Heart

Nicotinamide Adenine Dinucleotide Phosphate Oxidase-4–Dependent Upregulation of Nuclear Factor Erythroid–Derived 2-Like 2 Protects the Heart During Chronic Pressure Overload

Ioannis Smyrnias, Xiaohong Zhang, Min Zhang, Thomas V.A. Murray, Ralf P. Brandes, Katrin Schröder, Alison C. Brewer, Ajay M. Shah

See Editorial Commentary, pp 499–501

Abstract—The transcription factor nuclear factor erythroid–derived 2-like 2 (Nrf2) controls a network of cytoprotective genes. Neither how Nrf2 is activated in the heart under hemodynamic overload nor its role and mechanism of action are known. This study aimed to investigate the activation and role of Nrf2 during chronic cardiac pressure overload. We first compared the responses of Nrf2−/− mice and wild-type littermates to chronic pressure overload. Hearts of Nrf2−/− mice showed impaired antioxidant gene expression, increased hypertrophy, and worse function compared with those of wild-type littermates after overload. Hearts of Nrf2−/− mice had increased mitochondrial DNA damage, a caspase 8/BH3-interacting domain death agonist–related cleavage of mitochondrial apoptosis–inducing factor, nuclear DNA damage, and cell death. Nrf2 activation was under the control of the endogenous reactive oxygen species–generating enzyme nicotinamide adenine dinucleotide phosphate oxidase-4, both in vivo and in vitro. In mice with cardiac-specific overexpression of nicotinamide adenine dinucleotide phosphate oxidase-4, Nrf2 deletion significantly attenuated their protective phenotype during chronic pressure overload. This study identifies nicotinamide adenine dinucleotide phosphate oxidase-4–dependent upregulation of Nrf2 as an important endogenous protective pathway that limits mitochondrial damage and apoptosis–inducing factor–related cell death in the heart under hemodynamic overload. (Hypertension. 2015;65:547–553. DOI: 10.1161/HYPERTENSIONAHA.114.04208.) • Online Data Supplement

Key Words: cell death  ■  Nfe2l2 protein, mouse  ■  Nox4 protein, mouse  ■  oxidative stress

A chronically increased cardiac workload predisposes to heart failure. Heart function may initially be compensated, but sustained hemodynamic stress leads to contractile impairment, myocyte death, fibrosis, and ventricular dilatation. The balance between reactive oxygen species (ROS) production and cellular antioxidant levels is considered an important determinant of the response to chronic hemodynamic stress.1,2 Excessive ROS are derived from mitochondrial dysfunction and other cellular sources (eg, nicotinamide adenine dinucleotide phosphate oxidases and uncoupled nitric oxide synthases), and they induce detrimental effects, such as cardiomyocyte ionic abnormalities, energetic deficit, and cell death.2 However, ROS can also induce more specific changes by modulating intracellular signaling pathways, with associated beneficial or detrimental effects.2

Endogenous antioxidant and detoxification systems are a key determinant of ROS levels and effects. Although the cardiac activities of several antioxidant enzymes during hemodynamic stress have been quantified, the global mechanisms regulating myocardial antioxidant status are poorly understood. Nuclear factor erythroid–derived 2-like 2 (Nrf2) is a transcription factor that is a master regulator of a cytoprotective gene program, encoding not only antioxidant genes but also numerous detoxification enzymes that conjugate oxidation products and export them out of cells. It binds to antioxidant- or electrophile–response elements within the promoters of target genes to control basal and inducible expression.3 Nrf2 levels are normally low because of Nrf2 binding to an inhibitor protein, Kelch-like ECH–associated protein 1, that promotes its proteosomal degradation.3 Kelch-like ECH–associated protein 1 can be readily oxidized or react with electrophiles, leading to its dissociation from Nrf2 and allowing Nrf2 to activate target genes. Nrf2 may therefore be activated under cellular stress conditions associated with increased ROS production, such as organ injury and inflammation.4 Nrf2 was reported to protect against oxidative stress...
stress–induced injury in cultured cardiomyocytes and exert beneficial cardiac effects during pressure overload in vivo. However, neither how it is activated in the overloaded heart nor the mechanisms underlying its protective effects are known.

Recent studies indicate that the ROS-generating enzyme nicotinamide adenine dinucleotide phosphate oxidase-4 (Nox4) exerts beneficial cardiac and vascular effects during chronic hemodynamic stress. Nox4 is a member of the Nox family proteins that differ from most other ROS-generating enzymes in that ROS production is their primary function. Nox enzymes generate ROS using nicotinamide adenine dinucleotide phosphate as an electron donor and are involved in redox signaling in many tissues. The Nox isoenzymes present in the cardiovascular system, ie, Nox1/2/4/5, have distinct tissue distributions and subcellular localization. Among these, Nox4 is unique in being constitutively active and regulated predominantly by changes in abundance. Nox4 was shown to protect the heart during chronic pressure overload, at least in part through a hypoxia-inducible factor-1–mediated increase in myocardial capillary density. Intriguingly, the beneficial vascular effects of Nox4 in a model of inflammatory injury were attributed, at least in part, to an induction of Nrf2. Forced overexpression of Nox4 activates the Nrf2 pathway, as expected with maneuvers that increase ROS levels, but the endogenous source(s) of ROS responsible for activating Nrf2 during hemodynamic stress is unknown.

This study investigated the role of endogenous Nox4 in physiologically activating Nrf2 and the mechanisms through which Nrf2 activation influences functional outcome in the chronically overloaded heart.

Materials and Methods

Detailed Materials and Methods are provided in the online-only Data Supplement.

Animal procedures were performed in accordance with UK national guidelines. Nrf2−/−, Nox4−/−, and cardiomyocyte-targeted Nox4 transgenic (Nox4TG) mice were described previously. Aortic constriction or sham surgery and echocardiography were performed as described. Cardiomyocyte area, interstitial fibrosis, and capillary density were quantified in heart sections. Apoptosis was assessed by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling staining. Mitochondrial DNA (mtDNA) damage was assessed with a quantitative polymerase chain reaction–based assay in which the amount of a 10-kb mtDNA polymerase chain reaction product is inversely related to DNA damage. Protein carbonyl levels were measured using a spectrophotometric kit (Cell Biolabs, Cambridge, United Kingdom). Neonatal rat ventricular myocytes (NRVM) were prepared as described.

Data are presented as means±SEM. Comparisons were made by 1-way or repeated measures ANOVA, followed by Bonferroni test for multiple comparisons. *P<0.05 was considered significant.

Results

Upregulation of an Nrf2-Regulated Gene Program During Chronic Pressure Overload

Nrf2 protein levels were significantly upregulated in the myocardium of wild-type (WT) mice after aortic constriction (Figure 1A). Pressure overload significantly increased the mRNA expression of the Nrf2 targets glutamate-cysteine ligase (Gclc), glutathione-S-transferase α2 (Gsta2), and heme oxygenase 1 (Hmox1) in WT littermates but not in Nrf2−/− mice (Figure 1B–1D). Levels of nicotinamide adenine dinucleotide phosphate-quinone-oxidoreductase 1 (Nqo1), catalase (Cat), and thioredoxin reductase 1 (Txnrd1) were lower in Nrf2−/− animals than in WT littermates, but pressure overload per se did not alter expression levels (Figure 1E–1G).

Nrf2-Null Mice Develop Exacerbated Pressure Overload–Induced Cardiac Dysfunction

Nrf2−/− mice and WT littermates had similar baseline cardiac dimensions and contractile function by echocardiography groups (Figure S1 in the online-only Data Supplement). After 6 weeks of suprarenal abdominal aortic constriction, Nrf2−/− mice developed significantly greater cardiac dilatation and contractile impairment than WT littermates (Figure S1). Nrf2−/− mice developed greater cardiac hypertrophy than WT littermates, both at whole heart and cardiomyocyte level, as well as larger
Nrf2 Deficiency Increases Myocardial Oxidative Stress, mtDNA Damage, and Caspase 3–Independent Cell Death

To assess mechanisms underlying the protective effects of Nrf2 during hemodynamic stress, we first assessed oxidative stress. The myocardial protein carbonyl content was substantially higher in Nrf2−/− mice than in WT littermates after pressure overload (Figure 2A). We found significantly higher levels of mtDNA damage in Nrf2−/− hearts than in WT littermates (Figure 2B). Myocardium of Nrf2−/− mice also showed more terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling–positive cells than that of WT littermates under stress (Figure 2C).

To investigate how Nrf2-related oxidative stress and mitochondrial dysfunction modulate cell death, we first assessed the release of cytochrome c from mitochondria into the cytosol, which is associated with caspase 3–mediated apoptosis.12 There was difference neither in cytosolic cytochrome c levels between myocardium of Nrf2−/− mice and that of WT littermates nor in the levels of cleaved caspase 3 or Bcl-2 protein among groups (Figure S2). Apoptotic cell death pathways can be amplified through cleavage of the Bcl-2 family protein BH3-interacting domain death agonist (Bid) by caspase 8 to form truncated Bid, which contributes to outer mitochondrial membrane events.13,14 Pressure-overloaded hearts of Nrf2−/− mice showed significantly increased caspase 8 levels compared with that of Nrf2−/− sham-operated animals, whereas caspase 8 levels were unaffected by overload in WT littermates (Figure 3A). The truncated Bid:uncleaved Bid ratio was also significantly increased in Nrf2−/− hearts under stress (Figure 3B).

A cytochrome c–independent mechanism downstream of caspase 8/truncated Bid activation is the cleavage of mitochondrial apoptosis–inducing factor (AIF) and its nuclear translocation to mediate damage through binding to DNA.15,16 There was a substantial increase in cleaved AIF in nuclear and total fractions from hearts of Nrf2−/− mice compared with WT littermates after pressure overload (Figures 3C and S2D, respectively). Nuclear DNA damage, assessed by the levels of phosphorylated H2AX (γ-H2AX) that is activated in response to DNA double-strand breaks,17 was also significantly higher in Nrf2−/− hearts (Figure 3D).

Endogenous Nox4 Activates Cardiomyocyte Nrf2 During Overload Stress

We next investigated the relationship between cardiomyocyte Nrf2 activation and the increase in Nox4 levels that also occurs during hemodynamic overload.7 In cultured NRVM treated with phenylephrine to simulate hemodynamic stress, there was a significant increase in Nox4 and Nrf2 protein levels (Figure 4A and 4B). Phenylephrine-induced increases in Nrf2 were abrogated by shRNA-mediated knockdown of Nox4 (Figure 4B). Phenylephrine upregulated the Nrf2 target genes Hmox1, Gclc, and Gsta2, and these increases were abolished in cells in which either Nox4 or Nrf2 had been knocked down (Figures 4C–4E and S3A–S3D, respectively). We tested the effects of knocking down Nox2, a Nox isoform that is also upregulated by phenylephrine, but this did not affect the changes in Nrf2 protein levels or Nrf2 target genes in NRVM (Figure S3E–S3I). In NRVM in which Nox4 levels had been knocked down (and where the phenylephrine-induced increase in Nrf2 target genes was blunted), the activation of Nrf2 by knockdown of Kelch-like ECH–associated protein 1 resulted in significant upregulated of Nrf2 target genes (Figure S4), indicating that the Nrf2 pathway downstream of Nox4 remained intact.

To extend these results to the in vivo setting, we studied Nox4−/− mice and WT littermates. Basal Nrf2 levels were slightly higher in Nox4−/− mice than in WT littermates, but the stress-induced upregulation of Nrf2 observed in WT littermates after aortic constriction was completely abolished in Nox4−/− mice (Figure 5A). The basal mRNA levels of Gclc, Hmox1, and Gsta2 were similar in WT and Nox4−/− mice, but the upregulation observed in WT littermates after aortic constriction was inhibited in Nox4−/− mice (Figure 5B–5D). No differences were observed in Cat, Nqo1, or Txnr1 mRNA levels in either group (Figure S5).

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**Figure 2.** Effect of nuclear factor erythroid–derived 2-like 2 (Nrf2) deficiency on myocardial oxidative stress, mitochondrial DNA damage, and cell death. A, Myocardial protein carbonyl content; n=4 to 6 per group. B, Mitochondrial DNA (mtDNA) damage shown relative to wild-type (WT) control hearts; n=3 per group. C, Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL)–positive cells per square millimeter of heart tissue; n=5 per group; *P<0.05 and **P<0.01.
Taken together, these results identify Nox4 as a crucial endogenous upstream regulator of Nrf2 activation in the heart during hemodynamic stress.

**Role of Nrf2 Activation in the Cardioprotective Effects of Nox4 During Pressure Overload**

We previously reported that myocardial Nox4 is protective against the detrimental consequences of chronic pressure overload, and we report here that Nrf2 also exerts similar protective effects. To determine the contribution of Nrf2 to the protective effects of Nox4, we undertook crosses of cardiomyocyte-specific Nox4TG and Nrf2+/− mice to generate Nox4TG/Nrf2+/+ and Nox4TG/Nrf2−/− mice. These animals, as well as WT and Nrf2−/− mice, were subjected to chronic aortic constriction.

Echocardiography showed that Nox4TG/Nrf2+/+ mice developed less cardiac dysfunction, dilatation, and hypertrophy than WT littermates (Figures 6A–6D and S6). Genetic deletion of Nrf2 in Nox4TG significantly blunted the protective effects of Nox4; thus, Nox4TG/Nrf2−/− mice exhibited worse contractile dysfunction and hypertrophy than Nox4TG/Nrf2+/+ mice (Figures 6 and S6). These results suggest that Nrf2 activation downstream of myocardial Nox4 contributes significantly to the protective effects of Nox4 during pressure overload.

**Figure 3.** Nuclear factor erythroid–derived 2-like 2 (Nrf2) modulates a caspase 8/truncated BH3–interacting domain death agonist (tBid)/apoptosis-inducing factor (AIF)–dependent pathway. A, Representative immunoblots and mean data for changes in protein levels of caspase 8. B, Bid, tBid, and the ratio of tBid to uncleaved Bid (tBid/Bid). C, Total and cleaved AIF in cytosolic (C) and nuclear (N) fractions. Nuclear AIF levels were expressed relative to lamin B; GAPDH was used as a cytosolic marker. D, γ-H2Ax histone as readout of nuclear DNA damage in hearts from wild-type (WT) and Nrf2−/− mice under stress conditions; n=4 per group; *P<0.05, **P<0.01, and ***P<0.001. Nuc indicates nuclear.

**Figure 4.** Endogenous nicotinamide adenine dinucleotide phosphate oxidase-4 (Nox4) regulates the activation of cardiomyocyte nuclear factor erythroid–derived 2-like 2 (Nrf2). A, Representative immunoblots and mean data for changes in Nrf2 levels after treatment of neonatal rat ventricular myocytes (NRVM) with phenylephrine (PE). B, Effect of Nox4 knockdown on PE-induced upregulation of Nrf2. C–E, Effect of Nox4 knockdown on mRNA levels of Hmox1, Gclc, and Gstα2 in NRVM treated with PE; n=4 per group; *P<0.05 and ***P<0.001.
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Discussion

Nrf2 controls the basal and inducible expression of numerous antioxidant proteins, detoxifying enzymes, and cytoprotective molecules, thereby exerting protective effects in disease settings. Previous work suggested that Nrf2 exerts cardioprotective effects,\textsuperscript{5,6,18} but the mechanisms underlying its upregulation during hemodynamic overload or its protective effects are unknown. Here, we report the novel findings that Nrf2 activation is under the regulation of endogenous Nox4 and it mediates protective effects in the pressure-overloaded heart via a caspase 8/Bid/AIF pathway to promote cell survival. These data identify a novel endogenous

Figure 5. Inhibition of nuclear factor erythroid–derived 2-like 2 (Nrf2) activation in nicotinamide adenine dinucleotide phosphate oxidase-4 (Nox4) knockout hearts subjected to chronic pressure overload. A, Representative immunoblots and mean data for changes in Nrf2 protein levels. B–D, Changes in the mRNA levels of Nrf2 target genes in wild-type (WT) and Nox4\textsuperscript{−/−} mice; n=3 per group; \( *P<0.05 \).

Figure 6. Contribution of nuclear factor erythroid–derived 2-like 2 (Nrf2) activation to the cardioprotective effects of nicotinamide adenine dinucleotide phosphate oxidase-4 (Nox4) during in vivo chronic pressure overload. A and B, Echocardiography data for fractional (Fr.) shortening and septal thickness; n=7 to 9 per group. C, Heart weight/body weight (HW/BW) ratio; n=9 to 14 per group. D, Cardiomyocyte cross-sectional area; n=6 per group; \( *P<0.05 \), \( **P<0.01 \), and \( ***P<0.001 \). IVSd indicates interventricular septal end diastolic dimension; Nox4TG, Nox4 transgenic; and WT, wild-type.
cytoprotective pathway involving specific ROS-antioxidant signaling that regulates the cardiac response to chronic overload stress. Our study confirms the results of a previous study in Nrf2-deficient mice that reported the protective effects of Nrf2 during hemodynamic overload, but additionally it sheds light on mechanisms. We found that Nrf2 deficiency was associated with increased oxidative stress and a substantial increase in mtDNA damage during pressure overload, which is consistent with the fact that mitochondria are targets of oxidative damage.19 Interestingly, not all Nrf2 target genes were affected either by pressure overload or by Nrf2 deficiency, most likely because additional transcription factors may be involved in regulating these genes.1 We found that the cell death accompanying mtDNA damage was independent of caspase 3/cytochrome c and instead related to a caspase 8/ Bid/AIF pathway. Protein levels of caspase 8 were lower at baseline in Nrf2−/− mice than in WT littermates but rose to a greater extent after stress, suggesting that the magnitude of stress-related increase may be more important than absolute levels. Nrf2−/− mice also demonstrated increased AIF cleavage and translocation to the nucleus, which is consistent with previous data that AIF release from mitochondria occurs downstream of caspase 8/Bid activation.15 Nuclear translocation of AIF mediates DNA damage,16,20 and we found evidence of increased nuclear DNA damage in Nrf2−/− mice after pressure overload.

In the previous work, we showed that upregulation of cardiomyocyte ROS–generating Nox4 beneficially affects cardiac remodeling during chronic pressure overload.7 Because Nrf2 may be activated by ROS, we investigated whether Nox4 is involved in activating Nrf2. Remarkably, both phenylephrine-induced Nrf2 activation in NRVM and pressure overload–induced cardiac activation in vivo required endogenous Nox4. Notably, the knockdown of a different Nox isoform, Nox2, failed to inhibit Nrf2 activation. Whereas diverse ROS sources can activate Nrf2, these findings indicate that cardiomyocyte Nox4 serves as a specific and indispensable endogenous activator of Nrf2 signaling during pressure overload. This could be related to the specific subcellular localization of Nox4 or specific signaling linking Nox4 and Nrf2, although this is speculative at this stage. We noted that although Nrf2 activation was inhibited in Nox4-null mice after aortic constriction, basal Nrf2 levels were slightly higher in Nox4-null mice than in WT littermates. This may be because of adaptive responses to a chronically compromised redox state in Nox4-null animals8 because the Nrf2 pathway itself remains intact. Having identified Nox4 as an upstream regulator of Nrf2 activation, it was of interest to assess the contribution of Nrf2 activation to the protective effects of Nox4. Using a genetic approach to ablate Nrf2 activation in mice with cardiomyocyte-targeted Nox4 overexpression, we found that Nrf2 plays a significant role in Nox4-dependent protection. Previously, we found that an important mechanism contributing to the Nox4 protective effect was an increase in myocardial capillary density.7 In the current study, Nrf2 deficiency had no effect on myocardial capillary density during pressure overload, indicating that the protective effects of Nox4 are mediated by multiple mechanisms (including Nrf2 activation). Indeed, other Nox4–mediated beneficial cardiac effects were reported in the context of ischemia-reperfusion, although detrimental effects of Nox4 related to excessive ROS production were also reported.21,22 Therefore, the effects of Nox4 and the signaling pathways engaged downstream of its activation may vary depending on the precise disease condition.

Perspectives
Increased oxidative stress is considered detrimental in the chronically overloaded heart or failing heart, but clinical trials of antioxidant vitamins were unsuccessful.23 One reason may be that such approaches provide only simple antioxidant supplementation, whereas the endogenous cytoprotective program activated by Nrf2 enhances not only antioxidant enzymes but also conjugation and detoxification enzymes that deal with toxic electrophiles, transporters that efflux toxic conjugates, and many other proteins. The recent development of small molecule activators of the Nrf2 pathway24 may be a promising alternative approach. Furthermore, simple antioxidant approaches may paradoxically disrupt endogenous protective pathways, such as the Nox4-mediated Nrf2 activation identified in the current study. Therefore, the current results support targeted interventions to modulate endogenous antioxidant systems as a better therapeutic approach than simple antioxidant supplementation.

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Disclosures
None.

References


**What Is New?**

- The transcription factor nuclear factor erythroid–derived 2-like 2 (Nrf2) is regulated by endogenous nicotinamide adenine dinucleotide phosphate oxidase-4 in the hemodynamically overloaded heart.
- Nrf2 activation prevents mitochondrial DNA damage and modulates cardiomyocyte death via a caspase 8/truncated BH3–interacting domain death agonist/apoptosis–inducing factor–dependent pathway.
- Cardiac Nrf2 activation contributes to the protective effects of nicotinamide adenine dinucleotide phosphate oxidase-4 during pressure overload.

**What Is Relevant?**

- Nrf2 is cardioprotective during pressure overload by regulating cyto-

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**Novelty and Significance**

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**What Is Relevant?**

- Nrf2 is cardioprotective during pressure overload by regulating cyto-

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**Summary**

This study identifies a crucial endogenous protective pathway involving nicotinamide adenine dinucleotide phosphate oxidase-4 and Nrf2 signaling that plays an important role in the cardiac response to pressure overload.
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NOX4-DEPENDENT UPREGULATION OF NRF2 PROTECTS THE HEART DURING CHRONIC PRESSURE OVERLOAD

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MATERIALS AND METHODS

Animal studies
Nrf2⁻/⁻, Nox4⁻/⁻ and cardiomyocyte-targeted Nox4 transgenic (Nox4TG) mice were described previously ¹,². To ensure that any effects observed in Nox4TG were not related to the site of integration of the transgene, we generated two independent Nox4 transgenic lines which both showed similar phenotypes at baseline and after aortic banding.¹ Male mice and littermate wild-type (WT) controls underwent aortic constriction or sham surgery under 2% isoflurane anesthesia.¹

Aortic constriction
All procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (United Kingdom). We studied male mice and respective matched wild-type (WT) littermates, all on a C57BL/6 background. Aortic constriction was performed in 16-18g mice by a single surgeon, using suprarenal banding with a 27 gauge band under 2% isoflurane anesthesia.³ Sham constriction involved identical surgery apart from band placement. Increases in invasively measured peak aortic systolic pressure ranged from 21.7±6.0 to 22.4±2.2 mmHg, consistent across groups. Animals were studied up to 6 weeks post-surgery.

Echocardiography
Echocardiography was performed under 1.5% isoflurane using a Visualsonics Vevo 2100 ultrasound system with a 40-MHz transducer.¹ Heart rates were above 400 bpm in all experiments. Analysis of data was performed using the VevoStrain software package (Visualsonics).

cDNA synthesis and real time qPCR
Total RNA was reversed-transcribed using random decamers with M-MLV RT (Promega, UK) for heart tissue or cardiomyocyte samples according to the manufacturer's protocol. Control reactions omitting the RT were also performed in all cases. An Eppendorf Mastercycle Realplex detection system (Eppendorf, UK) using SYBR Green and the comparative Ct method was used, with cytoskeletal β-actin levels employed for normalization. Primer sequences are provided in Table S1.

Histology
Hearts were arrested in diastole with KCl and fixed with 2% paraformaldehyde either for 6 hrs at room temperature or overnight at 4°C. For all histology experiments, 6 μm transverse cross-sections were used. FITC-conjugated wheat germ agglutinin (FITC-WGA, Vector RL-1022) was used to outline cardiomyocytes. Interstitial fibrosis was assessed by blinded quantitative image analysis (Volocity, Perkin Elmer) of Picrosirius red-stained sections 4. Capillaries were immunostained with isolectin B4 (Vector B-1205) and capillary density quantified as the number of capillaries per square millimeter 5. Apoptosis was assessed by TUNEL staining (Millipore S7110 kit).

Western blotting
Snap-frozen heart tissue samples or pelleted cardiomyocytes were homogenized and lysed in hypotonic lysis buffer [50 mmol/L Hepes, pH 7.4, 10 mmol/L KCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 2 mmol/L MgCl2, 2 mmol/L DTT, 0.1% NP-40, protease inhibitor cocktails and Ser/Thr and Tyr phosphatase inhibitor cocktails (Sigma, UK)]. Protein concentration for each sample was measured using BCA assay (Pierce, UK). Heart tissue homogenates or cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Nuclear proteins were extracted using the NE-PER nuclear and cytoplasmic extraction kit (Abcam, UK). Antibodies used were the following: Nrf2 (Santa Cruz Biotechnology c-20); Nox4 6; cleaved caspase 3, Bcl-2, caspase 8, Bid, AIF and γ-H2Ax (NEB, UK). GAPDH (Sigma), Lamin B (nuclear protein marker) and VDAC (mitochondrial protein marker) (NEB, UK) were used as a loading control. For Nrf2 immunoblotting, based on a recent report from Lau et al. 7, the biologically relevant species of Nrf2 migrates at ~110 kDa and that was the migratory species of Nrf2 that was quantified in this study (see Fig. 1A). Protein band quantification was undertaken using ECL reagent (GE-Healthcare, UK) or an Odyssey Li-Cor imaging system (Li-Cor Biosciences, UK).

Isolation and culture of neonatal rat ventricular myocytes (NRVM)
Primary cultures of NRVM were prepared as described previously 8. Adenoviral vectors expressing shNrf2 or control short hairpin sequence were purchased from Welgen, USA. Adenoviral vectors expressing shNox4/GFP and the appropriate shGFP control have been described elsewhere 9. Cells were cultured for 24 hrs before infection with virus at appropriate multiplicity of infection for 48-72 hrs in serum-free media before harvesting or stimulation with phenylephrine (Ad.shNrf2 and Ad.Control: 300 MOI; Ad.shNox4 and Ad.shGFP: 20 MOI).

Mitochondrial DNA damage
A qPCR-based assay was used for the assessment of mitochondrial DNA damage as described 10. Briefly, the technique involves measuring DNA sequence-specific damage by quantifying the decrease in amplification of DNA compared to control samples. This assay is based on the principle that damaged DNA will impede the progression of the DNA polymerase when analysed by PCR, resulting in a decrease in the amount of PCR product. High-quality genomic DNA was isolated from heart tissue samples using the Qiagen blood and tissue DNA extraction kit (Qiagen, UK). To accurately assess the number of induced DNA lesions, template DNA was quantified and diluted to the same concentration with minimal variation between samples prior to qPCR. Typically, 15 ng of total DNA per sample were amplified. A long target of mitochondrial DNA (10 kb) (Table S2 for primer
sequences) was amplified to increase the probability that the polymerase will encounter damaged DNA regions. QPCR was performed with the GeneAmp XL PCR system (Applied Biosystems), which uses rTth DNA polymerase XL enzyme designed to amplify target DNA sequences up to 40 kb. All reactions were initiated by a hot start (75°C, 2 min) prior to the addition of the rTth enzyme (Table S2 for QPCR conditions). We normalised QPCR results for mitochondrial copy number to ensure that changes in amplification levels are accurate reflections of DNA damage and not affected by fluctuation in the amount of mitochondrial DNA present in each sample. This was performed via amplification of a small mitochondrial target (~200 bp) whose amplification would not be significantly affected by DNA damage.

DNA damage was quantified by comparing the amount of the large mitochondrial DNA fragment (~10 kb) amplified from heart samples of interest compared to amplification obtained from heart samples of WT sham controls. Large and small mitochondrial QPCR products were semi-quantified on 0.8% and 2% agarose gels respectively and the resulting values were expressed as relative probability of mtDNA lesions by application of the Poisson distribution [lesions/amplicon = -ln(sample of interest/WT Sham)].

Other assays

Protein oxidation was assessed by measurement of protein carbonyl levels using a spectrophotometric kit (Cell Biolobs, UK). Cytochrome c translocation from the mitochondria to the cytosol was measured with a cytochrome c release assay kit (Abcam, UK).

Statistics

Data are presented as mean ± SEM. Comparisons were made by 1-way or repeated measures ANOVA, followed by Bonferroni test for multiple comparisons. P < 0.05 was considered significant.
References (Online Supplement)


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<td>AGTAGAGTCCGAGCAATGGAGG</td>
<td>TATAGAGTTTCCGAGCAGAGG</td>
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<tr>
<td>Gsta2 (r)</td>
<td>AAGCTAAAGAAAGACGGGAATTG</td>
<td>GTAGTTGAGAATGCTCTGCTC</td>
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<td>Hmox1 (r)</td>
<td>CGACAGCATGTCGCCAGCATT</td>
<td>TCTGAAAGTTCCTCATGAAAC</td>
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<tr>
<td>Nqo1 (r)</td>
<td>AGCATTTTCAGGGTCTCCTTG</td>
<td>TCTTCTACGCCCATGCC</td>
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<tr>
<td>Txnrd1 (r)</td>
<td>ACTCAGCAGAGCGGGTTCCCT</td>
<td>AAAAGATCGTCACTGCTGATGC</td>
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<tr>
<td>Cat (r)</td>
<td>AGTACAACCTCCAGAAGCCCTAAGAAT</td>
<td>CCGTGCCTTTACAGGTTAGCTT</td>
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<tr>
<td>Nppa (m)</td>
<td>CGTGGCCGCCGACCACGCCAGCATGGGCCTC</td>
<td>GGCTCGAGGGCCAGCGAGCAGAGG</td>
</tr>
<tr>
<td>Myh7 (m)</td>
<td>AGCAGCAGTGGGATGAGCGACT</td>
<td>CCAGCTCCTCGATGCGTGCC</td>
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</table>

**Table S1:** Mouse (m) and rat (r) primer sequences used in QPCR experiments
<table>
<thead>
<tr>
<th>Large mitochondrial fragment (10 kb)</th>
<th>Primers</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: 5’-3’ GCCAGCCTGACCCATAGCCATAATAT</td>
<td>1) 75°C 2 min 2) 94°C 1 min 3) 94°C 15 sec 4) 72°C 10 min</td>
<td></td>
</tr>
<tr>
<td>R: 5’-3’ GAGAGATTATGCGGTGTAATCGG</td>
<td>65°C 12 min (20 cycles)</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small mitochondrial fragment (200 bp)</td>
<td>Primers</td>
<td>PCR conditions</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>---------</td>
<td>---------------</td>
</tr>
<tr>
<td>F: 5’-3’ CCCAGCTACTACCATCATTCAAGT</td>
<td>1) 75°C 2 min 2) 94°C 30 sec 3) 72°C 45 sec (18 cycles)</td>
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<tr>
<td>R: 5’-3’ GATGGTTGGGAGATGTTGATG</td>
<td>60°C 45 sec</td>
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</table>

**Table S2:** Mouse primer sequences and QPCR conditions used to amplify large (10kb) and small (200bp) mitochondrial fragments
Figure S1: Effect of Nrf2 on cardiac structure and function after chronic pressure overload. A-D) Echocardiography data for left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), fractional shortening and interventricular septal thickness; n=10-16/group. E) Heart-weight/body-weight ratio; n=11-17/group. F) Cardiomyocyte cross-sectional area; n=6/group. G-H) mRNA levels of Nppa and Myh7; n=4/group. I) Intersitial fibrosis; n=8-14/group. J) Myocardial capillary density; n=4-6/group. * P<0.05, ** P<0.01 and *** P<0.001
Figure S2: Effect of Nrf2 deficiency on cytochrome c release and protein levels of caspase 3 and Bcl-2. A-C) Representative immunoblots and mean data for cytochrome c release, cleaved caspase 3 and Bcl-2 in WT and Nrf2−/− hearts; n=4/group. NS=not significant. Mt and Cyt denote mitochondrial and cytosolic fractions respectively. D) Representative immunoblot for total AIF and cleaved AIF (arrow) in total protein lysates from WT and Nrf2−/− hearts.
Figure S3: Effects of Nrf2 or Nox2 knockdown on response to phenylephrine (PE) in neonatal cardiomyocytes. A) Representative immunoblot and mean data for changes in Nrf2 protein levels after Nrf2 knockdown; n=3/group. B-D) Effect of Nrf2 knockdown on mRNA levels of Hmox1, Gclc and Gsta2 in NRVM treated with PE; n=4/group. E) Representative immunoblot showing knockdown of Nox2 by Ad.shNox2. F) Effect of Nox2 knockdown on PE-induced upregulation of Nrf2; n=3/group. G-I) Effect of Nox2 knockdown on mRNA levels of Hmox1, Gclc and Gsta2 in NRVM treated with PE; n=5/group. *P<0.05, **P<0.01 and ***P<0.001.
Figure S4: The Nrf2 pathway can still be activated by knockdown of KEAP1 after Nox4 depletion in cardiomyocytes. A-D) Effect of KEAP1 knockdown on mRNA levels of Keap1, Hmox1, Gclc and Gsta2 in NRVM; n=3/group. E-G) Effect of siRNA against Keap1 or scrambled control (Scr) on mRNA levels of Hmox1, Gclc and Gsta2 against a background of Nox4 depletion in NRVM. Experiments were performed with and without PE stimulation. n=6-9/group. *P<0.05 and **P<0.01.
Figure S5: Inhibition of Nrf2 activation in Nox4 knockout hearts subjected to chronic pressure overload. A-C) Changes in the mRNA levels of selected Nrf2 target genes in WT and Nox4^- mice; n=3/group. NS=not significant.
Figure S6: Contribution of Nrf2 activation to the cardioprotective effects of Nox4 during *in vivo* chronic pressure overload. A-B) Echocardiography data for LVEDV and LVESV; n=7-9/group. *P*<0.05, **P*<0.01 and ***P*<0.001.