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Original article

Nrf2 enhances myocardial clearance of toxic ubiquitinated proteins

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ABSTRACT

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a master transcription factor that controls the basal and inducible expression of a battery of antioxidant genes and other cytoprotective phase II detoxifying enzymes. While knockout of Nrf2 exaggerates cardiac pathological remodeling and dysfunction in diverse pathological settings, pharmacological activation of Nrf2 protects against cardiomyocyte injury and cardiac dysfunction. In contrast, there is also a concern that the chronic activation of Nrf2 secondary to oxidative stress is a contributing mechanism for the reductive stress-mediated heart failure. However, a direct link between cardiac specific activation of Nrf2 and cardiac protection or dysfunction in vivo remains to be established. Therefore, we investigated the effect of cardiomyocyte-specific transgenic activation of Nrf2 ($Nrf2^{ctg}$) on cardiac pathological remodeling and dysfunction. We found that the cardiomyocyte-specific activation of Nrf2 suppressed myocardial oxidative stress as well as cardiac apoptosis, fibrosis, hypertrophy, and dysfunction in a setting of sustained pressure overload induced by transverse aortic arch constriction (TAC) in mice. Notably, the constitutive activation of Nrf2 increased the steady level of autophagosomes while decreasing the ubiquitinated protein aggregates in the heart after TAC. Nrf2 gene gain- and loss-of-function approaches revealed that Nrf2 enhances autophagosome formation and autophagic flux in cardiomyocytes. Unexpectedly, while Nrf2 minimally regulated apoptosis, it suppressed significantly the proteotoxic necrosis in cardiomyocytes. In addition, Nrf2 attenuated the proteocytotoxicity presumably via enhancing autophagy-mediated clearance of ubiquitinated protein aggregates in cardiomyocytes. Taken together, we demonstrated for the first time that cardiac specific activation of Nrf2 suppresses cardiac maladaptive remodeling and dysfunction most likely by enhancing autophagic clearance of toxic protein aggregates in the heart.

1. Introduction

Heart failure is the consequence of sustained, abnormal neurohormonal and mechanical stress and remains a leading cause of death worldwide. Pathological stress, such as hypertension, results in cardiac hypertrophy, myocardial apoptosis and fibrosis, altered microvascular structure, and chamber dilation, culminating in cardiac dysfunction and heart failure [1]. However, the underlying mechanisms are far from clear.

Notably, a causative role of oxidative stress in the pathogenesis of cardiovascular disease has been established [2,3]. However, the antioxidant approaches of non-selective reactive oxygen species (ROS) scavenging for the treatment of cardiovascular disease are ineffective or even harmful [3]. Accordingly, an effective therapy may require more specific targeting of either the source of oxidative stress or the endogenous antioxidant defense system [3]. In this context, we have demonstrated that Nrf2, a master transcription factor in controlling the basal and inducible expression of a battery of antioxidant genes and other cytoprotective phase II detoxifying enzymes, is a negative regulator of cardiac pathological remodeling and dysfunction in diverse pathological settings [4–7]. While it has been documented that Nrf2 plays a mediator role in hydrogen sulfide-mediated cardioprotection [8], we and others have demonstrated that Nrf2 might be a drug target for the treatment of cardiomyocyte injury and cardiac dysfunction [4,7,

Abbreviations: ANF, atrial natriuretic factor; Ang II, angiotensin II; αMHC, alpha-myosin heavy chain; βMHC, beta-myosin heavy chain; BNP, brain natriuretic factor; BafA1, bafilomycin A1; LC3, microtubule-associated protein 1 light chain 3; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, nuclear factor erythroid-2 related factor 2; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum calcium ATPase2a; TAC, transverse aortic arch constriction; TXN-1, thioredoxin-1; UPS, ubiquitin proteasome system.

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9–12]. Despite the prominent contribution of Nrf2 in cardiac protection, a direct link between cardiac specific activation of Nrf2 and cardiac protection in vivo remains to be established.

Recently, emerging evidence has indicated that reductive stress due to an increase in reduced glutathione may causally contribute to cardiomyopathy induced by protein aggregation [13]. In the setting of protein aggregation cardiomyopathy, there is a concern that the chronic activation of Nrf2 secondary to oxidative stress is a contributing mechanism for the reductive stress-mediated heart failure [14]. Although the 'dark' side of Nrf2 has not yet been demonstrated, Nrf2 appears to be involved in the regulation of protein aggregation in autophagic substrate selection [15] as well as the macroautophagy (commonly known as autophagy) per se [16–18]. Autophagy is an evolutionarily conserved process that mediates the lysosome-dependent turnover of macromolecules and entire organelles [19]. Importantly, autophagy and the ubiquitin proteasome system (UPS) are the major routes for the complete degradation/clearance of abnormal protein products in cells [20,21]. UPS is usually effective in clearing soluble misfolded or damaged proteins via ubiquitination of the target proteins whereas autophagy is generally efficient in clearing less soluble or insoluble ubiquitinated protein aggregates. Upon a functional impairment of UPS, the ubiquitinated soluble abnormal proteins accumulate and aggregate into insoluble and toxic protein aggregates, and then autophagy is activated to be a major clearance route of the ubiquitinated proteins by default. Moreover, myocardial protein aggregation is often associated with cardiac proteasome insufficiency in various cardiomyopathies and heart failure [22,23] while the activation of autophagy may result in either protective or detrimental consequences in the heart [24,25]. These results raise an intriguing question whether Nrf2 plays a role in the control of protein quality via regulating autophagy in the heart thereby contributing to cardiac remodeling and heart failure.

Thus, we sought to investigate the impact of cardiac specific activation of Nrf2 on cardiac maladaptive remodeling and dysfunction and explore the possibility of a novel role for Nrf2 in regulating autophagy-mediated clearance of protein aggregates and preventing cardiac dysfunction. The results herein indicate that constitutive activation of Nrf2 facilitates autophagic clearance of protein aggregates in the heart thereby protecting against cardiac dysfunction. In addition, Nrf2 primarily suppresses necrosis rather than apoptosis in cardiomyocytes in a setting of toxic ubiquitinated protein overload via facilitating autophagic clearance of the toxic protein aggregates.

2. Methods

2.1. Animals

Transgenic mice with cardiomyocyte-specific overexpression of Nrf2 ($Nrf2^{ctg}$) were generated in a FVB/NJ background using the transgene cassette containing a murine 5.5 kb alpha myosin heavy chain (α-MHC) promoter [26] (a gift from Dr. Jeffrey Robbins at Cincinnati Children's Hospital Medical Center, Cincinnati, Oho) in the Medical University of South Carolina (MUSC) Transgenic Core. Nrf2 knockout ($Nrf2^{-/-}$) mice were generated using heterozygote breeding pairs ($Nrf2^{+/-}$ in an ICR/Sv129 background) as previously described [4]. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Institution of Health), and all protocols were approved by the University of South Carolina Institutional Animal Care and Use Committee.

2.2. Transverse aortic arch constriction (TAC)

Male mice at 8–9 weeks of age were subjected to sham or TAC operations as described [4]. Mice were humanely euthanized after 4 weeks of TAC. Echocardiography, blood pressure measurement, pathological and biochemical analysis were performed as described [4]. Ubiquitinated protein aggregates were determined by filter trap assay as described [27].

2.3. Cell cultures, virus preparations, infection, autophagy flux, and cell death assay

Rat neonatal cardiomyocytes and mouse adult cardiomyocytes were isolated and cultured as described [4,28]. Rat cardiac myocyte-like H9C2 cells were purchased from ATCC. Virus preparation, stable infectants, autophagic flux assay, and cell death assay were performed as described in the Online Data Supplement.

2.4. Statistics

Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance with the Student *t* test when the sample size was appropriate and the population was distributed normally. When differences among >3 groups were evaluated, results were compared by ANOVA followed by Bonferroni test for multiple comparisons. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Generation of Nrf2 cardiomyocyte-specific transgenic ($Nrf2^{ctg}$) mice

Three founders of $Nrf2^{ctg}$ mice were identified by PCR (Supplementary Figs. 1A–C). Two lines of $Nrf2^{ctg}$ mice with relative high or modest overexpression of inserted human Nrf2 gene have been established by standard breeding (Supplementary Fig. 1D). $Nrf2^{ctg}$ mice with modest overexpression of hNrf2 were expanded. Upregulation of Nrf2 protein expression in the heart of $Nrf2^{ctg}$ mice was confirmed by Western blot (Supplementary Fig. 1E). These mice were viable and fertile, and had no observable health problems when housed in pathogen-free conditions. Adult $Nrf2^{ctg}$ mice did not develop any apparent cardiovascular system functional abnormalities (Supplementary Tables 1 and 2), suggesting that constitutive activation of Nrf2 in cardiomyocytes is not detrimental to the heart.

3.2. Cardiomyocyte-specific overexpression of Nrf2 suppresses cardiac maladaptive remodeling and dysfunction in response to chronic pressure overload

To determine the effect of cardiac specific activation of Nrf2 on cardiac dysfunction, we created TAC in littermates of male wild type (WT) and $Nrf2^{ctg}$ mice. We have previously demonstrated that the TAC-induced pressure overload initially results in an adaptive cardiac remodeling with preserved cardiac function (days 1–14) in mice [3]. However, the ability of the heart to compensate for this sustained elevation in stress is limited and, as a result, the remodeling becomes maladaptive and left ventricular dysfunction ensues (days 14–28) [3]. The decrease in fractional shortening FS (%) appears on day 14, and thereafter is escalated over time [3]. There is a ~40% decrease in FS (%) in survived 4 week-TAC mice, which develops apparently myocardial oxidative stress, hypertrophy, fibrosis, and cell death [3]. Importantly, compared with TAC WT control, the decreases in FS (%) on day 14 and day 28 were less in TAC $Nrf2^{ctg}$ mice, demonstrating that chronic activation of Nrf2 in cardiomyocytes protects against TAC-induced cardiac dysfunction (Table 1 and Supplementary Table 3). WT mice after TAC developed myocardial oxidative stress, hypertrophy, fibrosis, and apoptosis (Fig. 1, Supplementary Figs. 2–4, and Table 1); however, the increased fibrosis in left ventricles (LVs) (25%) was excessively high compared with the previous findings [7,29]. Of interest, there was a substantial fibrosis (11%) in right ventricles (RAs) (Table 1 and Supplementary Fig. 3), which is usually around 2% in WT C57BL/6J mice 4 weeks after TAC [29,30]. A careful review of previous studies regarding TAC-induced pathological cardiac remodeling and heart failure

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