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Loss of Nrf2 promotes rapid progression to heart failure following myocardial infarction

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ABSTRACT

Nrf2 gene encodes a transcription factor regulating the expression of antioxidant and detoxification genes. We test here whether Nrf2 plays a role for cardiac protection during ischemic injury in an effort to establish Nrf2 as a target for cardiac protection therapies. Cardiac ischemia induced by the left anterior descending (LAD) coronary artery ligation results in myocardial infarction (MI). Young mice surviving MI show minimal signs of heart failure. Mice lacking Nrf2 experience an accelerated progression to heart failure that occurs within 10 days following induction of MI. Nrf2 knockout (Nrf2 KO) mice have a survival rate similar to wild type (WT) mice at 24 h after MI, but a significantly higher mortality rate within 10 days after MI (50% vs 86%). Morphological examination revealed maladaptive remodeling, including cardiac hypertrophy and dilated left ventricle in Nrf2 KO mice, which were absent in WT mice. Measurements of cardiac function revealed increased left ventricular mass and decreases in cardiac output in Nrf2 KO mice. In addition, Nrf2 KO mice show biomarkers of heart failure, such as elevated levels of β-MHC, ANF, and BNP mRNA in the myocardium. These data support that Nrf2 plays an important role in protecting the myocardium from ischemic injury. Lack of Nrf2 leads to rapid development of heart failure.

1. Introduction

Heart disease is the leading cause of natural death worldwide. Coronary heart disease alone is responsible for 1 of every 6 deaths in the United States (Mozaffarian et al., 2016). Blockage of coronary artery results in myocardial infarction (MI) and death of cardiomyocytes within ischemic area. Early on after a MI, a robust immune response is initiated shown as recruitment of neutrophils and macrophages to the infarcted region for removal of necrotic tissue (Frangogiannis, 2014). Myofibroblasts and fibroblasts are activated for collagen deposition, and formation of fibrous scar tissue serves as a means of wound repair and preventing the rupture of ventricular wall (Sutton and Sharpe, 2000; Liehn et al., 2011). In response to loss of contractile force due to death of cardiomyocytes, the non-infarcted area of the ventricles usually develops hypertrophy by enlargement of cardiomyocytes (Frey and Olson, 2003). Initially, hypertrophy of ventricular wall can be compensatory for maintaining the contractile force to meet the demand of the workload of whole body metabolism. However, hypertrophy becomes decompensatory overtime and progressive myocardial remodeling involving dilated hypertrophy along with insufficiency of contractile force occurs during heart failure. Those surviving MI are at a risk of developing heart failure. Heart failure is a main cause of morbidity and mortality yet lacks effective pharmacological treatment.

Animal models have been developed for studying the biochemical and molecular events associated with wound healing and progression towards heart failure after MI. Surgical occlusion of the left anterior descending (LAD) coronary artery is a well-established model for initiating an MI in rodent models. However, young mice (2–3 months) typically recover from MI induced by LAD coronary occlusion, surviving for months without showing significant signs of heart failure (Michael et al., 1999; Gould et al., 2002). Older mice, e.g. 14-months old, develop heart failure after MI (Gould et al., 2002), making the model relevant to humans, since the occurrence of MI is of a particular concern to the elderly, who is at the risk of developing heart failure and has a higher mortality rate (St John Sutton et al., 1997; DeGeare et al., 2000; Haase et al., 2000). Economically, because of the expenses of keeping experimental animals to old age, a new model that can develop heart failure within a short period of time following MI is useful for studying the pathophysiology and for drug development against heart failure.

Nrf2 is a basic leucine zipper transcription factor best known as a master regulator of antioxidant and detoxification genes. In response to oxidative or electrophilic stress, Nrf2 protein is induced and translocates from the cytosol to the nucleus to bind to the Antioxidant Response Element located in the promoter of a number of antioxidant and detoxification genes, such as NAD(P)H: quinone dehydrogenase, superoxide dismutase 1, glutathione transferases, glutamate cysteine

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ligase, and heme oxygenase-1 (Jeong et al., 2006; Kensler et al., 2007; Maher and Yamamoto, 2010). Genome wide profiling of genes downstream of Nrf2 regulation revealed a long list of genes involved in pleiotropic action ranging from tissue repair to proliferative signaling (Lee et al., 2003; Malhotra et al., 2010). Nrf2 regulation of such a wide array of antioxidant, detoxification, proliferation, and repair genes has led to a broad interest of Nrf2 as an important target for cytoprotection. Although the cytoprotective effects of Nrf2 have been well studied in numerous tissues and cell lines, limited studies have investigated the role of Nrf2 in modulating myocardial physiology, injury, or disease states. These limited studies involve the use of small molecule Nrf2 inducers (He et al., 2009; Piao et al., 2010; Ashrafian et al., 2012; Bai et al., 2013; Deng et al., 2013; Cheng et al., 2015). Previous works from our laboratory indicated that genetic knockout of Nrf2 results in enhanced acute cardiac injury due to ischemic reperfusion, and loss of cardiac protection by ischemic preconditioning. Since prolonged ischemic injury can cause heart failure over time, we address here whether genetic knockout of Nrf2 results in an increased incidence of heart failure.

2. Materials and methods

2.1. Chemicals

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Reagents for histology, Hematoxylin GX3, Clear View CV1, Bluing Reagent BX1, and Eosin EX1 were obtained from Creative Waste Solutions, Inc., Tualatin, OR.

2.2. Surgical procedure

Male C57BL/6J mice, age 8–12 weeks, were housed, cared for and used for experiments according to National Institutes of Health Guidelines on the Use of Laboratory Animals. Animal breeding, handling and surgery protocol were reviewed, approved and monitored by the University of Arizona Institutional Animal Care and Use Committee. The founder pair of Nrf2 KO mice was obtained from Dr. Donna Zhang's laboratory at the University of Arizona with the permission from Dr. Jefferson Y. Chan at the University of California Irvine (Chan et al., 1996). Heterozygous male founders were mated to heterozygous female and offsprings were genome typed. Male wild type or homozygous Nrf2 KO at 8–12 weeks old were used for LAD occlusion (Michael et al., 1995; Michael et al., 1999; Xu et al., 2011; Xu et al., 2014). After induction of deep anesthesia with Avertin (240 mg/kg, i.p.) by frequent assessments of withdrawal from toe pinch, decreased respiration rate and mucous membrane condition, a tracheotomy was performed to ventilate the animals. The heart was exposed by a left thoracotomy at the third intercostal space, and a 8–0 suture was used to ligate the left anterior descending (LAD) coronary artery 1–2 mm below the atria. The chest cavity was closed by bringing together the second and third ribs with a 6–0 nylon suture. Slight pressure is applied on the chest with the needle holder to reduce the volume of free air in the chest cavity while tying a knot. All layers of muscle and skin are closed with 6–0 continuous absorbable and nylon sutures, respectively. The animal was recovered under a heated pad with a heat lamp until ambulatory, before being returned to the home cage. Analgesia is extended with subcutaneous 0.1 mg/kg buprenorphine every 8–12 h for the first 48 h. The mice recovered overnight in warm housing. At 10 days after the procedure, the mice were euthanized by 100 mg/kg Sodium Penobarbital following echocardiography, and the hearts were excised and the wet weight of each heart was recorded for calculating heart/body weight ratio.

2.3. Echocardiogram analysis

The mice were anesthetized using 1.5% isoflurane and digital images were recorded at a frame rate of 200 frames/s with a linear 35-MHz probe (Model 707B Scanhead, Visualsonics, Canada) for left ventricular function analysis by echocardiogram (ECHO). 2-D M-mode analysis was used to calculate ejection fraction, fractional shortening, ventricle wall thickness, and intra-ventricle diameter. Aortic outflow tract velocity time integral was calculated using a Doppler.

2.4. Histology

Excised hearts were fixed in 10% formalin for 24 h and embedded in paraffin. Transverse sections of the heart, about 5 μm thick, were generated from paraffin blocks and mounted on microscope slides. After deparaffinization by xylene (three changes, 3 min each) and sequential washes from 100% to 70% ethanol, heart tissue sections were rinsed in water before 2 mins incubation in Hematoxylin GX3. After washing with running tap water, the tissue sections were rinsed in Clear View CV1 to remove non-specific staining. After one minute in Bluing Reagent BX1 to facilitate the staining intensity of Hematoxylin G3, and 10 quick dips in 80% ethanol, the slides were incubated with Eosin EX1 for one minute followed by 3 one minute washes with 100% alcohol and 3 one minute washes with Xylene before mounting with coverslips. Stained slides were observed under an Olympus BX53 microscope with an attached DP72 digital camera. Images were acquired by Olympus cellSens software using the size of lens as indicated in the figures.

2.5. Real-time RT-PCR

Total RNA extracted using TRIzol was used as templates for RT-PCR. cDNA syntheses were performed using a commercial cDNA synthesis kit (Fermentas) with random hexamers. PCR primers were purchased from Sigma with the sequences of 5′-CAATGCGACCAAGCTGTGTG-3′ (forward) and 5′-CCGATAGATCTTCCCTCTTG-3′ (reverse) for ANF; 5′-TGGATTCTCAAACGTGTCTAGTGA-3′ (forward) and 5′-GCATTCTCCTGCTGTTTCCTT-3′ (reverse) for β-MHC; 5′-CTATCCTTTTGTTTTCCATCCTG-3′ (forward) and 5′-CAGGATGGAAAACAAAAGGATAG-3′ (reverse) for BNP; 5′-TGTGATGGTGGGAATGGGTCA-3′ (forward) and 5′-TGTGGTGCCAGATCTTCTCCA-3′ (reverse) for β-actin (reference 1); 5′-CCAGTAAGTGCGGGTCATAAG-3′ (forward) and 5′-GGCCTCACTAAACCATCCAA-3′ (reverse) for 18S rRNA (reference 2); and 5′-AACTTTGGCATTGTGGAAGG-3′ (forward) and 5′-ACACATTGGGGGTAGGAACA-3′ (reverse) for GAPDH (reference 3). Quantitative real time PCR was performed using the CFX96 Thermal Cycler (Bio-Rad) and Cyber Green dye (Fermentas). Real-Time PCR was performed with initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s for denaturation, 60 °C for 30 s for annealing and 72 °C for 30 s for extension. Melting curve analysis was performed at the end of PCR to verify the specificity of the product. Bio-Rad CFX Manager software was used for data analyses. The expression values were calculated as Relative Quantity$_{gene}$ = E$_{gene}$^(C$_{T(control)}$ − C$_{T(sample)}$), Normalized Expression$_{sample}$ = RQ$_{sample}$/(RQ$_{reference\ 1}$ × RQ$_{reference}$ × … × RQ$_{reference\ n}$)^(1/n), with E = efficiency of primer set calculated by (% Efficiency × 0.01) + 1, where 100% efficiency = 2, and RQ = relative quantity of sample.

2.6. Statistical analysis

Student's *t*-test was used to compare two means of data with standard deviation (SD). One-way analysis of variance (ANOVA) with a Bonferroni correction was used to compare multiple (>2) means. Survival curves were compared using the Mantel-Cox and Gehan-Breslow-Wilcoxon tests. A p-value of <0.05 was used as the cutoff for statistical significance.

3. Results

Nrf2 knockout (KO) mice and wild type (WT) littermates were subjected to permanent ligation of the LAD coronary artery. Following the