 8. Briefly, cultured neonatal rat myocytes were suspended in hypotonic lysis buffer (10 mmol/L K-HEPES (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L DTT, 0.2 mmol/L Na3VO4, 1% Protease Inhibitor Cocktail), incubated for 15 min on ice and homogenized. Whole cell lysates were centrifuged at 60xg for 5 min to collect unbroken cells. The supernatants were collected and the homogenization and centrifugation were repeated on the pellets. The total homogenates were centrifuged at 1,200xg to separate crude nuclei and unbroken cells (pellet) from cell membrane and cytosolic proteins (supernatant). The supernatants of the total homogenates were centrifuged at 3,500xg for 20 min to separate mitochondrial fractions (pelleted in tube) from cytosolic and microsomal fractions (supernatant). The supernatants were then further centrifuged at 100,000xg for 60 min to separate microsomal fractions (pelleted in tube) from cytosolic fractions (supernatant).

**Lucigenin Chemiluminescence Assay**

Mouse whole heart homogenates, or mitochondrial or microsomal fractions, were suspended in 200 μL of an assay buffer composed of 100 mmol/L potassium phosphate (pH 7.0), 10 μmol/L flavin adenine dinucleotide (FAD), 1 mmol/L NaN3, and 1 mmol/L EGTA. After preincubation with 5 μmol/L lucigenin, NADH or NADPH was added to a final concentration of 500 μmol/L.9 The chemiluminescence was continuously monitored using a luminometer. The
reaction was terminated by the addition of SOD (100 µg/mL) 6.

**H$_2$O$_2$ Measurement**

H$_2$O$_2$ production was measured with an Amplex Red H$_2$O$_2$ assay kit (Molecular Probes; Invitrogen) according to the manufacturer’s instructions. In brief, left ventricular blocks (30–50 mg) were incubated with Amplex Red (100 µmol/L) and horseradish peroxidase (1 U/mL) for 30 min at 37°C in Krebs–HEPES buffer protected from light. The supernatant was then transferred to a 96-well plate, and absorbance was measured (560 nm). Background fluorescence, determined in a control reaction without sample, was subtracted from each value. H$_2$O$_2$ release was calculated using H$_2$O$_2$ standards and expressed as micromoles per milligram of dry tissue.

**DHE Staining**

After harvest, heart tissues were immediately embedded in an optimum cutting temperature (OCT) compound in ethanol–dry ice and stored at −80°C. Unfixed frozen samples were cut into 5 µm-thick sections and placed on glass slides. DHE (10 µmol/L) was applied to each tissue section, and then the sections were coverslipped. The slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Ethidium fluorescence (excitation at 490 nm, emission at 610 nm) was examined by fluorescence microscopy.

**Assays for Measurement of Oxidative Stress**

Cardiac tissue homogenates were assessed for MDA+4HAE content (LPO-586; Oxis International Inc.) according to the manufacturer’s instructions.
**NADP/NADPH Ratio**

NADP/NADPH ratio of lysates from mouse hearts was measured using the EnzyChrom NADP/NADPH Assay Kit according to the protocol of the manufacturer (ECNP-100, Bioassay Systems, Hayward, Calif).

**Antioxidant Capacity**

Antioxidant capacity of the lysates from mouse hearts was measured using an Antioxidant Assay Kit according to the protocol of the manufacturer (Cayman).

**Measurement of ATP Content**

ATP content was measured with an ATP Bioluminescent Assay kit (Sigma). In brief, the ischemic region of the mouse heart was suspended and homogenized with homogenate buffer (0.25 mol/L Sucrose, 10 mmol/L HEPES pH 7.8). After centrifuging at 10,000xg for 10 min, the supernatant was added to the ATP Assay Mix solution. Luciferase activity was monitored using a luminometer.

**Evaluation of mitochondrial membrane potential**

In order to evaluate mitochondrial membrane potential, staining of cultured cardiac myocytes with 5,5’ ,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolocarbocyanin iodide (JC-1) was conducted using MitoTP JC-1 (ImmunoChemistry Technologies), according to the manufacture’s instructions.

**Mitochondrial Swelling Assay**

Fifty µg of isolated mouse heart mitochondria from from WT, cNox4 KO, or Nox2 KO
mice subjected to sham operation or I/R were suspended in a swelling buffer (250 mmol/L sucrose, 10 mmol/L MOPS, 5 μmol/L EGTA, 2 mmol/L MgCl₂, 5 mmol/L KH₂PO₄, 5 mmol/L pyruvate, and 5 mmol/L malate) and incubated with 150 μmol/L calcium chloride (CaCl₂) in a final volume of 200 μL in a 96-well plate for 20 min. Absorbance was read at 520 nm.

**Myocardial Triglyceride Content**

Myocardial triglyceride content was assayed as described. Briefly, left ventricular tissues were homogenized with an ice-cold chloroform/methanol/water mixture (2:1:0.8) for 2 min. Additional chloroform and water were added to separate the organic and aqueous layers. After centrifugation, the aqueous layer was removed, the chloroform layer was decanted and the mixture was evaporated at 70°C. The residue was dissolved in 0.5 ml of isopropanol, and triglycerides were assayed with a triglyceride measurement kit from Sigma.

**Oil Red O Staining**

Left ventricular tissues were used for frozen sections and slides from the ischemic area were stained with oil red O staining as previously described.

**Quantitative RT-PCR**

Total RNA was prepared from left ventricles or cultured cardiac myocytes using the RNeasy Fibrous Tissue Mini Kit (QIAGEN), and then cDNA was generated using M-MLV Reverse transcriptase (Promega). Real-time RT-PCR was performed using Maxima SYBR Green qPCR master mix (Fermentas). β-actin was used as an internal control. PCRs were carried out using the following oligonucleotide primers:
Pcg1a 5’-ATGAATGCAGCGGTCTTAGCACTC-3’
5’-TTGCTGTTGACAAATGCTCTTCGCTTTA-3’
Ppara 5’-CCTGGCCCTTCTAAACATAGG-3’
5’-TCCCTGCTCTCGTGATATGGG-3’
Pdk4 5’-AGGTTATGGGACAGACGCTATCATCTACTT-3’
5’-GTTCCTCGTTCCCTGCTTGGG-3’
Acox1 5’-ATGAATCCCGATCTGCGCAA-3’
5’-TTCTCGATTCTCGACGGCG-3’
Cd36 5’-GAATCTGAAGAGACCTTACATTGTACC-3’
5’-CACTCCAATCCCAAGTAAGGCCAT-3’
Cpt1b 5’-TTGGGAACCACATCCGCAA-3’
5’-TTATGCCTGTGAGCTGGCCAC-3’
Mcad 5’-GAAGCTGASTGAGGGACGCCA-3’
5’-GCTTGGAGCTTAGTTACACGAGG-3’
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receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. *Nat Med.* 2004;10:1245-1250
Online Figure I

Systemic Nox4 KO inhibits I/R injury. A. Expression levels of Nox4 and tubulin in the LVs of WT and systemic Nox4 KO mice at baseline were evaluated by immunoblotting. B. WT, cardiac-specific Nox4 KO, and systemic Nox4 KO mice were subjected to ischemia (30 min) and reperfusion (24 h). Infarct size was evaluated by TTC and Alcian blue staining. *P<0.05, N.S. not significant. C. O$_2^-$ production in LV sections from the indicated mice was evaluated by SOD-inhibitable chemiluminescence 24 h after reperfusion. *P<0.05, N.S. not significant.
Online Figure II

A

WT  cNox4KO  Nox2KO

sham

I/R 24h

B

Total cell density (nuclei/mm²)

I/R 24h

Online Figure II

The cell density in the ischemic area after I/R. A and B. The cell density in the ischemic area was evaluated by HE staining. *P<0.05, N.S. not significant. Bar=30 μm.
Online Figure III

A

Cardiac specific overexpression of Nox4 does not exacerbate I/R injury. A. NTg and Tg-Nox4 mice were subjected to ischemia (30 min) and reperfusion (24 h). Infarct size was evaluated by TTC and Alcian blue staining. N.S. not significant. Bar=1 mm. B. Apoptotic cells in the ischemic area were evaluated by TUNEL staining 24 h after reperfusion. N.S. not significant. Bar=10 μm. C. O$_2^-$ production in LV sections was evaluated by SOD-inhibitable chemiluminescence 24 h after reperfusion. *P<0.05.
Online Figure IV

Necrotic cells were evaluated by hairpin2 staining in NTg and Tg-DN-Nox mice. The number of hairpin2-positive nuclei in the ischemic area was increased in Tg-DN-Nox mice compared to in NTg mice. *P<0.05. Bar=10 μm.
Online Figure V

Functional recovery during reperfusion is attenuated in Tg-DN-Nox mouse hearts. Cardiac function in NTg and Tg-DN-Nox mice was evaluated by isolated perfused heart assay using the Langendorff system. *P<0.05 vs. NTg at same time point. LVP: left ventricular pressure, HR: Heart rate.
Online Figure VI

The effect of broad suppression of Nox on the sources of ROS and antioxidants. A. Expression levels of Nox1, Nox2, Nox3, Nox4, NOS, XO, COX IV, and tubulin in the LVs of WT, Tg-DN-Nox, and DKO mice were evaluated by immunoblotting. B. Expression levels of SOD1, SOD2, Trx, catalase, and tubulin in the LVs of WT, Tg-DN-Nox, and DKO were evaluated by immunoblotting. C. Antioxidant capacity was measured in the LVs of WT, Tg-DN-Nox, and DKO. *P<0.05 vs WT. D. NADP/NADPH ratio was measured in the LVs of WT, Tg-DN-Nox, and DKO mice. *P<0.05 vs WT.
Online Figure VII

Echocardiographic data at baseline in NTg, Tg-DN-Nox, WT, and DKO mice. LVDs: systolic left ventricular dimension, LVDd: diastolic left ventricular dimension, FS: Fractional shortening.
Online Figure VIII

Superoxide production at baseline is mediated through Noxs in the heart. A. Superoxide production in the LV sections was evaluated with DHE staining at baseline. Bar=20 μm. *P<0.05 vs. WT, #P<0.05 vs. cNox4KO, ## P<0.05 vs. Nox2KO. B. H₂O₂ production in LV sections was evaluated with the Amplex Red Assay. C. O₂⁻-producing activity of mitochondrial fraction from hearts of the indicated mice was evaluated with lucigenin chemiluminescence assays. *P<0.05
Online Figure IX

A. Mitochondrial membrane potential of myocytes treated with the indicated adenoviruses was evaluated with JC-1 staining after 12 h of hypoxia and 24 h of reoxygenation. Bar=50 μm. *P<0.05, N.S. not significant. B. Expression of mRNA for PGC-1α was evaluated with quantitative RT-PCR. *P<0.05. C. Expression levels of TFAM and tubulin were evaluated by immunoblotting.
ATP content in the ischemic area was evaluated in the indicated mice 6h after reperfusion. *P<0.05.
Capillary density in the LV at baseline in NTg and Tg-DN-Nox4 mice was evaluated by immunostaining with CD31 and Troponin T antibody. Nuclei were stained with DAPI. N.S. not significant. Bar=20 μm.
Online Figure XII

**A**

<table>
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<tr>
<th>sham</th>
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<tbody>
<tr>
<td>NTg</td>
<td>NTg</td>
</tr>
<tr>
<td>Tg-DN-Nox</td>
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HIF-1α

PPARα

Tubulin

Expression of HIF-1α and PPARα during ischemia/reperfusion and hypoxia/reoxygenation. A. Expression of HIF-1α, PPARα, and tubulin in NTg and Tg-DN-Nox mice 30 min after reperfusion was evaluated by immunoblotting.

**B**

I/R 24h

<table>
<thead>
<tr>
<th>NTg</th>
<th>Tg-Nox4</th>
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PPARα

Tubulin

Expression levels of PPARα and tubulin in the LVs of NTg and Tg-Nox4 mice with I/R were evaluated by immunoblotting.

**C**

<table>
<thead>
<tr>
<th>Normoxia</th>
<th>Hypoxia/reoxygenation</th>
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<tbody>
<tr>
<td>sh-Scr</td>
<td>DN-Nox</td>
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<tr>
<td>sh-Nox4α</td>
<td>sh-Nox2</td>
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</tbody>
</table>

HIF-1α

PPARα

Tubulin

Expression levels of HIF-1α, PPARα, and tubulin in myocytes transduced with the indicated adenoviruses with or without hypoxia/reoxygenation were evaluated by immunoblotting.
Online Figure XIII

Expression levels of HIF-1α, PPARα, MCAD, and Tubulin at baseline were evaluated by immunoblotting in WT, Tg-DN-Nox, and DKO mice. *P<0.05.
DN-Nox upregulates FAO-related genes through PPARα after I/R. A. Expression of mRNA for PPARα and FAO-related genes was measured by quantitative RT-PCR. *P<0.05, N.D. not detectable. B. Expression of PPARα, HIF-1α and tubulin in WT and PPARα -/- mice was evaluated by immunoblotting.
Online Figure XV

A. ATP content in the ischemic area was evaluated in the indicated mice 6 and 24 h after reperfusion. *P<0.05.

B. Expression levels of CHOP and tubulin in the indicated mice after I/R were evaluated by immunoblotting.
Online Figure XVI A hypothetical model of the role of Nox2 and Nox4 in response to I/R. I/R induces ROS production by stimulating Nox2 and Nox4, leading to cytotoxicity via mitochondrial dysfunction and ER stress. Downregulation of Nox2 or Nox4 mildly suppresses ROS production and attenuates I/R injury. ROS from either Nox2 or Nox4 is essential for upregulation of HIF-1α and stimulation of glycolysis. Downregulation of both Nox2 and Nox4 strongly suppresses ROS production, and thereby downregulates HIF-1α and upregulates PPARα. A metabolic shift from glycolysis to FAO during I/R induces myocardial TG deposition, which leads to lipotoxicity. The presence of a physiological level of Nox-derived ROS protects the heart from I/R injury. PHD: prolyl hydroxylase, HIF-1α: hypoxia-inducible factor-1α, PPARα: peroxisome proliferator-activated receptor α, MCAD: Medium-chain Acyl-CoA Dehydrogenase