Elimination of NADPH Oxidase Activity Promotes Reductive Stress and Sensitizes the Heart to Ischemic Injury

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Background—The NADPH oxidase family (Nox) produces reactive oxygen species by adding the electron donated by NADPH to oxygen. Excessive reactive oxygen species production under a variety of pathological conditions has been attributed to increased Nox activity. Here, we aimed at investigating the role of Nox in cardiac ischemic injury through gain- and loss-of-function approaches.

Methods and Results—We modulated Nox activity in the heart by cardiac-specific expression of Nox4 and dominant negative Nox4. Modulation of Nox activity drastically changes the cellular redox status. Increasing Nox activity by cardiac-specific overexpression of Nox4 imposed oxidative stress on the myocardium [increased NAD(P)+/NAD(P)H and decreased glutathione/glutathione disulfide ratio] and worsened cardiac energetics and contractile function after ischemia-reperfusion. Overexpression of the dominant negative Nox4 (DN), which abolished the Nox function, led to a markedly reduced state [decreased NAD(P)+/NAD(P)H and increased glutathione/glutathione disulfide ratio] at baseline and paradoxically promoted mitochondrial reactive oxygen species production during ischemia resulting in no recovery of heart function after reperfusion. Limiting the generation of reducing equivalent through modulating carbon substrates availability partially restored the NAD+/NADH ratio and protected dominant negative Nox4 hearts from ischemic injury.

Conclusions—This study reveals an important role of Nox in cardiac redox regulation and highlights the complexity of developing therapies that affect the intricately connected redox states. (J Am Heart Assoc. 2014;3:e000555 doi: 10.1161/JAHA.113.000555)

Key Words: cardiac ischemic injury • NADPH oxidase • oxidative stress • reductive stress

The roles of reactive oxygen species (ROS) as physiological signaling mechanisms as well as pathological stresses have been demonstrated repeatedly in the cardiovascular system.1–4 The NADPH oxidase family (Nox) is a major and dedicated cellular ROS generation system in cardiac myocytes and many other cell types, including neurons.5,6 Nox2 and Nox4 are the major catalytic subunits expressed in cardiac myocytes. The binding of p22phox to the catalytic subunit is required for the Nox enzyme activity. Unlike Nox2, Nox4 enzyme activity is readily regulated via transcription without agonist stimulation.7,8 Many studies have demonstrated the detrimental role of Nox2/4-mediated ROS generation in a variety of cardiac stresses.9–12 However, recent studies also suggested that Nox could play important protective roles in the cardiac response to chronic stresses.13 The physiological role of Nox—specifically, the function of the Nox in the regulation of cellular redox state—has not been investigated.

Cellular and mitochondrial redox homeostasis is intricately connected and regulated by several redox-sensitive couples, such as glutathione (GSH)/glutathione disulfide (GSSG), NADP+/NADPH, and NAD+/NADH, as well as by ROS.14 NADH is generated by glycolysis in the cytosol or via the tricarboxylic acid cycle in the mitochondria. The mitochondrial and cytosolic NAD+/NADH pools are linked by malate/aspartate shuttle. Being the major substrate for oxidative phosphorylation, the majority of NADH produced in cardiac myocytes enters the electron transport chain to drive the generation of ATP. NADPH can be produced in both cytosol and mitochondria.15 The NADH pool in mitochondria...
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previously described. Hearts were perfused with phos-
NADPH binding site as described previously. Age- and sex-
tized with sodium pentobarbital (150 mg/kg intraperitone-
Mice were heparinized (100 U intraperitoneally) and anesthe-
Lasers were calculated using the ratio of their peak area to the
Myocardial Infarct Size Measurement
Reperfused hearts were

Materials and Methods
Animal Models
We used 3-month-old mice with cardiac-specific overexpres-
sion of TG or DN, which has a Phe437His mutation within the
NADPH oxidase in cardiac ischemia/reperfusion (I/R) injury. We
found that the increased NADPH oxidase activity in TG is
associated with a highly oxidative state, while eliminating
its activity in DN resulted in excessively reduced state.
Interestingly, both oxidative and reductive stresses exacer-
bated I/R injury in the heart. The TG hearts generated more
ROS during reperfusion and showed poor recovery in
cardiac function and energetics. Unexpectedly, the DN
hearts exhibited severe mitochondrial damage via paradox-
ical ROS generation during ischemia and no recovery of
contractile function on reperfusion. These observations raise
caution against targeting NADPH oxidases as antioxidant
therapies.

Isolated Perfused Heart Experiment and
31P Nuclear Magnetic Resonance Spectroscopy
Mice were heparinized (100 U intraperitoneally) and anesthe-
tized with sodium pentobarbital (150 mg/kg intraperitone-
ally). The hearts were excised and perfused in a Langendorff
mode at 37°C with oxygenated perfusate (pH 7.4) as
previously described. Hearts were perfused with phos-
phate-free Krebs–Henseleit buffer containing (in mmol/L):

EDTA 0.5 and 1 of the following 2 different combinations of
substrates: “mixed-substrate buffer” (containing glucose 5.5
mmol/L, mixed long-chain fatty acids 0.4 mmol/L, bound to
1% albumin); or "glucose+pyruvate buffer" (containing glucose 10 mmol/L and pyruvate 0.5 mmol/L). A
water-filled balloon was inserted into the left ventricle (LV) to
record ventricular pressure and heart rate. Isovolumic
contractile function was estimated by the product of LV
developed pressure and heart rate (rate-pressure product). All
hearts were stabilized for 20 minutes at a constant perfusion
pressure of 80 mm Hg and then subjected to low-flow global
ischemia (3% of baseline) for 25 minutes and reperfused for
60 minutes.

All hearts were placed in a 10-mm nuclear magnetic
resonance (NMR) sample tube and inserted into a 1H/31P
double-tuned probe situated in an 89-mm-bore, 14-T super-
conducting magnet (Bruker Avance III). Dynamic changes in
cardiac high-energy phosphate content and intracellular pH
were monitored by 31P NMR spectroscopy, simultaneously
with the continuous LV functional recording. Spectra were
collected at 60° flip angle, 22.8-μs pulse, and 2.14-second
delay and analyzed with an ACD/1D NMR Processor
(Advanced Chemistry Development, Inc). Two consecutive 5-
minute baseline 31P NMR spectra (120 scans) were collected
after stabilization, followed by 10 consecutive 2-minute–
30-second spectra (60 scans) during ischemia and twelve
5-minute spectra (120 scans) during reperfusion. The 31P
NMR resonance areas corresponding to ATP, phosphocreatine
(PCr), and inorganic phosphate (Pi) were fit to Lorentzian
function and, by comparing the peak areas of fully relaxed
(recycle time of 20.4 seconds) and those of partially
saturated spectra (recycle time of 2.54 seconds), the correc-
tion factors for saturation were calculated for ATP (1.0), PCr
(1.33), and Pi (1.3). The mean value of ATP concentration
of WT hearts previously measured with HPLC was used to
 calibrate the ATP peak area of the baseline 31P NMR spectrum
for the respective groups. Concentrations of other metab-
olites were calculated using the ratio of their peak area to the
average of β- and γ-ATP peak areas, and intracellular pH (pHi)
was determined by the chemical shift of Pi relative to PCr. At
the end of the experiments, hearts were either processed for
infarct size measurement or freeze-clamped and stored in
−80°C for further analysis.

Myocardial Infarct Size Measurement
Reperfused hearts were first frozen at −20°C and then cut
manually into 6 transverse slices of approximately equal
thickness (≈0.8 mm). Tissue sections were weighed and
incubated in 1% triphenyl tetrazolium chloride (TTC) for
15 minutes at 37°C and then fixed in 10% formaldehyde for

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12 hours at room temperature, followed by digital photography. The areas of the infarct and ventricle in each slice were measured with computer planimetry. The weight of the infarct tissue in each section is calculated as (infarct area/LV area) × LV weight, as described previously. The infarct size was expressed as (total infarct weight/total LV weight) × 100%.

**ROS and Mitochondrial Membrane Potential Measurements of Perfused Hearts**

ROS production and mitochondrial membrane potential of the perfused heart were measured during baseline perfusion and early reperfusion, respectively, by adapting a method of fluorescent labeling and confocal microscopy as previously described. To ensure the signals between the groups are comparable, we used a Zeiss LSM 510 confocal microscope equipped with a 63x, 1.3-NA oil immersion objective. Dual excitation imaging of Mitosox Red was achieved by alternating excitation at 405 and 514 nm and collecting emissions between 560 and 615 nm, and the signal ratio of Mitosox Red at 405-nm and 514-nm excitation was used to indicate specific mitochondrial O$_2^-$ production. Similarly, simultaneous imaging of Mitosox Red (ex: 405, 514 nm; em: 560 to 615 nm), Mitotracker Green (ex: 488 nm; em: 505 to 530 nm), and Vybrant Ruby (ex: 633 nm; em: 657 to 700 nm) was performed by tandem excitation.

**Lactate Output Measurement**

Coronary effluents were collected before and during I/R, and the flow rate, volume of coronary effluents, and heart weight were recorded so as to calculate the rate of total lactate output from the hearts. Total lactate content in the coronary effluent was measured with a Sigma assay kit. Total lactate output was measured from the start of ischemia to the first 5 minutes of reperfusion; it was calculated through subtraction of the lactate content in the perfusate to the first coronary flow rate and heart weight.

**NAD$^+$/NADH and NADP$^+$/NADPH Measurements**

Myocardial levels of NAD$^+$ and NADH were measured using tissue homogenates with the EnzyChrom NAD$^+$/NADH Assay Kit, according to the manufacturer’s protocol (Bioassay Systems). NADP$^+$ and NADPH levels were measured with EnzyChrom NADP$^+$/NADPH Assay Kit (Bioassay Systems). The NAD$, NADH, NADP$, and NADPH concentrations in the sample were determined by colorimetric measurement at 565 nm and the NAD$^+$ and NADP$^+$ standard curves.

**Glutathione Levels**

Total (GS), reduced (GSH), and oxidized (GSSG) glutathione concentrations were measured using the Calbiochem Glutathione Assay Kit II, based on a 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)–glutathione reductase recycling system. Kinetic measurement of the absorbance of a yellow DTNB-reduced product at 405 nm measured the total GS in the sample. Detection of GSSG was performed on duplicate samples with an extra reaction with 2-vinylpyridine.

**Measurements of Enzyme Activities and Metabolites**

Aconitase activities were measured spectrophotometrically with the use of an assay kit from Cayman. Malate/aspartate shuttle imaging. Confocal imaging was performed using a Zeiss LSM 510 confocal microscope equipped with a 63x, 1.3-NA oil immersion objective. Dual excitation imaging of Mitosox Red was achieved by alternating excitation at 405 and 514 nm and collecting emissions between 560 and 615 nm, and the signal ratio of Mitosox Red at 405-nm and 514-nm excitation was used to indicate specific mitochondrial O$_2^-$ production. Similarly, simultaneous imaging of Mitosox Red (ex: 405, 514 nm; em: 560 to 615 nm), Mitotracker Green (ex: 488 nm; em: 505 to 530 nm), and Vybrant Ruby (ex: 633 nm; em: 657 to 700 nm) was performed by tandem excitation.
was reconstituted by adding cytosolic components of heart homogenates in saturating concentrations to intact cardiac mitochondria, and the shuttle capacity was calculated by measuring the rate of oxidation of NADH monitored at 340 nm at 37°C as previously described.\textsuperscript{23,24} The rate of the change in absorbance with and without added substrates for the shuttle was normalized to the amount of added mitochondrial protein. The lactate content in the coronary effluent was measured with the use of a Sigma assay kit. Lactate output was calculated by subtracting the lactate content in the perfusion buffer and normalized to coronary flow rate and heart weight. The total lactate production during ischemia was estimated by summing the lactate output during ischemia and at the onset of reperfusion as previously described.\textsuperscript{20}

### Mitochondria Isolation and Respiration Assay

Mitochondria from WT, DN, and TG hearts were isolated as described by Boehm et al.\textsuperscript{25} Briefly, freshly isolated hearts were rinsed with sucrose-containing mitochondrial isolation buffer. Heart tissue was chopped into pieces, trypsinized, and homogenized with a homogenizer. Mitochondria were purified through centrifugations. Mitochondria respiration assay was performed using Oxytherm (Hansatech) with pyruvate, malate, and succinate as substrates.

### ROS Measurements in Cultured Cardiomyocytes

Adult cardiac myocytes were isolated from WT, TG, or DN mouse heart (3 to 6 months old) following the standard enzymatic protocol as reported previously.\textsuperscript{26} Freshly isolated myocytes were plated at a density of 0.5 to 1 x 10^4/cm² on coverslips precoated with 20 μg/mL laminin (Invitrogen) for 2 hours. Adenoviruses containing mitochondria-targeted Hyper (mt-Hyper) or nontargeted Hyper (cyto-Hyper) were added on the attached myocytes at a multiplicity of infection of 100. The Hyper is a newly developed and genetically encoded fluorescent indicator for intracellular hydrogen peroxide.\textsuperscript{27} Myocytes were cultured in M199 medium (Sigma) containing 10 μmol/L blebbistatin for up to 72 hours. Hyper fluorescence with adequate intracellular localization can be observed 48 hours after gene transfer.

For confocal imaging, we used a Zeiss LSM 510 Meta confocal microscope equipped with a 40×, 1.3-NA oil immersion objective. For Hyper imaging, we used dual excitation by alternating excitation at 405 and 488 nm and collecting emissions at >505 nm. The ratio of emission fluorescence at 488-nm and 405-nm excitation was used to indicate the intracellular or intramitochondrial hydrogen peroxide level. Real-time monitoring of Hyper fluorescence during I/R was achieved through serial 2-D imaging at a sampling rate of 30 s/frame. Cellular ischemia was achieved by perfusing the cells with oxygen-deprived solution\textsuperscript{28} containing (in mmol/L) NaCl 137, KCl 4.9, CaCl₂ 1, MgSO₄ 1.2, NaH₂PO₄ 1.2, HEPES 20, and Na₂S₂O₄ 2, pH 7.4. Reperfusion was achieved with perfusion with the same solution without Na₂S₂O₄ and with 15 mmol/L glucose. Solution oxygen level was monitored with an oxygen probe (microelectrode). For measurements of mitochondrial membrane potential, cells were loaded with TMRM (20 nmol/L) and images were taken with a Zeiss LSM 510 confocal microscope.

To monitor mitochondrial NADH level, we used an Olympus FV1000 2-photon microscope with a ×25 water immersion objective. Serial 2-dimensional scanning images were taken with 710-nm excitation and at a sampling rate of 30 s/frame during cellular I/R treatment.

### Western Blot Analysis

Heart tissues were lysed with RIPA buffer with a protease inhibitor cocktail. Protein samples were denatured with Lammeli buffer with the use of β-mercaptoethanol, separated via SDS-PAGE, and transferred to PVDF membranes according to standard procedures. The blots were probed with specific antibodies according to the manufacturer’s instructions. Anti-GAPDH and anti–hypoxia inducible factor 1α (HIF1α) antibodies were obtained from Cell Signaling, and anti-PPARα antibodies were from Cayman.

### RNA Isolation and Real-Time PCR

Total RNA was isolated from frozen LV tissue using the RNeasy Kit (Qiagen), and cDNA was synthesized using Omniscript reverse synthase and random hexamers according to manufacturers’ guidelines. Real-time PCR was performed using SYBR green (Bio-Rad). mRNA levels of peroxisome proliferator-activated receptor –α (Pparα), medium-chain acyl-coenzyme A dehydrogenase (Mcad), pyruvate dehydrogenase kinase isoform 4 (Pdk4), and glucose transporter-1 (Glut1) were detected using primers described in the Table. The real-time PCR results for the mRNA levels of each gene were normalized to 18S rRNA levels.

### Statistical Analyses

All data are presented as mean±SEM. One-way ANOVA with Newman–Keuls post-test is used. Two-way repeated-measures ANOVA was used to compare the responses to I/R. Nonparametric analysis was performed for data not shown to be normally distributed. Comparisons among the groups at all time points during the ischemia or reperfusion period were made by using Friedman’s 2-way ANOVA. Analyses were
performed with GraphPad Prism (GraphPad Software), and a value of $P<0.05$ was considered statistically significant.

**Results**

**Gain- or Loss-of-Function of Nox Altered Redox States in the Heart With No Significant Changes in Myocardial Contractile Function and Energetics**

We compared oxidative stress and redox state in WT and TG or DN mouse hearts at 3 months of age. We first examined the cellular ROS levels and mitochondrial membrane potential ($\Delta\psi$) in isolated hearts under normal perfusion conditions by using fluorescent indicators (DCFH-DA and TMRE) and confocal imaging. No significant difference in cellular ROS level or $\Delta\psi$ was observed in either TG or DN hearts under baseline conditions (Figure 1A through 1C). It is known that the DCFH fluorescent method is not specific and is inadequate for quantitation.\(^{29}\) Therefore, we also examined the basal mitochon- 
drial or cytosolic H$_2$O$_2$ levels with another indicator, Hyper, in cultured adult cardiac myocytes isolated from the 3 groups of mice. We found no difference in H$_2$O$_2$ levels among the 3 genotypes in either cellular compartment (Figure 1D through 1F). Interestingly, the aconitase activity was decreased by $\approx$30% in TG hearts (Figure 1G), suggesting that mild oxidative damage might have accumulated in TG hearts despite the undetectable changes of the basal ROS levels.

We then determined the intracellular redox pairs in the TG and DN hearts. In DN hearts, a marked decline in NAD(P)$^+$/NAD(P)H and an increase in GSH/GSSG were observed, indicating a significantly reduced state (Figure 1H through 1J). Noticeably, similar changes were observed in the hearts with Nox2/4 double deletion (DKO, Figure 1I and 1J), suggesting DN inhibits both Nox2 and Nox4. This is consistent with the observation that the dominant-negative Nox4 mutant competes with the endogenous Nox for the interaction with p22$^{phox}$ and that overexpression of DN inhibits activities of both Nox2 and Nox4 by sequestering the p22$^{phox}$.\(^{30}\) On the other hand, TG hearts exhibited a remarkable increase in NAD(P)$^+$/NAD(P)H and a decrease in GSH/GSSG, indicating significant oxidative stress (Figure 1H through 1J). In addition, the total NAD(P)$^+$/NAD(P)H pool was increased in the TG hearts (Figure 1K and 1L), likely due to a chronic adaptive response in attempt to restore the NAD(P)$^+$/NAD(P)H ratio. Furthermore, the malate/aspartate shuttle capacity increased by nearly 2-fold in TG and DN hearts, suggesting an increased exchange of cytosolic and mitochondrial NADH pools (Figure 1M).

To elucidate the functional consequences, we evaluated cardiac contractile function and myocardial energetics in isolated perfused heart using $^{31}$P NMR spectroscopy as previously described.\(^ {31}\) All hearts were supplied with a mixture of substrates containing fatty acids, glucose, lactate, and a physiological level of insulin (50 $\mu$U/mL) to mimic in vivo conditions. Both TG and DN hearts showed comparable heart weights (Figure 2A) and normal contractile function at 3 months of age (Figure 2B). Moreover, myocardial ATP content in DN and TG hearts was not different from that of the WT hearts (Figure 2C). Interestingly, TG hearts displayed a small but significant reduction in PCR content reflected as a lower PCR/ATP ratio (Figure 2D), suggesting a moderate decrease of energy reserve. This is consistent with the lower NADH level and a trend of lower mitochondrial respiration rate in this group of hearts (Figures 1I and 2E).

**I/R Injury Was Exacerbated in Both TG and DN Hearts**

We next examined the functional outcome of TG and DN hearts in response to I/R by subjecting the isolated perfused hearts to low-flow ischemia for 25 minutes (3% of normal coronary flow) followed by 1-hour reperfusion. The contractile function of both transgenic hearts was identical to that of the WT hearts at baseline. However, TG hearts showed significantly impaired posts ischemic contractile performance, as evidenced by increases in LV end-diastolic pressure, decreases in LV developed pressure, and heart rate after reperfusion (Figure 3A through 3C). The overall functional recovery, estimated by rate-pressure product, was 17% of the baseline in TG compared with 69% in WT hearts (Figure 3D). Unexpectedly and contrary to our prediction of increased resistance to I/R injury, the DN hearts displayed the poorest...
recovery among the 3 groups (Figure 3A through 3D). All DN hearts failed to resume beating during the entire period of reperfusion (Figure 3C). Consistent with the functional response, TTC staining at the end of reperfusion revealed infarct size/LV area in the order of DN>TG>WT hearts (Figure 3E and 3F).

Figure 1. Overexpression of Nox4 (TG) promoted oxidative stress, while overexpression of dominant-negative Nox4 (DN) increased reductive stress in mixed-substrate perfused hearts at baseline, without significant changes in cellular ROS levels. (A and B) Cellular ROS levels and mitochondrial membrane potential were quantified by the fluorescent intensities of DCFH-DA and TMRE normalized against DAPI. (C) Representative confocal micrographs of WT, TG, and DN heart sections stained with DAPI, DCFH-DA, and TMRE. (D) Confocal images of cultured adult cardiac myocytes from WT, TG, and DN mice loaded with mitochondrial membrane potential indicator (TMRE) or expressing the hydrogen peroxide indicator in mitochondria (mt-Hyper) or cytosol (cyto-Hyper). Baseline fluorescence (488/405 ratio) of (E and F) mt-Hyper or cyto-Hyper in cultured adult cardiac myocytes from WT, TG, and DN mouse (n=5 to 11 cells from 3 hearts). (G) Aconitase activities, (H) GSH/GSSG ratio, (I) NAD+/NADH, and (J) NADP+/NADPH ratio were measured in tissue homogenates. (K) NAD+ and (L) NADP+ pools in the WT, TG, double knockout (DKO), and DN hearts under baseline perfusion were measured. (M) Malate/aspartate shuttle was reconstituted by adding cytosolic components of heart homogenates to intact cardiac mitochondria, and the shuttle capacity was estimated by measuring the rate of oxidation of NADH monitored at 340 nm at 37°C (n=3 to 7 hearts/group for A through C and G through L). *P<0.05, vs WT. DAPI indicates 4′,6-diamidino-2-phenylindole; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DKO, double knockout; DN, dominant negative; GSH, glutathione; GSSG, glutathione disulfide; ROS, reactive oxygen species; TG, transgenic; TMRE, tetramethylrhodamine ethyl ester; TMRM, tetramethylrhodamine methyl ester; WT, wild-type.

Poor Recovery of the DN Hearts After Ischemia Was Associated With Severe Impairment of Myocardial Energetics

To determine whether changes in cardiac bioenergetics contribute to the differential functional responses during I/
we measured the content of high-energy phosphates and pH by \(^{31}\text{P}\) NMR spectroscopy simultaneously with the recording of contractile function in all 3 groups of hearts. Adequate perfusion before ischemia was evidenced by normal levels of PCr, ATP, Pi, and pH in all 3 groups of hearts (Figure 4). During ischemia, PCr was depleted and ATP declined in all groups, whereas Pi increased substantially (Figure 4A, 4B, and 4D). The TG hearts displayed a similar decline in energetic profile as that of the WT hearts during ischemia, but the recovery of PCr during reperfusion was significantly impaired (Figure 4A through 4C). In contrast, the DN hearts showed a rapid and substantial decline of ATP and a greater accumulation of Pi during ischemia compared with WT and TG hearts (Figure 4B and 4D). During reperfusion, the recovery of PCr, ATP, and pH in the DN hearts were also attenuated and failed to reach the level of WT or TG hearts.
The data collectively suggested that DN hearts developed an earlier and greater impairment of myocardial energetics during the ischemia period. These findings corroborated with the failure to resume beating on reperfusion and the greater infarct size in the DN hearts (Figure 3) and suggested that the DN hearts suffered substantial injury during ischemia.

Mitochondrial Dysfunction and ROS Production in TG and DN During I/R

It is well recognized that increased ROS production during I/R may lead to mitochondrial dysfunction and energetic defects.32 To determine whether oxidative stress underlies excessive I/R injury in TG and DN hearts, we monitored ROS levels in perfused hearts and isolated cardiac myocytes subjected to simulated I/R. Isolated perfused hearts were loaded with ROS indicators (Mitosox Red and DCFH-DA) at the onset of reperfusion and the greater infarct size in the DN hearts (Figure 3) and suggested that the DN hearts suffered substantial injury during ischemia.

(Figure 4A through 4C). The data collectively suggested that DN hearts developed an earlier and greater impairment of myocardial energetics during the ischemia period. These findings corroborated with the failure to resume beating on reperfusion and the greater infarct size in the DN hearts (Figure 3) and suggested that the DN hearts suffered substantial injury during ischemia.

Interestingly, cardiac myocytes from DN mice presented robust increases in both mitochondrial and cytosolic H$_2$O$_2$ levels while demonstrating a small decline of the NADH level during ischemia (Figure 5F). Since the preischemic NADH level was twice as high in the DN compared with WT and TG groups (Figure 1K), the DN myocytes remained in a highly reduced state despite the small decline of NADH level. The decline rather than further accumulation of NADH in DN myocytes is
highly suggestive of NADH consumption for excessive ROS production in the mitochondria during ischemia.

Collectively, the TG hearts demonstrated increased oxidative burden throughout the protocol and a greater cardiac dysfunction after I/R compared with the WT hearts. The DN hearts presented the most reduced state under normal perfusion but robust mitochondrial ROS production during ischemia. Thus, both oxidative and reductive stresses could sensitize the hearts to I/R injury through increased ROS production.

**Increasing the NAD^+/NADH Ratio Rescued Cardiac Dysfunction in the DN Hearts Subjected to I/R**

To test if the elevated NADH level observed in DN hearts underlies the greater ROS generation and cardiac dysfunction when subjected to ischemic injury, we sought to lower the NADH production through substrate metabolism. We removed fatty acids, lactate, ketone, and insulin from the perfusate and supplied the hearts with glucose (10 mmol/L) supplemented with a low concentration of pyruvate (0.5 mmol/L). Mouse hearts perfused with 10 mmol/L glucose in the absence of insulin were energetically unstable. The addition of 0.5 mmol/L pyruvate is essential to meet the minimum requirement of substrate supply. Indeed, DN hearts perfused with glucose/pyruvate buffer had a lower NADH level and a higher NAD^+/NADH ratio compared with hearts perfused with mixed substrate buffer at baseline and end-ischemia (Figure 6A through 6C). Similar changes in GSH/GSSG were observed (Figure 6D and 6E). We next assessed ROS production at early reperfusion by using Mitosox Red and DCFH-DA. Mitochondrial O_2^- production was not affected in WT or TG hearts by switching the substrates but was significantly lowered in DN hearts perfused with glucose/pyruvate (Figure 6F). The cellular ROS production was significantly reduced in both TG and DN hearts with glucose/pyruvate perfusate (Figure 6G).

Although switching the substrates did not affect myocardial energetics or contractile function in any of the 3 groups at baseline (data not shown), functional recovery after I/R was dramatically improved in TG and DN hearts (Figure 7A). Consistently, the infarct sizes of TG and DN hearts perfused with glucose/pyruvate buffer were also significantly reduced.
The lactate production of perfused hearts was similar for the 2 substrates (Figure 7C), suggesting that the beneficial effect of glucose/pyruvate perfusion is unlikely due to altered glycolysis or uncoupling of glycolysis to oxidation. $^{31}$P NMR analysis showed that glucose/pyruvate perfusate markedly improved the recovery of high-energy phosphate content and pH in DN hearts (Figure 7D through 7F).

Figure 5. DN hearts showed a greater ROS formation during I/R. (A) Representative confocal micrographs of WT, TG, and DN heart sections stained with Vybrant ruby/Mitotrackr green and Mitosox red during early reperfusion with mixed substrate buffer. (B) Representative confocal micrographs of WT, TG, and DN heart sections stained with DAPI, DCF-DA, and TMRE during early reperfusion with mixed substrate buffer. (C) Mitochondrial $O_2^-$ production was quantified as fluorescent signal ratio of Mitosox red at 405-nm and 514-nm excitation. (D) Cellular ROS production and (E) mitochondrial membrane potential were quantified as fluorescent intensities of DCFH-DA and TMRE normalized to DAPI signal, respectively ($n=3$ to 5/group). *$P<0.05$ vs WT. (F through H) Representative traces showing the real-time changes of single-cell NADH autofluorescence, mt-Hyper, or cyto-Hyper signals during I/R treatment in DN (F), TG (G), and WT (H) myocytes. For each trace, data are normalized to its own baseline (F/F₀). Similar results were obtained from 2 experiments. DCFH-DA indicates 2',7'-dichlorofluorescein diacetate; DN, dominant negative; ROS, reactive oxygen species; TG, transgenic; TMRE, tetramethylrhodamine ethyl ester; WT, wild-type.
was eliminated (Figures 4B and 7E). Collectively, the data showed that lowering NADH production at baseline by limiting carbon substrate supply reduced mitochondrial ROS production and rescued cardiac dysfunction during I/R in TG and DN hearts.

It has been suggested that cellular ROS level regulates the accumulation of HIF1α, which in turn promotes glucose metabolism and enhances the resistance to ischemic/hypoxia damages. We therefore examined whether the loss of NADPH oxidase activity in DN hearts affected HIF1α level. Under baseline conditions, HIF1α is undetectable in the nuclear fraction of the cardiac tissue of any genotypes (Figure 8A). We also observed no difference in the expression of PPARα, an HIF1α-responsive gene, or its downstream targets at baseline in the 3 groups of hearts (Figure 8B and 8C). At the end of ischemia, accumulation of HIF1α in the nucleus was

Figure 6. Glucose + pyruvate buffer perfusion elevated NAD+/NADH ratio and decreased ROS production in TG and DN hearts, compared with mixed substrate buffer. NAD+/NADH ratio of (A) DN, (B) TG, and (C) WT hearts perfused with the 2 types of buffers were measured at BL, end of ischemia, and end of reperfusion. *P<0.05 glucose + pyruvate vs mixed-substrate perfused hearts. GSH and GSSG levels were measured in the 3 groups of hearts perfused with (D) mixed substrate or (E) glucose + pyruvate buffer, which were harvested at baseline, end-ischemia, and end-reperfusion. *P<0.05 vs WT under the same condition. (F) Mitochondrial O$_2^-$ and (G) cellular ROS production of hearts perfused with the mixed-substrate or glucose + pyruvate buffers after I/R were measured as described in Methods. *P<0.05 vs WT perfused with mixed substrates; #P<0.05 glucose + pyruvate vs mixed-substrate perfused hearts of the same genotype. For all experiments, n=5 to 6/group. BL indicates baseline; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DN, dominant negative; GSH, glutathione; GSSG, glutathione disulfide; ROS, reactive oxygen species; TG, transgenic; WT, wild-type.
observed in all genotypes but at a reduced level in DN (Figure 8A). However, the lower HIF1α was not associated with impaired glycolysis as the lactate production during ischemia was not decreased in DN hearts, suggesting that it might be too soon for the HIF1α accumulated during ischemia to affect glucose metabolism via transcriptional mechanisms in our experimental setting. It is possible that HIF1α-mediated changes become effective during a longer reperfusion period in vivo as we have recently shown.30

Discussion

Using mouse models with cardiac-specific overexpression of NADPH oxidase Nox4 in the WT (TG) and dominant-negative (DN) mutant forms, we have observed novel roles of Nox in cellular redox regulation and in acute responses to I/R. Overexpression of Nox4 or dominant negative Nox4 markedly impacted the redox states of the heart under normal perfusion conditions. TG showed oxidative stress [low GSH/GSSG, high NAD(P)̄/NAD(P)H], while DN displayed reductive stress [high GSH/GSSG, low NAD(P)̄/NADPH]. As expected, the elevated oxidative stress in TG was associated with a moderate increase in I/R injury. It was unexpected, though, that the DN, which presented a highly reduced state due to broad inhibition of Nox, sustained even greater damage by I/R. We have further shown that the I/R injury in the DN was attributable to increased mitochondrial ROS production during ischemia, loss of mitochondrial membrane potential, and exacerbated impairment of myocardial energetics. These defects could be rescued by lowering the NADH production in the DN hearts. Our results, therefore, revealed a critical role of reductive stress in promoting oxidative I/R injury.

Although increased Nox4 activity has been associated with increased oxidative stress, we did not detect significant increases of the ROS levels in the TG hearts from the young mice and under normal perfusion. However, the aconitase activity was decreased in the TG hearts, suggesting that cumulative oxidative damage had occurred despite the modest changes of the ROS level. We also found marked decreases in NAD(P)H and GSH levels in TG hearts. The total NAD(P)̄/NAD(P)H pool was also increased with enhanced exchange between the mitochondria and cytosolic pools.
These changes collectively reflect that chronically increased Nox activity altered metabolism and induced a rigorous antioxidative response at the expense of redox balance. Previous studies have shown that the NADH and NADPH levels can be linked through multiple reactions in the mitochondrial and cytosolic compartments, including reactions catalyzed by nicotinamide nucleotide transhydrogenase, isocitrate dehydrogenase, tricarboxylate carrier, and malate/aspartate shuttle. Increased NADPH consumption in TG may therefore shift the redox equilibrium toward a higher NAD(P)⁺/NAD(P)H ratio. These changes have also promoted the communication of cytosolic and mitochondrial NADH pool, as evidenced by the doubling of the malate/aspartate shuttle capacity, leading to a global change of cellular redox state.

Figure 8. (A) Protein levels of HIF1α in the nuclear and cytosolic fractions of heart tissues harvested at baseline (BL) and end-ischemia. (B) Protein levels of PPARα in the tissue homogenate of WT, TG, and DN hearts were analyzed by Western blot. (C) The mRNA expression levels of PPARα, MCAD, PDK4, and GLUT1 in WT, TG, and DN hearts harvested at baseline were determined by real-time PCR (n=4 to 6/group). *P<0.05 vs WT. DN indicates dominant negative; GLUT1, glucose transporter-1; HIF1α, hypoxia inducible factor 1α; MCAD, medium-chain acyl-coenzyme A dehydrogenase; PCR, polymerase chain reaction; PDK4, pyruvate dehydrogenase kinase isoform 4; PPARα, peroxisome proliferator-activated receptor-α; TG, transgenic; WT, wild-type.
Our study did not address the potential effects of Nox activity on other pathways for NADH production and consumption, but we previously reported that Nox4 was localized on mitochondria and consumes NADH.11 Unlike the TG mice, overexpression of a dominant negative Nox4 protein (DN) resulted in a highly reduced state. We recently showed that the DN robustly bound to p22phox and that transgenic expression of DN exhausted endogenous p22phox in cardiac myocytes, indicating that DN competes not only with endogenous Nox4 but also with Nox2 for p22phox. Consistent with the notion, increases in NAD(P)H level in the DN are greater than those with single deletion of Nox2 or Nox4 but comparable to those with double deletion of Nox2/4.30 Therefore, the DN heart models a general elimination of Nox activity rather than specific loss of function of the Nox4.

The TG hearts demonstrated an increased sensitivity to I/R injury, which was associated with a greater cellular but not mitochondrial ROS production during ischemia and at early reperfusion. This finding is consistent with the detrimental role of Nox4 previously observed in cerebral ischemia36 and supports the notion that activation of Nox4 contributes to I/R injury. Surprisingly, elimination of Nox activity in the DN hearts leads to even more severe I/R damage, raising the possibility of reductive stress. The reductive stress describes an abnormally high reducing environment in the cells with high GSH/GSSG, low NADP+/NADPH,37,38 or low NAD+/NADH ratio.39 It has been suggested that decreases in NAD+/NADH ratio, which have been observed in mitochondrial diseases or metabolic diseases such as diabetes, promote reductive stress.40,41 Increased NADPH level facilitates the reduction of GSSG to GSH, which serves as an important antioxidant mechanism. However, it has been shown that excessive increases in GSH/GSSG ratio paradoxically elevate mitochondrial oxidation and cytotoxicity in cultured cells and cause cardiomyopathy in mice.42–44 Our data also raise a cautionary note in targeting the NADPH oxidase activity for cardioprotection as the reductive stress resulting from the excessive suppression of the system leads to greater I/R injury than activation of the system.

The DN hearts show a unique increase in mitochondrial ROS production during ischemia compared with the TG hearts, which produce more ROS in the cytosol during I/R. The greater mitochondrial ROS production is accompanied by the loss of mitochondrial membrane potential, accelerated ATP depletion, and a stable NADH level during ischemia, suggesting that the ROS generation and mitochondrial function are reciprocally linked under this condition. It has been hypothesized that very low NAD+/NADH ratio associated with severely reduced state in combination with slow electron flow through the electron transport chain can facilitate the direct transfer of the electrons to oxygen (leaking) to produce superoxide.45–47 Although this notion is largely based on in vitro experiments using isolated mitochondria, our data suggest that such a scenario is the case for the DN heart subjected to ischemia in which excessive NADH level meets the slow electron transport chain flow. It should be mentioned that ischemia elevates NADH level (NAD+/NADH ratio declines) in WT and TG hearts, as we do detect increased NADH autofluorescence and decreased NAD+ /NADH ratio, but does not cause electron leak in these hearts during ischemia. Therefore, a threshold of the NAD+/NADH ratio (or NADH level) may exist above which significant electron leaks can happen. The high level of NADH in DN hearts during normal perfusion likely renders it close to the threshold, which allows NADH to donate electrons for robust ROS production during slowed electron transport chain flow. Damages caused by the ROS may lead to further electron leak and form a vicious circle of mitochondrial injury leading to the severe insult as we have observed in DN but not in WT and TG during ischemia.

If the reductive stress worsens ischemic injury, approaches that restore the redox balance would be protective. Indeed, we were able to improve the posts ischemic recovery in the DN by partially restoring the NAD+/NADH ratio through modulating substrate supply in the perfused hearts. It has been shown that the mitochondrial NADH level can be modulated by altering substrate supply in isolated perfused hearts without affecting MVO2 or contractile function.24,48 By removing fatty acids, lactate, ketone, and insulin from the perfusate, we aim to restrict substrate availability to the tricarboxylic acid cycle, hence reducing the NADH production. This approach has successfully increased NAD+/NADH ratio in DN hearts before ischemia and lowered their vulnerability to I/R injury. We have recently demonstrated that decreased NAD+/NADH ratio leads to an increase in protein acetylation and sensitization of the mitochondrial permeability pore transition.49 Therefore, restoration of the NAD+/NADH ratio may improve post I/R recovery by stabilizing mitochondrial permeability pore during reperfusion.

It has been suggested that the lack of Nox-mediated ROS generation will prevent the accumulation of HIF1α and consequently impair the switch of fatty acid to glucose utilization during ischemia, thus causing more severe damage. This is unlikely in our experimental setting, because we did not observe any downregulation of glycolytic activity in DN hearts during ischemia or a heightened uncoupling of glycolysis and glucose oxidation, which occurs during excessive fatty acid oxidation. Furthermore, the lactate production during ischemia is similar in each genotype with either set of substrates despite the dramatic differences in the functional phenotype. These observations, however, do not rule out the possibility that fatty acid oxidation could promote more mitochondrial ROS formation. Fatty acids generate not only reducing equivalent NADH but also FADH2 that enters the electron transport system through electron transfer...
flavoproteins. The FADH$_2$ flux to the electron transport system in mixed substrate perfused hearts could compete for the electron transfer at coenzyme Q with NADH, further enhancing reverse electron flow and O$_2^-$ generation.$^{14,50,51}$

In summary, the present study provides strong evidence that elimination of Nox activity causes reductive stress and renders the heart vulnerable to ischemic injury through paradoxical oxidative damage of the mitochondria. On the other hand, overexpression of Nox4 leads to oxidative stress and contractile dysfunction after I/R, suggesting that the imbalance of redox status toward either extreme in the heart deteriorates the outcome of I/R injury. Studies using genetic models are for proof-of-concept rather than defining quantitative relationships. Nevertheless, the findings highlight the complexity of developing therapeutic strategies by modulating the cellular redox states. Antioxidant therapies or specific NADPH oxidase inhibitors have been developed to treat diseases attributed to oxidative stress. Our study has demonstrated that redox couples are intricately linked to maintain the delicate balance of cellular redox status. Any substantial disturbance of the redox status, including excessively reduced state, will ultimately lead to oxidative damage. This warrants caution, especially for pathological conditions such as metabolic diseases and mitochondrial dysfunction, in which both redox imbalance and oxidative stress are observed.

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Disclosures

None.

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