Nrf2 Protects Against Maladaptive Cardiac Responses to Hemodynamic Stress

Jinqing Li, Tomonaga Ichikawa, Luis Villacorta, Joseph S. Janicki, Gregory L. Brower, Masayuki Yamamoto, Taixing Cui

Background—Reactive oxygen species (ROS) play an important role in the maintenance of cardiovascular homeostasis. The present study sought to determine whether nuclear factor erythroid-2 related factor 2 (Nrf2), a master gene of the endogenous antioxidant defense system, is a critical regulator of the cardiac hypertrophic response to pathological stress.

Methods and Results—Cardiac hypertrophy and dysfunction were established in mice by transverse aortic constriction (TAC). Nrf2 expression was transiently increased and then declined to the basal level while impairment of cardiac function proceeded. The knockout of Nrf2 (Nrf2

Conclusions—These findings demonstrate that activation of Nrf2 provides a novel mechanism to protect the murine heart against pathological cardiac hypertrophy and heart failure via suppressing oxidative stress. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: ●●●

In response to stress from neurohumoral activation, hypertension, or other myocardial injury, the heart initially compensates with an adaptive enlargement of the myocardium (ie, cardiac hypertrophy that is characterized by an increase in the size of individual cardiac myocytes and whole-organ mass). However, sustained cardiac hypertrophy is detrimental and leads to stroke, heart failure, and sudden death.1–3 The latest epidemiological data has revealed that cardiac hypertrophy is a major predictor of heart failure, with a mortality as high as 80% for men and 70% for women within 8 years.4 Despite the prominent contribution of cardiac hypertrophy to heart failure, the molecular mechanisms responsible for the transition from compensated hypertrophy to failure are poorly understood.

It is firmly established that oxidative stress plays a causative role in the pathogenesis of cardiovascular disease including pathological cardiac hypertrophy and heart failure.5–10 Surprisingly, larger clinical trials have shown that ROS scavengers of antioxidant vitamins for treatment of cardiovascular disease are ineffective or even harmful.5–8 Because these studies have not examined hypertrophic heart disease or heart failure per se, additional studies with specific targeting of the source of oxidative stress or enhancing intrinsic antioxidant pathways are needed. Such studies will not only further extend our understanding of the role of oxidative stress but also potentially lead to therapeutic interventions for the prevention of pathological cardiac hypertrophy.

Nrf2 belongs to the Cap ‘n’ Collar (CNC) family of basic leucine zipper (bZip) transcription factors that include NF-E2, Nrf1–3, and Bach1–2.11–13 Nrf2 is a pleiotropic protein that binds to a cis-acting enhancer sequence known as the antioxidant response element (ARE) with a core nucleotide sequence of 5′-RTGACNNNGC-3′ to control the basal and inducible expression of a battery of antioxidant genes and other cytoprotective phase II detoxifying enzymes, such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferases (GST), NAD(P)H:quinone oxidoreductase (NQO1), NQO2, γ-glutamylcysteine synthase (γ-GCS), and glucuronosyl-
transferase. In fact, it has been demonstrated that several Nrf2 target genes, including HO-1,14,15 SOD,16 and GPx,17 protect the heart against maladaptive remodeling. Interestingly, emerging evidence has revealed that Nrf2/ARE signaling plays an important role in preventing oxidative cardiac cell injury in vitro.18,19 Nevertheless, the role of Nrf2 in the regulation of cardiac hypertrophy and the prevention of heart failure in vivo remains to be determined.

In the present study, we explore the role of Nrf2 in the pathogenesis of cardiac hypertrophy and subsequent heart failure utilizing Nrf2 knockout (Nrf2−/−) mice. Our results demonstrate that Nrf2 protects the heart against maladaptive hypertrophic remodeling and failure in response to a sustained pathological hemodynamic stress via the suppression of oxidative stress.

Materials and Methods

Animals, cell culture, adenoviral infection, transverse aortic constriction (TAC) model of pressure overload–induced cardiac hypertrophy and dysfunction, echocardiographic and hemodynamic analysis, RT-PCR and quantitative real-time (Q-PCR), histological, and immunohistochemical analysis, as well as statistical analysis are detailed in the supplemental Materials and Methods (available online at http://atvb.ahajournals.org).

Results

Upregulation of Nrf2 in Hypertrophied Hearts

To study the potential role of Nrf2 in the pathogenesis of cardiac hypertrophy and heart failure, we initially examined Nrf2 expression profile in murine hypertrophied hearts after TAC, a well-established model of pressure overload-induced cardiac hypertrophy and dysfunction in rodents. In wild-type ICR mice, TAC gradually induced cardiac hypertrophy that was characterized by an increase in the atrial natriuretic factor (ANF), and brain natriuretic factor (BNP) fetal genes, and thickening of the diastolic left ventricle posterior wall (LVPWd; supplemental Figure III). Cardiac hypertrophy was evident by day 7 and progressive up to the experimental end point of 28 days (supplemental Figure III). Fractional shortening FS (%) was preserved until day 14 and significantly decreased on day 28 (supplemental Figure III). Sano et al recently reported similar changes of TAC-induced cardiac hypertrophy and dysfunction in C57BL/6 mice.20 These results suggest that pressure overload initially induced adaptive hypertrophy (days 1 to 14) with preserved cardiac function; however, this adaptive mechanism could not protect the hypertrophied heart against sustained pressure overload, resulting in maladaptive cardiac remodeling and left ventricular dysfunction (days 14 to 28) in murine TAC model. Interestingly, we observed that TAC caused a transient enhancement of Nrf2 expression at both mRNA and protein levels in the heart. The Nrf2 expression was rapidly increased initially, reached a peak on day 7, and thereafter decreased to near to the basal level on day 28 after TAC (supplemental Figure IVA). The expression of Nrf2 downstream genes, such as GPx, HO-1, NQO-1, thioredoxin-1 (Txn-1), thioredoxin reductase-1 (Txnrd-1), SOD-2, and SOD-3, was also significantly increased after TAC reaching a peak on day 7 of TAC and thereafter decreasing to a value near to the pre-TAC level on day 28 after TAC (supplemental Figure IVB). Of note, nuclear translocation of Nrf2 proteins was dramatically enhanced in the cardiomyocytes of hypertrophied hearts after TAC (supplemental Figure IVB). These results indicate that Nrf2 expression and activity are enhanced during the earlier stage of cardiac adaptive hypertrophy and decreased in the process of maladaptive responses to the sustained hemodynamic stress. Nrf2 might play a critical role in the regulation of maladaptive cardiac remodeling and the transition of cardiac hypertrophy to heart failure.

Characterization of Cardiac Function in Nrf2−/− Mice at Physiological Normal Status

To further investigate the pathological relevance of Nrf2 signaling, we included Nrf2−/− mice. At 8 weeks of age, Nrf2 deficiency did not cause any apparent structural defects and functional abnormalities in heart (supplemental Figure V and supplemental Table II), suggesting that Nrf2 is not an essential mediator for normal cardiac development or functional integrity at nonstressed physiological conditions.

Overt Heart Failure in Nrf2−/− Mice in Response to Pathological Hemodynamic Stress

To test whether Nrf2 is required for adaptive responses to pathological hemodynamic overload, we performed TAC on Nrf2−/− and WT littermate mice. As previously reported,21 survival rate of WT mice to 2 weeks of TAC was more than 90% (WT; 92%, 23 of 25). However, the survival rate was decreased in Nrf2−/− mice (Nrf2−/−; 49%, 18 of 37) after 2 weeks of TAC (Figure 1A). The dead Nrf2−/− mice had apparent signs of heart failure at autopsy (data not shown). Compared to the WT that survived after 2 weeks of TAC, the surviving Nrf2−/− mice exhibited enhanced cardiac hypertrophy, including increases in LVPWd and systolic LV internal dimension (LVIDs) (Figure 1B and D; Table), and increased lung weight to body weight ratio, an index of heart failure (Table), demonstrating a worse pathological ventricular remodeling in Nrf2−/− mice. Most importantly, FS (%) was significantly decreased only in Nrf2−/− mice but not in WT mice (Figure 1C), suggesting that loss of Nrf2 function results in earlier onset of cardiac dysfunction in response to pressure overload. Of note, Nrf2 deficiency per se did not affect the blood pressure or heart rate response to pathological pressure overload. After TAC, blood pressure of left and right carotid arteries of WT mice was 96±4.6 and 141.1±12.9 mm Hg, respectively, whereas blood pressure of left and right carotid arteries of Nrf2−/− mice was 90±1.8 and 133.5±5.9 mm Hg, respectively. TAC induced a similar significant pressure gradient between the right and left carotid arteries in WT (44 mm Hg) and Nrf2−/− (43 mm Hg) mice (supplemental Figure 1). Echocardiography-derived heart rates of anesthetized (isoflurane) WT and Nrf2−/− mice 2 weeks after TAC were also similar.
Taken together, the Nrf2 deficiency exaggerates the decompensation in Nrf2−/− mice which is not attributable to an inappropriate increase in heart or myocyte size per se.

**Fetal Gene Induction**

Pressure overload and other pathological stresses typically result in the activation of the fetal hypertrophic gene program including an upregulation of ANF, BNP, and β-myosin heavy chain (βMHC), as well as a downregulation of α-myosin heavy chain (αMHC) and sarcoplasmic reticulum calcium ATPase2a (SERCA), which is correlated with pathological ventricular remodeling. The upregulation of βMHC and downregulation of αMHC (αMHC/βMHC switch) contribute to the reduction of myofibrillar ATPase activity and cardiac myofiber shortening and velocity of shortening thereby leading to eventual contractile dysfunction. In addition to the αMHC/βMHC switch, a downregulation of the gene for encoding the sarcoplasmic reticulum Ca2+ ATPase (SERCA) is involved in pathological myocardial hypertrophy and heart failure, thus the role of Nrf2 in regulating fetal hypertrophic gene program was examined by determining the fetal gene expression using Q-PCR. In WT mice, TAC robustly increased the mRNA levels of ANF, BNP, and βMHC, and significantly decreased the mRNA levels of αMHC and SERCA (supplemental Figure VII and Table). In Nrf2−/− mice, TAC resulted in a further increase in the mRNAs of ANF, BNP, and βMHC as well as a further decrease in the mRNAs of αMHC and SERCA (supplemental Figure VII and Table). Therefore, the further increase in the ratio of αMHC/βMHC and decrease in SERCA in the heart of Nrf2−/− mice could be a potential link to the overt heart failure in these mice secondary to the pathological pressure overload. In summary, the Nrf2 deficiency exaggerates the pressure overload–induced transcripational responses by turning on the αMHC/βMHC switch and decreasing SERCA expression that then lead to pathological cardiac hypertrophy and cardiac dysfunction.

**Fibrosis**

Cardiac fibrosis plays a critical role in the development of abnormal myocardial stiffness and ultimately ventricular dysfunction in response to pathological stimuli. Fibrosis determined by Masson trichrome staining for collagen was negligible in hearts of sham-operated WT and Nrf2−/− mice (Table). Interstitial fibrosis in WT hearts after TAC was 12.6±1.57% in the LV free wall endocardium and papillary muscle and 9.8±1.57% in the base and midportion of the interventricular septum (IVS) which is similar to the findings of others in this model, whereas in the TAC Nrf2−/− hearts it was increased to 24.4±3.42% of LV free wall and 20.0±3.40% of IVS; supplemental Figure VIII and Table). Fibrosis in the perivascular area in TAC Nrf2−/− hearts was also larger than that in the TAC WT hearts (data not shown). Collectively, these results indicate that the upregulation of fibrosis is associated with the Nrf2 deficiency.

**Heart and Myocyte Size**

Increased size of hearts is thought to be an adaptive response to increased workload, and inadequate hypertrophy causing heart failure is observed in animal models and patients with aortic stenosis and left ventricular failure. According to previous observations that indicate isoflurane suppresses the basal heart rate and TAC could further induce slight decreases in heart rates (supplemental Figure I), taken together, the Nrf2−/− mice that survived 2 weeks had worse cardiomyopathy and overt heart failure after TAC. These results demonstrate that Nrf2 is a critical regulator of adaptive cardiac remodeling, and cardioprotective against the transition to heart failure.
indicate that Nrf2 deficiency exaggerates left ventricular fibrosis in response to the pathological pressure overload.

**Apoptosis**

Because myocardial apoptosis is also a causative factor in cardiomyopathy,27 we examined the apoptotic changes by TUNEL and cleaved caspase-3 staining in hearts of WT and Nrf2−/− after TAC. In WT hearts 2 weeks after TAC, TUNEL-positive cells were increased 10-fold compared with the sham-operated WT mice (supplemental Figure IXA and IXB and Table), similar to the observations of others in this model.26 In Nrf2−/− hearts after TAC, the apoptotic cells were further increased by about 20-fold compared with the sham-operation (Figure 3 and Table). Although hearts from sham-operated WT and Nrf2−/− mice had the same numbers of apoptotic cells, a 3-fold increase of myocardial apoptotic cells was observed in the TAC Nrf2−/− hearts compared with the TAC WT hearts (supplemental Figure IXA and IXB and Table). These observations were further supported by similar changes in caspase-3 activities that were assessed by immunochemical staining of cleaved caspase-3 in the heart sections (supplemental Figure IXC and IXD and Table). Although the immunochemical staining could not precisely distinguish the apoptotic cell types such as cardiomyocytes or cardiac fibroblasts, these results indicate that Nrf2 deficiency increases overall myocardial apoptosis after pressure overload.

**Cardiac Oxidative Stress of Nrf2−/− Mice After Pathological Pressure Overload**

We also investigated the possibility that Nrf2 deficiency exaggerates oxidative stress thereby contributing to the maladaptive cardiac remodeling after pressure overload. Accordingly, we examined oxidative stress status as well as the expression of Nrf2 downstream genes in the hearts of Nrf2−/− mice and their WT littermates after TAC.

Myocardial levels of 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA oxidation,28,29 were significantly increased in WT mice 2 weeks after TAC (Figure 3A and 3B), suggesting that pathological pressure overload induces oxidative stress in the heart. Importantly, the TAC-induced oxidative stress was further exaggerated in the hearts of Nrf2−/− mice (Figure 3). There were no apparent differences of 4-HNE and 8-OHdG levels in the hearts of sham-operated WT and Nrf2−/− mice (Figure 3). These results demonstrate that Nrf2 deficiency leads to increased oxidative stress in the heart secondary to pathological pressure overload. As shown in Figure 3C and supplemental Figure XC through XE, TAC upregulated myocardial expression of HO-1, NQO-1, Txn-1, Txnrd-1, SOD-2, and SOD-3 mRNAs in WT mice but not in

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**Table. Echocardiography and Pathology of WT and Nrf2−/− Mice After TAC**

<table>
<thead>
<tr>
<th>Echocardiography, WT</th>
<th>TAC</th>
<th>P</th>
<th>Nrf2−/−</th>
<th>Sham</th>
<th>TAC</th>
<th>P</th>
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<tr>
<td>lVsd, mm</td>
<td>0.71±0.11</td>
<td>0.93±0.11</td>
<td>A</td>
<td>0.76±0.10</td>
<td>1.10±0.12</td>
<td>B C</td>
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<tr>
<td>LVIDd, mm</td>
<td>4.30±0.28</td>
<td>4.20±0.28</td>
<td>A</td>
<td>4.50±0.24</td>
<td>4.31±0.56</td>
<td>B C</td>
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<tr>
<td>LVIDs, mm</td>
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<td>3.15±0.34</td>
<td>A</td>
<td>3.13±0.31</td>
<td>3.48±0.35</td>
<td>B C</td>
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<tr>
<td>LVPWd, mm</td>
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<td>1.05±0.12</td>
<td>A</td>
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<td>B C</td>
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<tr>
<td>FS, %</td>
<td>28.7±4.76</td>
<td>25.0±4.30</td>
<td>A</td>
<td>30.7±4.68</td>
<td>18.8±5.91</td>
<td>B C</td>
</tr>
</tbody>
</table>

**Pathology, n**

| BW                  | A | 37.1±3.89 (8) | 29.9±3.40 (11) | B |
| HW/BW               | A | 5.70±0.85 (8) | 9.78±1.34 (11) | B C |
| MC CSA, μm²         | A | 179±10.7 (6)  | 312±14.4 (6)  | B C |
| Lung wt/BW          | A | 8.04±2.77 (8) | 16.1±4.11 (11) | B C |

**Fibrosis, % area**

| LV                  | A | 0 (6) | 24.4±3.42 (6) | B C |
| IVS                 | A | 0 (6) | 20.0±3.40 (6) | B C |
| RV                  | A | 0 (6) | 1.71±0.33 (6) | B |
| mRNAs               | 3 | 4 |
| ANF                 | 0.92±0.25 | 14.23±5.33 | A | 2.16±1.61 | 23.3±6.67 | B C |
| BNP                 | 1.14±0.23 | 5.63±1.53 | A | 1.30±0.54 | 7.95±1.88 | B C |
| αMHC                | 0.82±0.05 | 0.56±0.09 | A | 0.75±0.09 | 0.42±0.04 | B C |
| βMHC                | 1.25±0.53 | 8.91±2.81 | A | 1.62±0.90 | 17.6±4.43 | B C |
| SERCA               | 0.93±0.12 | 0.65±0.21 | A | 0.89±0.11 | 0.45±0.11 | B |
| TUNEL, % cells      | 0.04±0.04 (7) | 0.42±0.14 (6) | A | 0.06±0.05 (6) | 1.24±0.23 (6) | B C |
| Cleaved caspase-3   | 0.05±0.07 (7) | 0.23±0.17 (6) | A | 0.04±0.04 (6) | 0.64±0.20 (6) | B C |
Nrf2−/− mice. Also, TAC-induced regulation of PGx was partly inhibited by Nrf2 deficiency (Figure 3C). Although TAC did not alter myocardial expression of γGCLc in either WT or Nrf2−/− mice, it downregulated myocardial expression of SOD-1 in both WT and Nrf2−/− mice to a similar extent (supplemental Figure XA through XC). Taken together, these results indicate that endogenous Nrf2 coordinates a group of its downstream antioxidant genes, including GPx, HO-1, NQO-1, Txn-1, Txnrd-1, SOD-2, and SOD-3, to suppress cardiac oxidative stress in the heart in response to pathological pressure overload.

**Role of Nrf2 in the Regulation of Cardiac Myocyte Hypertrophy and Cardiac Fibroblast Proliferation**

To further examine the role of Nrf2 in the regulation of cardiac hypertrophy and fibrosis, we applied adenoviral Nrf2 gain- and loss-of-function approaches in cultured cardiac myocytes and fibroblasts.

Efficacy of adenoviral overexpression of Nrf2 and Nrf2 shRNA was established in primary cultures of rat neonatal cardiomyocytes and cardiac fibroblasts (Figure 2). Cardiac hypertrophy of WT and Nrf2−/− mice 2 weeks after TAC. A, Left panel was the representative pictures of hearts of WT and Nrf2−/− mice after TAC. Right panel was the HW/BW ratio. B, Upper panel was the representative left ventricular cross-sectional sections. Lower panel was the quantitative left ventricular cardiac myocyte cross-sectional area from 2000 to 5000 myocytes per heart.

**Figure 2.** Cardiac hypertrophy of WT and Nrf2−/− mice 2 weeks after TAC. A, Left panel was the representative pictures of hearts of WT and Nrf2−/− mice after TAC. Right panel was the HW/BW ratio. B, Upper panel was the representative left ventricular cross-sectional sections. Lower panel was the quantitative left ventricular cardiac myocyte cross-sectional area from 2000 to 5000 myocytes per heart.

Nrf2−/− mice. Also, TAC-induced regulation of PGx was partly inhibited by Nrf2 deficiency (Figure 3C). Although TAC did not alter myocardial expression of γGCLc in either WT or Nrf2−/− mice, it downregulated myocardial expression of SOD-1 in both WT and Nrf2−/− mice to a similar extent (supplemental Figure XA through XC). Taken together, these results indicate that endogenous Nrf2 coordinates a group of its downstream antioxidant genes, including GPx, HO-1, NQO-1, Txn-1, Txnrd-1, SOD-2, and SOD-3, to suppress cardiac oxidative stress in the heart in response to pathological pressure overload.

**Figure 3.** Oxidative stress in the hearts of WT and Nrf2−/− 2 weeks after TAC. A, Representative confocal microscopic images of LV 4-HNE staining. 4-HNE-positive is shown red. Nuclei are shown blue. Magnification, ×630. B, Representative confocal microscopic images of LV 8-OHdG staining. 8-OHdG-positive is shown red. Nuclei are shown blue. Cardiomyocytes are green (anti-Tropomyosin I antibody to mark cardiac myocyte tropomyosin). Magnification, ×630. Images were representatives of 6 to 7 hearts in each experimental group. C, Effect of Nrf2 deficiency on antioxidant gene expression. Expression of GPx, HO-1, Txn-1, NQO-1, Txnrd, and SOD2 was assessed by Q-PCR. Numbers of hearts are indicated. Nonsignificant, ns.
cardiac myocytes and fibroblasts (supplemental Figure II). Adenoviral overexpression of Nrf2 completely suppressed norepinephrine (NE)- and phenylephrine (PE)-induced ROS production in both cell types, whereas adenoviral overexpression of Nrf2 shRNA dramatically enhanced basal ROS production without further increases in the NE- or PE-stimulated cells (Figure 4 and supplemental Figure XIA and XI B). These results indicate that Nrf2 is a critical endogenous suppressor of oxidative stress in both cardiomyocytes and cardiac fibroblasts. In addition, the Nrf2 overexpression inhibited both basal as well as PE- and NE-induced \(^{[3}H\)leucine incorporation and cell size increases in cardiomyocytes (Figure 5 and supplemental Figure XII). Moreover, the Nrf2 knockdown enhanced basal cardiomyocyte hypertrophy, whereas it further increased the cardiomyocyte hypertrophy induced by PE and NE (Figure 5). These results suggest that Nrf2 acts as an endogenous inhibitor of cardiomyocyte hypertrophy. On the other hand, not the basal but the PE- and NE-induced cardiac fibroblast proliferation was blocked by the Nrf2 overexpression (supplemental Figure XIC). Of interest, the Nrf2 knockdown dramatically enhanced PE- and NE-induced proliferation of cardiac fibroblasts while only slightly increasing the basal proliferation (supplemental Figure XIC). These results indicate that Nrf2 is an essential negative regulator of cardiac fibroblast proliferation. Because it has been demonstrated that ROS plays a critical role in mediating hypertrophic growth in either cardiomyocytes or cardiac fibroblasts, it is conceivable that Nrf2 inhibits cardiomyocyte hypertrophy and cardiac fibroblast proliferation via the suppression of oxidative stress. Collectively, we have demonstrated that Nrf2 acts as a novel endogenous inhibitor of hypertrophic growth in both cardiomyocytes and cardiac fibroblasts via its ability to suppress oxidative stress.

**Discussion**

In response to inflammatory or oxidative insults, Nrf2 coordinately upregulates a compensatory transcriptional program that drives the induction of a network of intracellular antioxidative associated pathways and detoxification enzymes, providing a defense against pathological damages including hepatotoxicity, chemical carcinogenesis, pulmonary inflammatory diseases, neurodegenerative diseases, inflammatory and innate immune response, and aging. In the present study, we clearly demonstrate that Nrf2 is a protective regulator that prevents the maladaptive cardiac remodeling and heart failure associated with a sustained pathological hemodynamic stress via at least partly the suppressing of oxidative stress. Of note, Nrf2 deficiency results in an earlier onset of cardiac dysfunction induced by pressure overload. Accordingly, Nrf2 might play a critical role in preventing the transition from compensated to decompensated hypertrophy. Thus our results have provided solid evidence of cardioprotective role of Nrf2 in vivo.
From a mechanistic viewpoint, we have identified several potential cellular mechanisms of the stress-related myocardial abnormalities in Nrf2<sup>−/−</sup> mice, involving pathological cardiomyocyte hypertrophy, myocardial apoptosis, and fibrosis. Although each of these cellular mechanisms could be predicted from the known actions of Nrf2 in cardiovascular cells, suggesting a direct link between the Nrf2 deficiency and the phenotype, our data have further provided direct evidence to support the notion that Nrf2 orchestrates redox homeostasis for the maintenance of the functional integrity of cardiomyocytes and cardiac fibroblasts, thereby protecting against pathological cardiac remodeling.

It should be noted that a precise molecular mechanism of the Nrf2-mediated cardiac protection has not been fully dissected in the present study. The cause of a more severe cardiac remodeling and dysfunction in Nrf2<sup>−/−</sup> mice is likely multifactorial, including impaired cardiomyocyte hypertrophy, activated cardiac fibroblasts of a more proliferative and synthetic phenotype, increased cardiac cell apoptosis, and probably enhanced inflammatory responses, because of the following reasons: Firstly, we have demonstrated for the first time that Nrf2 is a novel endogenous inhibitor in the control of cardiomyocyte hypertrophy and cardiac fibroblast proliferation via the regulation of redox status in vitro and Zhu et al have documented that the loss of Nrf2 sensitizes cardiomyocytes and cardiac fibroblasts to oxidative stress–mediated cell death, supporting an antiapoptotic effect of Nrf2 at least for cardiomyocytes and cardiac fibroblasts in the pressure-overloaded heart in vivo. Secondly, whereas Nrf2 is a critical mediator for the resolution of inflammatory and fibrotic responses in lung tissue, it has been demonstrated that chronic cardiac inflammation leads to an altered myocyte phenotype and the activation of matrix metalloproteinases, which results in disruption of interstitial matrix and eventual augmentation of interstitial fibrosis. Therefore, it is conceivable that Nrf2 signaling is critical for suppressing these proinflammatory responses which leads to pathological remodeling of the heart. Thirdly, because it has been demonstrated that genetic background, aging, and gender combined with Nrf2 deficiency contribute to autoimmunity, the gender or aging may influence the Nrf2-mediated cardiac pathophysiology. However, the specific strain of male Nrf2<sup>−/−</sup> (ICR/Sv129) mice used in our experiments grow normally and do not exhibit any manifestations of illness such as reduced body weight, edema, or cutaneous ulcers. Therefore, it is unlikely that the Nrf2 deficiency–induced detrimental effects on cardiac remodeling in the setting of TAC were related to an altered autoimmunity. Nevertheless, because our results do not specifically exclude the potential impact of Nrf2 on autoimmune-mediated cardiac dysfunction, this issue deserves further investigation using different strains of Nrf2 transgenic mice. Finally, several lines of evidence have recently revealed that cardiac cell interactions, including that between fibroblasts and cardiomyocytes, as well as others such as vascular cells, immune cells, and circulating cardiac progenitor stem cells, are essential for the progression of pathological cardiac remodeling via paracrine or autocrine mechanisms. Of interest, we have observed that increased mortality, cardiac dysfunction, and increased cardiac fibrosis are out of proportion to the slight increase in cardiac hypertrophy in Nrf2<sup>−/−</sup> mice after pathological pressure overload. Thus, whether the net effect of Nrf2 in regulating cardiac remodeling will be its ability to prevent cardiac fibrosis needs to be further determined.

From a clinical viewpoint, our results emphasize the notion of enhancing endogenous antioxidant systems as a novel therapeutic strategy for the treatment of heart disease. First, our data have demonstrated that Nrf2 expression is transiently upregulated during the earlier stage of cardiac adaptive hypertrophy with preserved cardiac function; however, it decreased in the later stage of maladaptive cardiac remodeling associated with systolic dysfunction. Actually, the activity of antioxidant enzymes is decreased in the human failing hearts. Second, large clinical trials have suggested that the scavenging of ROS using antioxidant therapy for cardiovascular disease is dangerous. Emerging evidence has revealed that the physiological role of ROS is largely neglected and nonselective elimination of ROS is detrimental. In this regard, pharmacological targeting Nrf2 has a great therapeutic potential for the treatment of cardiovascular disease.

In conclusion, our present work demonstrates that Nrf2 is a critical regulator for maintaining structural and functional integrity of the heart that is abnormally stressed. A further dissection of Nrf2-regulated signaling pathways in the heart will provide new perspectives for the rational design and development of a novel class of antioxidative derivatives for the treatment of cardiovascular disease.

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**Disclosures**

None.

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Supplement Material

Animals
Breeding pairs of Nrf2+/− (ICR/Sv129) mice were obtained from a colony at Tsukuba University and housed under standard conditions in the Institution’s AAALAC approved animal facility. Nrf2+/+ and Nrf2−/− mice were generated according to the breeding procedures previously described 1. It has been demonstrated that genetic background, aging and gender combined with nrf2 deficiency contribute to autoimmunity; e.g., female Nrf2−/− (C57B6/129SVJ) mice at 12 months of age and female Nrf2−/− (ICR/Sv129) at 15 months of age develop systemic lupus erythematosus (SLE) 2,3 whereas Nrf2−/− (SVJ129) mice at 2 months of age exhibit a lupus-like autoimmune syndrome with a marked female predominance 4. However, male Nrf2−/− (ICR/Sv129) mice grow normally and do not exhibit any clinical manifestations of illness such as reduced body weight, edema, or cutaneous ulcers. Therefore, male Nrf2−/− (ICR/Sv129) at 8 weeks of age were used in the present study to avoid the potential impact of aging and female susceptibility to Nrf2-mediated cardiac pathophysiology. All of the animal procedures were approved by the University’s Institutional Animal Care and Use Committee.

Pressure Overload Cardiac Hypertrophy
Mice at 8 weeks of age with an average of body weight of 30 ± 2 g were subjected to transverse aortic arch constriction (TAC) under anesthesia, as previously described 5. Briefly, male Nrf2−/− and male Nrf2+/+ litter mate mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The use of a horizontal incision at the level of the suprasternal notch allows direct visualization of the transverse aorta without entering the pleural space and thus obviates the need for mechanical ventilation. The transverse aorta was banded between the right innominate and left carotid arteries to the diameter of a 27-gauge needle using a 7-0 silk suture. Sham operations on sex- and age-matched mice were only omitted the actual aortic banding and served as a control for all experimental groups. Blood pressure changes in the left and right carotid arteries were measured after TAC in WT and Nrf2−/− mice by invasive catheterization. TAC led to increase in blood pressure in right carotid arteries by ~45 mmHg in both WT and Nrf2−/− mice (Supplementary Figure 1B). Cardiac hypertrophy was determined by heart weight–to–body weight (HW/BW) ratio, myocardial cross-sectional area, and expression levels of cardiac hypertrophy marker genes including atrial natriuretic factor (ANF), brain...
natriuretic peptide (BNP), alpha-myosin heavy chain (α-MHC), beta-myosin heavy chain (β-MHC), sarcoplasmic reticulum calcium ATPase2a (SERCA) 6.

**Measurement of Blood Pressure in Carotid Artery**
The measurement of mean arterial pressure (MAP) was performed as described previously 5. Briefly, mice were anesthetized with pentobarbital sodium (Nembutal, Abbott Laboratories, Chicago, IL) and the carotid arteries were catheterized with mouse jugular vein catheters (MJC-02, SAI Infusion Technologies, Libertyville, IL) which were connected to a pressure sensor as illustrated in supplementary Figure 1A. The pressure signal was amplified with Powerlab/800 (ADInstruments Inc., Colorado Springs, CO), processed with a Millar pressure conductance unit (MPCU-200; Millar Instruments Inc., Houston, Texas), and recorded by a Chart 4.0, AD system (ADInstruments Inc., Colorado Springs, CO).

**Echocardiographic and Hemodynamic Analysis**
Blood pressures were measured in conditioned, unanesthetized mice using the tail-cuff method (Hatteras Instruments, MC4000 Blood Pressure Analysis System). Echocardiography was performed on anesthetized (isoflurane) mice, using the Vevo 770 High-Resolution Imaging System (VisualSonics Inc.) with a 37.5-MHz high-frequency linear transducer, as previously described 7,8. Briefly, mice were anesthetized with 3% isoflurane and maintained with 1.5% isoflurane in room air supplemented with 100% O₂. After the anterior chest was shaved, the animals were placed on a warming pad to maintain normothermia. The echocardiographic gel was warmed before use to avoid hypothermia. Care was taken to avoid excessive pressure on the thorax, which can induce bradycardia. Two-dimensionally (2D) long axis images of left ventricle (LV) were obtained at the plane of the aortic and mitral where the LV cavity is largest and visualization of the LV apex is adequate, and a short-axis image was recorded at level of the papillary muscles. A 2D guided M-mode echocardiogram was recorded through the anterior and posterior LV walls at 21 frames/s. Images were obtained at the level of the papillary muscle tips, and measurements were then performed to obtain the LV internal dimension (LVID; in mm), interventricular septum thickness (IVS), and LV posterior wall thickness (LVPW; in mm) according to the leading-edge method of the American Society of Echocardiography 8. LV percent fractional shortening FS (%) was calculated via VisualSonics Measurement Software.
Histological and Immunochemical Analysis
Hearts were cannulated via the left ventricular apex, cleared by perfusion with PBS at 90 mmHg, fixed by perfusion with 10% formalin, and embedded in paraffin. Paraffin sections were prepared (5 μm, Leica RM2030, rotary microtome) and stored at room temperature until staining. For left ventricular cardiomyocyte cross-sectional area, coronal sections were deparaffinized and stained for membranes with Texas Red-X conjugated wheat germ agglutinin (WGA) (Invitrogen Corp., Carlsbad, CA) and for nuclei with DAPI, and observed under the fluorescence microscope (Nikon Eclipse E600; Nickon In, Melville, NY) at 400 × magnification. Twenty fields of each section were randomly photographed using Qcapture software (MAG Corp., Pleasanton, CA) and cardiomyocyte area was measured using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). For myocardial fibrosis, coronal sections were stained for collagen with a Masson’s Trichrome Kit (Poly Scientific, Bay Shore, NY) according to the protocol provided by the manufacturer. Sections were observed under light microscope (Nikon Optiphot-2; Nikon Inc., Melville, NY) at 200 × magnification. Twenty fields of each section were randomly photographed using Axio Vision 3.1 software (Carl Zeiss Inc., Maple Grove, MN). The percentage of fibrosis (the blue stained area) was measured by Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). Apoptosis was measured by TUNEL assays on tissue sections using In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN) according to the protocol provided by the manufacturer. Briefly, the sections were deparaffinized, rehydrated, microwaved in 0.01 mol/L citrate buffer for 30 minutes, and incubated in 0.3% triton X-100 in PBS for 30 minutes. After incubation with 50 μl TUNEL Reaction Mixture at 37ºC for 1 hr, the sections were incubated with Alexa Fluor® 488 phalloidin (Invitrogen Corp., Carlsbad, CA) for 1 hour at RT, and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen Corp., Carlsbad, CA). A positive control section was digested with DNase (RNase-Free DNase Set, QIAGEN Inc., Valencia CA) for 30 minutes and a negative control section was only incubated with labeling solution (without enzyme solution). The apoptotic nuclei were labeled with TUNEL (red) all nuclei were counterstained with DAPI (blue), and the F-actin was stained by Alexa Fluor® 488 phalloidin (green). In addition, caspase-3 activity was assessed as a biomarker of apoptosis by staining cardiac tissue sections with an anti-cleaved caspase-3 antibody (Cat# 9661, Cell Signaling Technology Inc., Danvers, MA), as previously described.
Cardiomyocytes were stained with anti-cardiac myosin heavy chain antibody (Cat#ab15, Abcam Inc., Cambridge, MA). Sections were observed under fluorescence microscope at 200 × magnification (Nikon Eclipse E600; Nikon In., Melville, NY) to count the apoptotic nuclei in the left ventricle, and the number of the nuclei of each LV section was counted as follows; the section was observed at 200 × magnification and photographed sequentially with little overlapping area between two images using Qcapture software (MAG Corp., Pleasanton, CA), then the images were combined together using Adobe Fireworks Software (Adobe Systems Incorporated, San Jose, CA), all nuclei of each LV section was counted with Image-Pro Plus. Representative images were acquired by confocal microscope (LSM510META, Carl Zeiss Inc., Maple Grove, MN) at 630 × magnification.

Nrf2 immunohistochemistry was performed using a rabbit ABC Staining System (Santa Cruz Biotechnology, INC., Santa Cruz, CA) according to the protocol provided by the manufacturer. Briefly, paraffin embedded sections were deparafinized, rehydrated, microwaved in 0.01 mol/L citrate buffer for 30 minutes for antigen retrieval, and incubated in 3% hydrogen peroxide for 15 minutes to quench endogenous peroxidase. The sections were further incubated in 1.5% blocking serum in PBS overnight, then incubated with a rabbit anti mouse Nrf2 polyclonal antibody (C-20, Santa Cruz Biotechnology, INC., Santa Cruz, CA) for 30 minutes at room temperature(RT), followed with a biotinylated secondary antibody for 30 minutes at RT and AB enzyme reagent for 30 minutes at RT. Thereafter, the sections were incubated with 150 μl peroxidase substrate for 45 seconds, mounted with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI), and observed under microscope (Nikon Eclipse E600; Nikon Inc., Melville, NY) at 1000 × magnification. Representative staining was acquired by Qcapture software (MAG Corporate, Pleasanton, CA).

Staining of 4-Hydroxy-2-Nonenal (4-HNE), a marker of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA oxidization, were performed with a mouse anti 4-HNE antibody (ab48506, Abcam Inc., Cambridge, MA) and a mouse anti 8-OHdG antibody (sc-660369, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively. Cardiomyocytes were stained with a rabbit anti-Tropomyosin I (ab55915, Abcam Inc., Cambridge, MA). Immunofluorescent staining was performed according to a standard protocol provided by Santa Cruz Biotechnology, Inc.. Images were acquired using a confocal microscope (LSM510META, Carl Zeiss Inc., Maple Grove, MN) at 630 × magnification.
Cell Culture and Adenovirus Infection

Rat neonatal cardiac myocytes and fibroblasts were isolated and cultured as previously described. Briefly, the hearts from 1- to 3-day old SD rats were finely minced and digested with type II collagenase (120 units/ml; Worthington Biochemical Corp., Lakewood, NJ). Dispersed cells were placed in culture flask for 30 minutes at 37°C in a CO₂ incubator. During this time, only the fibroblasts became attached to the culture flask. To further purify the cardiomyocytes, the non-adherent cells were carefully layered on percoll (Sigma-Aldrich, St. Louis, MO) and centrifuge at 2000 rpm for 20 minutes. The purified cardiomyocytes, the viability of which was >85% determined by trypan blue exclusion, were seeded onto laminin-coated plastic culture dishes at a density of 5 × 10⁴ cells/cm² in high glucose DMEM supplemented with 8% horse serum, 5% new-born calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and cytosine-β-D-arabinofuranoside (2 μg/ml). Purity of the cardiomyocytes was determined by the staining of cardiomyocytes with cardiac myosin heavy chain (MHC) antibody (ab15, Abcam Inc., Cambridge, MA). Over 90% of the cells were MHC positive 6 days after the culture. Nrf2 gain-or loss-of-function approaches were achieved by infecting the cells with adenovirus of GFP, murine Nrf2, scramble shRNA, or rat Nrf2 shRNA (Welgen Inc.) in serum free PC-1 media (Lonza Walkersville, Inc., Walkersville, MD) for 24 hours. Then the infected cells were treated as indicated below.

The fibroblasts attached to the culture flasks during the primary culture were maintained in high glucose DMEM supplemented with 10% new-born calf serum, 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cardiac fibroblasts at passage 2 or 3 were used. The purity of the fibroblasts was determined by the staining of a fibroblast marker vimentin using anti-Vimentin antibody (sc-7557-R, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Over 95% of the cultured cells were vimentin positive. The cells at a 70-80% confluent status were cultured for 48 hours in serum starvation media containing high glucose DMEM, 1% new-born calf serum, 0.5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) as described previously. The cells were then infected with adenovirus of GFP, Nrf2, scramble shRNA, or rat Nrf2 shRNA for 24 hours and followed various treatments as indicated below.

Adenoviral over-expression of Nrf2 (0-40 pfu/cell) resulted in dose-dependent increases in Nrf2 protein expression in both cardiac myocytes and fibroblasts, while adenoviral
over-expression of Nrf2 shRNA (0-50 pfu/cell) led to dose-dependent decreases in Nrf2 mRNA expression in these cells (Supplementary Figure 2). As can be seen, adenoviral over-expression of Nrf2 (20 pfu/cell) or Nrf2 shRNA (20 pfu/cell) resulted in dramatic Nrf2 protein expression or up to 80% knockdown of Nrf2 mRNA expression, respectively, in cardiac myocytes and fibroblasts (Supplementary Figure 2), without any apparent cytotoxic effects (data not shown). Accordingly, we used adenoviral over-expression of Nrf2 and Nrf2 shRNA at a dose of 20 pfu/cell to determine Nrf2-mediated cardiac biology in vitro.

[^3H]Leucine Incorporation and[^3H]Thymidine Incorporation
Cardiac myocytes were treated with DL-norepinephrine hydrochloride (NE, A7256, Sigma-Aldrich, St. Louis, MO) (20 μmol/L), or (R)-(−)-phenylephrine hydrochloride (PE, P6126, Sigma-Aldrich, St. Louis, MO) (20 μmol/L) in PC-1 media for 24 hours. [^3H]Leucine (Cat. No. 20032E01, MP Biomedicals, Solon, OH) was added to the media (final concentration 1 μCi/ml) 6 hours after NE or PE treatment. After being washed with ice-cold PBS two times, the cells were precipitated with ice-cold 5% trichloroacetic acid (TCA, T9159, Sigma-Aldrich, St. Louis, MO) for 20 minutes. They were further washed with ice-cold 5% TCA two times followed by two additional washes with ice-cold PBS and then lysed with 0.2 ml of 0.5M NaOH for 30 minutes at 37°C. The radioactivity of [^3H]leucine was measured by a Beckman LS6000 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Cardiac fibroblasts were treated with or without NE (10 μmol/ml) or PE (20 μmol/L) in a serum starvation media for 24 hours. [^3H]Thymidine (Cat. No. 2403095, MP Biomedicals, Solon, OH) was added to the media (final concentration 1 μCi/ml) 6 hours after NE or PE stimulation. The radioactivity of [^3H]thymidine was measured as above.

Measurement of Cardiomyocytes Surface Area
The cardiomyocytes seeded on Lab-Tek™ Chamber Slides (Cat. No. 177445, Thermo Fisher Scientific, Rochester, NY) were infected with adenovirus of Nrf2 and rat Nrf2 shRNA. The cells were stimulated with NE (20 μmol/L) or PE (20 μmol/L) in PC-1 media for 48 hours. The cells
were fixed with 4% paraformaldehyde (USB corp., Cleveland, OH) at 4°C overnight. The immunofluorescence staining of cardiac myosin heavy chain was performed according to a standard protocol of Santa Cruz Biotechnology, Inc.. Briefly, the cells were incubated subsequently in 0.1% Triton-X 100/PBS for 30 minutes, in 5% BSA/PBS for 10 minutes, and in 5% normal Goat serum/1% BSA/PBS for 10 minutes. Thereafter, they were incubated with rabbit anti cardiac myosin heavy chain antibody (1:100 diluted in 1% BSA/PBS, ab15, Abcam Inc. Cambridge, MA) overnight at 4°C. Then the cells were washed 3 times in 1% BSA/PBS with each time for 10 minutes, incubated in 5% normal Goat serum/1% BSA/PBS for 10 minutes, and with Alexa Fluor® 488 conjugated goat anti rabbit IgG (1:100 diluted in 1% BSA/PBS) for 1 hour at room temperature. The cells were washed 3 more times in PBS for 10 minute per wash, incubated in 4', 6-Diamidino-2-phenylindole (DAPI)/PBS (1:5000, Cat. No. D9542, Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature, washed 3 times in PBS for 5 minutes per wash, and mounted with Prolong® Gold antifade reagent (Cat. No. P36930, Invitrogen Corp., Carlsbad, CA). Finally, the cells were photographed using Qcapture software (MAG Corp., Pleasanton, CA). Triplicate experiments were repeated four times. For each well, 8 fields were randomly chosen to photograph. The cell size was quantified with Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD).

**Intercellular Reactive Oxygen Species (ROS) Detection**

Rat neonatal cardiac myocytes and fibroblasts were infected with the recombinant adenovirus as described above except ad-GFP was substituted with ad-βgalactoside (ad-βGal, Vector Biolabs). The intercellular ROS was determined by an Image-iT ™ LIVE Green Reactive Oxygen Species Detection Kit (I36007, Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. Briefly, the cardiomyocytes were stimulated with NE (200 μmol/L) or PE (100 μmol/L) in PC-1 media for 1 hour at 37°C, washed once with HBSS (H8264, Sigma-Aldrich, St. Louis, MO), loaded with the oxidant-sensitive fluorogenic probe carboxy-H₂DCFDA (25 mmol/L) working solution for 30 minutes at 37°C, and the nuclei were counterstained with Hoechst 33342 at a final concentration of 1.0 μmol/L for 5 minutes. Then the cells were washed 3 times with warmed HBSS, mounted with coverslips, and photographed using 40X object lens with Qcapture.
software (MAG Corp., Pleasanton, CA) under the same camera setting of Exposure time (286 ms), Gain (1.8), and Offset (-879). Intracellular ROS in cardiac fibroblasts were measured as described previously 13. Cardiac fibroblasts were washed with HBSS once, incubated with a carboxy-H$_2$DCFDA (25 mmol/L) working solution for 30 minutes at 37°C, stimulated with NE (200 μmol/L) or PE (100 μmol/L) in the serum starvation media for 1 hour, and counterstained with Hoechst 33342 at a final concentration of 1.0 μmol/L for 5 minutes. Then the cells were washed and photographed as described above with different same camera settings of Exposure time (6.5 s), Gain (1.0), and Offset (0). For each experiment, 8 fields were randomly chosen to photograph and integrated optical density (IOD)s of the images were quantified with Image Pro Plus software. Triplicate experiments were repeated four times.

**Western Blot Analysis**

Cell lysates were subjected to Western blot analysis using an anti-Nrf2 antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described previously 14.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time (Q-PCR)**

Total RNA from the left ventricles was extracted using RNeasy Fibrous Tissue Mini kit (Qiagen Inc., Valencia, CA), and reverse transcription reactions (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen Corp., Carlsbad, CA) were performed with 0.5 μg of DNase I (Qiagen)-treated RNA. PCR and quantitative real time PCR (Q-PCR) were carried out using the Mastercycler EP Realplex (Eppendorf, Westbury, NY). Genotypes (Nrf2$^+/+$, Nrf2$^{-/-}$, and Nrf2$^{+/−}$) of the animals were determined by PCR amplification of genomic DNA obtained from the tail. The PCR products were resolved on a 1% agarose gel. The genotypes of mice were verified by examining the size of the PCR products: Nrf2$^+/+$ (700 bp), Nrf2$^{-/-}$ (400 bp), Nrf2$^{+/−}$ (700 and 400 bp). Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as described by Pafaff 15. Primers for Genotypes: 5′-TGGACGGGACTATTGAAGGCTG-3′ (sense for Nrf2$^+/+$ and Nrf2$^{-/-}$), 5′-CGCCTTTTTCAGTAGATGGAGG-3′ (antisense for Nrf2$^{+/−}$), and 5′-
GCGGATTGACCGTAATGGGATAGG-3’ (antisense for LacZ). Primers that were used for Q-PCR are summarized in supplementary Table I.

**Statistical Analysis**

Data are shown as mean ± s.d.. Means were compared by ANOVA, followed by Bonferroni test for multiple comparisons. Differences were considered significant at P<0.05.
References


Figure legends

**Supplemental Figure 1.** A, Schematic depicting the TAC model and B, the measurement of mean blood pressure in the bilateral carotid arteries before and immediately after TAC. The mean blood pressure was measured as described in “Supplement Material”. The basal blood pressure of left and right carotid arteries of WT mice was 95 ± 11.3 and 96 ± 16.4 mmHg, respectively, while that in Nrf2−/− mice were 96.5 ± 7.9 and 98.3 ± 9.3 mmHg, respectively. After TAC, blood pressure of left and right carotid arteries of WT mice was 96 ± 4.6 and 141.1 ± 12.9 mmHg, respectively, while blood pressure of left and right carotid arteries of Nrf2−/− mice was 90 ± 1.8 and 133.5 ± 5.9 mmHg, respectively. TAC induced a similar, significant (*p<0.05) pressure gradient between the right and left carotid arteries in WT (44 mmHg) and Nrf2−/− (43 mmHg) mice. C, Heart rate of WT and Nrf2−/− mice two weeks after TAC. Heart rate was assessed by echocardiography in the mice anesthetized with 3% isoflurane. Due to the anesthesia, a decrease in the heart rate was observed in both WT and Nrf2−/− mice with sham or TAC operations as previously described 7. The heart rate of TAC mice was slightly lower that of sham mice 7, but there was no significant difference of the heart rate between TAC WT and Nrf2−/− mice.

**Supplemental Figure 2.** Nrf2 gain- and loss-of-function approach in primary cultures of rat neonatal cardiac myocytes and fibroblasts. A, Cardiac myocytes and fibroblasts were infected with several doses of adenovirus of Nrf2, and then subjected to Western blot analysis for Nrf2 protein expression. Results are representative of four separate experiments. B, Cardiac myocytes and fibroblasts were infected with several doses of adenovirus of rat Nrf2 shRNA, and then subjected to Q-PCR analysis for Nrf2 mRNA expression. *p<0.05 vs non-infected cells.

**Supplemental Figure 3.** Time course of cardiac hypertrophy in wild type ICR mouse. A, Heart weight/body weight (HW/BW) ratio. The 8-weeks-old wild type ICR/SV129 mice were randomly divided into two groups for TAC (n = 27) or Sham (n = 23) operations as described in “Supplement Material”. Mice were sacrificed at 1, 2, and 4 weeks after operation, and HW/BW ratio, cardiomyocyte size, and fetal gene expression and echocardiography were measured. Six
mice at age of 8 weeks without any treatments were subjected to various measurements as the basal data (n = 6, 0 week point). Numbers of animals at each end point were indicated in the figure. A, HW/BW ratio. *p<0.01 versus sham. B, Cardiac myocyte cross-sectional area. Left ventricular cross-sectional sections were stained with Texas Red-X conjugated wheat germ agglutinin (WGA). The left ventricular cardiac myocyte cross-sectional area was quantified from 2,000 -5,000 myocytes per heart in at least 20 randomly selected fields. *p<0.01 versus sham. C, & D, Expression of mRNA levels of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) in left ventricle. Hearts of mice were harvested, and left ventricles were dissected for RNA purification at the indicated time points. Expression of ANF and BNP was quantified by Q-PCR. *p<0.01 versus sham. E, Thickness of left ventricular posterior wall diastolic (LVPWd). F, Fraction shortening (FS). For E & F, mice were anesthetized by isoflurane and echocardiography was performed as described in “Supplement Material”. *p<0.01 versus sham.

Supplementary Figure 4. Expression profile of Nrf2 in murine heart after TAC. WT mice at age of 8 weeks were subjected to sham or TAC operations. The murine hearts were harvested at the times as indicated, and then used for analysis of Nrf2 mRNA and protein expression. A, Relative expression levels of Nrf2 mRNA in left ventricles of WT mice after TAC. The expression levels of Nrf2 mRNA were determined by Q-PCR. Numbers of hearts are indicated. *p<0.01 vs sham. B, Representative immunohistochemical staining of Nrf2 protein expression in left ventricles of WT mice after TAC. Left ventricle sections were processed by immunohistochemistry to determine Nrf2 protein expression. Nrf2 staining was performed at least in 4 sections per heart. Images were representatives of three hearts in each experimental group. Red arrow indicates nuclear translocated Nrf2. Brown color indicates Nrf2 protein expression. Scale bar = 50 μm; magnification, × 1000. C, Expression of Nrf2 downstream genes in murine heart after TAC. Expression of glutathione peroxidase (GPx), heme oxygenase 1 (HO-1), thioredoxin-1 (Txn-1), NAD(P)H:quinine oxidoreductase-1 (NQO-1), thioredoxin reductase (Txnrd), γ-glutamylcysteine ligase catalytic subunit γ-GCLc, and superoxide dismutase (SOD) 1-3 was assessed by Q-PCR. The number of hearts are indicated, *p<0.05 vs sham.
Supplementary Figure 5. Baseline characterization of Nrf2−/− mice. Measurements were made at age of 8 weeks of WT littermates and Nrf2−/− mice. A, Representative genotyping for WT and Nrf2−/− mice. The band for WT was about 700 bp and the band for Nrf2−/− mice was about 400 bp as described in “Supplement Material”. B, Representative pictures of hearts from WT and Nrf2−/− mice (top panel). Representative heart coronal sections stained with Masson’s trichrome (bottom panel). Scale bar = 5 mm. C, Heart weight to body weight (HW/BW) ratio of WT mice and Nrf2−/− mice. There was no significant difference of HW/BW ratio between WT and Nrf2−/− mice. D, Cardiomyocyte cross-sectional area. Left ventricular cross-sectional sections were stained with Texas Red-X conjugated WGA and quantified as supplementary Figure 3B. There was no significant difference of cardiomyocyte cross-sectional area between WT and Nrf2−/− mice.

Supplementary Figure 6. Representative long axis sections of hearts from WT and Nrf2−/− mice after TAC (n=6-7). Measurements were made two weeks after TAC. Scale bar = 5 mm. Masson’s trichrome staining was performed as described in “Supplement Material”. Collagen is blue, and nuclei are black. Cytoplasm, keratin muscle fibers, and intercellular fibers are red. Under higher magnification (> 100X), myocardial fibrosis could be clearly visualized. The fibrosis areas were significantly increased in TAC Nrf2−/− hearts, compared with the TAC WT hearts.

Supplementary Figure 7. Cardiac fetal gene expression in WT and Nrf2−/− mice two weeks after TAC. Expression of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), alpha-myosin heavy chain (α-MHC), and beta-myosin heavy chain (β-MHC) was quantified by Q-PCR as described in “Supplement Material”.

Supplementary Figure 8. Cardiac fibrosis in WT and Nrf2−/− mice two weeks after TAC. Left panel was the representative left ventricular (LV) or interventricular septum (IVS) sections stained with Masson’s trichrome. Right panel was the quantitative fibrosis area of LV and IVS interstitial fibrosis as a percentage of total microscopic area per heart.

Supplementary Figure 9. Apoptosis in the hearts of WT and Nrf2−/− two weeks after TAC. A, Representative TUNEL staining of apoptotic cells in left ventricle. TUNEL-positive, i.e.,
apoptotic nuclei in TAC hearts are stained red (arrows) and nuclei are blue. Myocardium is green (Alexa Fluor 488 Phalloidin to mark F-actin). Magnification, × 630. B, Apoptotic index. TUNEL-positive cells were quantified in all nuclei of whole LV per heart. Numbers of hearts are indicated. C, Representative staining of cleaved caspase-3 positive cells in left ventricle. Cleaved caspase-3-positive, i.e., cells with activated caspase-3 in TAC hearts are stained red (arrows) and nuclei are blue. Cardiomyocytes were green utilizing an antibody of anti-cardiac myosin heavy chain. Magnification, × 630. D, Relative caspase-3 activity. Cleaved caspase-3-positive cells were quantified in all nuclei of whole LV per heart. Numbers of hearts are indicated.

**Supplementary Figure 10.** Nrf2 downstream gene expression in the heart of WT and Nrf2−/− mice after TAC. Measurements were made two weeks after TAC. Expression of SOD-1, SOD-3 and γ-GCLc mRNAs was quantified by Q-PCR.

**Supplementary Figure 11.** Nrf2 gain- and loss-of-function on hypertrophic factor-induced ROS production and proliferation in rat neonatal cardiac fibroblasts. Representative images and quantitative analysis of intracellular ROS production in NE (200 μmol/L) or PE (100 μmol/L) treated cardiac fibroblasts that were infected with adenovirus of Nrf2 (A) or rat Nrf2 shRNA (B). *p<0.05 vs control cells that were treated with vehicles. C, [3H]Thymidine in NE (10 μmol/L) or PE (20 μmol/L) treated cardiac fibroblasts that were infected with adenovirus of Nrf2 or rat Nrf2 shRNA. *p<0.05 vs control cells that were treated with vehicle.

**Supplementary Figure 12.** Nrf2 gain- and loss-of-function on hypertrophic factor-induced enlargement of rat neonatal cardiac myocytes. Representative images of cell size enlargement in NE (20 μmol/L) or PE (20 μmol/L) treated cardiac myocytes that were infected with adenovirus of Nrf2 (A) or rat Nrf2 shRNA (B).

**Supplementary Table I.** Primers for Q-PCR.

**Supplementary Table II.** Echocardiography and Blood pressure of Nrf2−/− mice at basal physiological status. Measurements were made at age of 8 weeks of WT littermates and Nrf2−/− mice. Blood pressure was measured via tail cuff method and echocardiography was performed as
described in “Supplement Material”. BP, blood pressure; HR, heart rate; IVSd, interventricular septum diastolic; LVIDd, left ventricular internal dimension diastolic; LVIDs, left ventricular internal dimension systolic; LVPWd, left ventricular posterior wall diastolic; FS, fractional shortening. Non-significance, ns vs WT.
Li J et al., Supplementary Figure 1

A

Pressure Transducer

Right Carotid  Left Carotid
Right Subclavian  Left Subclavian
Aorta

B

Mean Blood Pressure (mmHg)

WT KO WT KO WT KO WT KO

Before TAC

After TAC

C

Heart Rate (min)

Sham TAC Sham TAC

WT Nrf2^-/-

ns

10 10 8 11

ns

* #
Li J et al., Supplementary Figure 2

A

Cardiomyocytes

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Cardiac fibroblasts

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Nrf2 mRNA relative levels (Nrf2 / GAPDH)

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n=4
Li J et al., Supplementary Figure 3

A) HW/BW

B) Myocyte Area

C) ANF mRNA

D) BNP mRNA

E) LVPWd

F) FS

- Sham
- TAC
Li J et al., Supplementary Figure 4A and B

A

Relative Nrf2 mRNA expression (Nrf2/GAPDH)

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<td>4</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

Sham

TAC
Li J et al., Supplementary Figure 4C
Li J et al., Supplementary Figure 5

A

WT  Nrf2<sup>−/−</sup>

700 bp  400 bp

B

WT  Nrf2<sup>−/−</sup>

Myocytes (μm<sup>2</sup>)

C

HW/BW (mg g<sup>−1</sup>)

WT  Nrf2<sup>−/−</sup>

n=6  n=4

D

Myocytes (μm<sup>2</sup>)

WT  Nrf2<sup>−/−</sup>

n=3  n=3

Li J et al., Supplementary Figure 5
Li J et al., Supplementary Figure 6

WT Nrf2−/−

Sham

TAC

5 mm
Li J et al., Supplementary Figure 7

**Relative ANF mRNA expression (ANF/GAPDH)**

- **WT**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

- **Nrf2−/−**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

**Relative BNP mRNA expression (BNP/GAPDH)**

- **WT**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

- **Nrf2−/−**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

**Relative βMHC mRNA expression (βMHC/GAPDH)**

- **WT**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

- **Nrf2−/−**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

**Relative αMHC mRNA expression (αMHC/GAPDH)**

- **WT**
  - Sham: n=3
  - TAC: n=4
  - p<0.01

- **Nrf2−/−**
  - Sham: n=3
  - TAC: n=4
  - p<0.01
  - p<0.05
Li J et al., Supplementary Figure 8

LV Fibrosis (% area)

WT Nrf2-/-
n=6 n=6

p<0.01

IVS Fibrosis (% area)

WT Nrf2-/-
n=6 n=6

p<0.01
Li J et al., Supplementary Figures 9A and B

A

WT

Nrf2^/-

Sham

TAC

B

TUNEL positive (%)

p<0.01

n=7

n=6

n=6

Sham TAC Sham TAC WT Nrf2^/-
Li J et al., Supplementary Figures 9C and D

C

WT

Nrf2−/−

Sham

TAC

D

Cleaved Caspase 3 positive (%)

WT

Nrf2−/−

n=7

n=6

n=6

n=6

p<0.05

p<0.01

p<0.01

0

0.3

0.6

0.9

1.2

Sham

TAC

Sham

TAC

n=7

n=6

n=6

n=6
A

\[ \frac{\gamma\text{-GCLc/GAPDH}}{} \]

- Sham TAC
- WT
- Nrf2\(^{-/-}\)
- n=6
- n=6
- n=5
- n=6

B

\[ \frac{\text{SOD-1/GAPDH}}{} \]

- Sham TAC
- WT
- Nrf2\(^{-/-}\)
- n=6
- n=6
- n=5
- n=6

C

\[ \frac{\text{SOD-3/GAPDH}}{} \]

- Sham TAC
- WT
- Nrf2\(^{-/-}\)
- n=6
- n=6
- n=5
- n=6

\[ p<0.05 \]

\[ p<0.01 \]

\[ \text{ns} \]
Li J et al., Supplementary Figure 11A and B

A

Vehicle | NE | PE
--- | --- | ---
Ad-βGal |  |  |
Ad-Nrf2 | | |

Intercellular ROS (X10^3 IOD)

- Ad-GFP
- Ad-Nrf2

p<0.01

Control | NE | PE
--- | --- | ---
4 | 4 | 4

B

Vehicle | NE | PE
--- | --- | ---
Ad-scramble shRNA |  |  |
Ad-Nrf2 shRNA | | |

Intercellular ROS (X10^3 IOD)

- Ad-scramble shRNA
- Ad-Nrf2 shRNA

p<0.01
Li J et al., Supplementary Figure 11C

C

[³H]-Thymidine uptake (CPM/well)

<table>
<thead>
<tr>
<th></th>
<th>Ad-GFP</th>
<th>Ad-Nrf2</th>
<th>p-value</th>
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<tbody>
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<td>4</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>4</td>
<td>4</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>PE</td>
<td>4</td>
<td>4</td>
<td>p&lt;0.01</td>
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</tbody>
</table>

[³H]-Thymidine uptake (CPM/well)

<table>
<thead>
<tr>
<th></th>
<th>Ad-scramble shRNA</th>
<th>Ad-Nrf2 shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NE</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PE</td>
<td>4</td>
<td>4</td>
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</tbody>
</table>
Li J et al., Supplementary Figure 12

A

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>NE</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-βGal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-Nrf2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>NE</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Scramble shRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-Nrf2 shRNA</td>
<td></td>
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### Supplementary Table I. Sequence of Primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Gene access #</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product length</th>
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</thead>
<tbody>
<tr>
<td>ANF</td>
<td>NM_008725.2</td>
<td>CATCACCCTGGGCTTTCTTCCCT</td>
<td>TGGGCTTCCAATCCTGTCAATC</td>
<td>405</td>
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<tr>
<td>BNP</td>
<td>NM_008726.4</td>
<td>CGGCACATGGATCTGAGTGAGGGGA</td>
<td>CCCAGGCGAGAGTGCAAACTG</td>
<td>418</td>
</tr>
<tr>
<td>α-MHC</td>
<td>NM_010856.3</td>
<td>CCAATGAGTACCCTGGGA</td>
<td>ACAGTCATGCCGGGATGTG</td>
<td>254</td>
</tr>
<tr>
<td>β-MHC</td>
<td>NM_080728.2</td>
<td>ATGTGGGCCGACCTTGGAA</td>
<td>CCTCGGGTTAGCTGAGAGATCA</td>
<td>170</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>NM_009722.3</td>
<td>CCATCTGGCTTGTCCATGCTACT</td>
<td>CAAATGGGTTAGGAACGGGTTACT</td>
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<tr>
<td>GAPDH</td>
<td>XM_001479322</td>
<td>ATGTTCCAGTATGACTCCACTCACG</td>
<td>GAAGACACCAGTAGACTCCACGACA</td>
<td>171</td>
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<td>Nrf2</td>
<td>NM_010902.3</td>
<td>ATGATGGACTTGGAGTGGCC</td>
<td>TCCTGGCTCCTTGGAGGTG</td>
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<td>NQO-1</td>
<td>NM_008706.5</td>
<td>CGGTATTACGATCTCTCCTCAAACA</td>
<td>AGCCTCTACAGCAGGCTCCTCAT</td>
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<td>γ-GCLc</td>
<td>NM_010295.1</td>
<td>CGGCATCGGAGAGGGAGGAGA</td>
<td>AGTGGCCAGCTGATCATAAGGTG</td>
<td>234</td>
</tr>
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<td>HO-1</td>
<td>NM_010442.2</td>
<td>AGGAGATAGAGCGCAACAAAGCAGA</td>
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<td>Trxnd-1</td>
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<td>GTTGTGCTGGCCGGTAGGAGGAGAGT</td>
<td>GTCACCGATGGCGTAGATGTAAGG</td>
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<td>SOD-1</td>
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<td>GPx</td>
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<td>GAGAATGGCAAGAATGAAGAG</td>
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<tr>
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<td>ATCTTTGGCCCGCTCTGATTGGTGGTC</td>
<td>GGTGCGCGAGGCTCCTGGAG</td>
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<td>Txn-1</td>
<td>NM_011660.3</td>
<td>CCCACCCTTTTGGACCCCTTTAT</td>
<td>AGCCCTTTCCCATTCCCTCTGT</td>
<td>147</td>
</tr>
</tbody>
</table>
**Supplementary Table II. Baseline characterization of Nrf2\(^{-/-}\) mice**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Nrf2(^{-/-})</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((n))</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td><strong>Hemodynamics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>systolic</td>
<td>119.89 ± 7.90</td>
<td>108.50 ± 6.57</td>
<td>ns</td>
</tr>
<tr>
<td>Diastolic</td>
<td>102.79 ± 8.81</td>
<td>96.06 ± 9.68</td>
<td>ns</td>
</tr>
<tr>
<td>HR</td>
<td>623.51 ± 49.78</td>
<td>562.41 ± 72.30</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((n))</td>
<td>19</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.71 ± 0.11</td>
<td>0.75 ± 0.10</td>
<td>ns</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>4.24 ± 0.22</td>
<td>4.46 ± 0.34</td>
<td>ns</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.95 ± 0.28</td>
<td>3.03 ± 0.34</td>
<td>ns</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.77 ± 0.08</td>
<td>0.79 ± 0.14</td>
<td>ns</td>
</tr>
<tr>
<td>FS (%)</td>
<td>34.29 ± 5.60</td>
<td>32.02 ± 3.27</td>
<td>ns</td>
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