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Research Paper

Constitutive activation of Nrf2 induces a stable reductive state in the mouse myocardium

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ABSTRACT

Redox homeostasis regulates key cellular signaling pathways in both physiology and pathology. The cell's antioxidant response provides a defense against oxidative stress and establishes a redox tone permissive for cell signaling. The molecular regulation of the well-known Keap1/Nrf2 system acts as sensor responding to changes in redox homeostasis and is poorly studied in the heart. Importantly, it is not yet known whether Nrf2 alone can serve as a master regulator of cellular redox homeostasis without compensation of the transcriptional regulation of antioxidant response element (ARE) genes through alternate mechanisms. Here, we addressed this question using cardiac-specific transgenic expression at two different levels of constitutively active nuclear erythroid related factor 2 (caNrf2) functioning independently of Keap1. The caNrf2 mice showed augmentation of glutathione (GSH), the key regulator of the cellular thiol redox state. The Trans-AM assay for Nrf2-binding to the antioxidant response element (ARE) showed a dose-dependent increase associated with upregulation of several major antioxidant genes and proteins. This was accompanied by a significant decrease in dihydroethidium staining and malondialdehyde (MDA) in the caNrf2-TG mice myocardium. Interestingly, caNrf2 gene-dosage dependent redox changes were noted resulting in generation of a multi-stage model of pro-reductive and reductive conditions in the myocardium of TG-low and TG-high mice, respectively. These data clearly show that Nrf2 levels alone are capable of serving as the master regulator of the ARE. These models provide an important platform to investigate the impact of the Nrf2 system independent of the need to regulate the activity of Keap1 and the consequent exposure to pro-oxidants or electrophiles, which have numerous off-target effects.

1. Introduction

Mal-adaptive, irreversible remodeling of cardiac muscle leads to structural and functional anomalies triggering heart failure, which is a growing cause of morbidity and mortality in humans across the globe [1,2]. A majority of the heart diseases are believed to be associated with oxidative stress; the concept of antioxidant induction is widely regarded as a plausible approach to protect the heart against oxidative stress [3,4]. Homeostasis of reduction and oxidation (redox homeostasis) in response to the cellular redox environment is essential for normal physiology. However, an increasing number of reports indicate that strategies involving activation of the cellular reductive status or the supplementation of dietary antioxidants have little or no efficacy [5]. There can be a number of explanations for these data but one possibility is that maintenance of a redox tone which is neither hyper-reduced nor oxidized is essential. Indeed we have proposed that there is a counterpart to oxidative stress which we term reductive stress in which physiological 'redox signaling' is perturbed by hyper-reduction resulting in pathophysiological effects in the heart and other organs [6–8].

Nrf2 is a master molecular switch and a central regulator of basal and inducible antioxidant mechanisms in cells [9–11]. In the quiescent state, Nrf2 is held inactive in the cytoplasm by strong binding of its repressor regulator, Keap1 which inactivates Nrf2 through sustained ubiquitination and subsequent proteasomal degradation [11–13]. Upon

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electrophilic stress, Keap1 cysteines are modified inducing dissociation from Nrf2 thus allowing nuclear translocation of Nrf2 to activate transcription of antioxidant genes [9–12]. Paradoxically, and in contrast to the oxidative stress paradigm, chronic activation of Nrf2 can be deleterious [14–16]. For example, using a genetic model of familial heart disease caused by mutant R120G αB-crystallin we have demonstrated an association of reductive stress with mutant protein aggregation cardiomyopathy [6,14,16]. This reductive stress could be attributed to a maladaptive compensatory response to the aggregation of mutant proteins. In these models and others in which activation of Nrf2 is induced by exposure to oxidants it is not possible to know the direct effect of changing the cellular redox state from its physiological set point. For example, to increase nuclear translocation of Nrf2 oxidative modification of Keap1 protein thiols is needed and the consequent exposure of cells to electrophilic or oxidative stress has numerous off-target effects on bioenergetics and autophagy [13,17,18]. Hence, establishing an exclusive model (independent of electrophilic or oxidative stress) for understanding the molecular impact of Nrf2 on cellular redox homeostasis is essential.

We hypothesize that a constitutive activation of Nrf2 signaling will promote the transcription and translation of antioxidants to cause a pro-reductive or hyper-reductive state independent of Keap1 or exposure to exogenous oxidants and without other compensatory mechanisms of transcriptional regulation of ARE genes. Here, we employed a genetic approach by which a hyper-reductive state is stably induced and maintained in the heart using constitutive overexpression of nuclear factor (erythroid-derived-2) – like 2 (NFE2L2 or Nrf2). This demonstrates that beyond Keap1, maintenance of the cellular redox state is predominantly under the control of Nrf2.

2. Methods

2.1. Preparation of constitutively active Nrf2 (caNrf2) plasmid construct and generation of heart-specific transgenic mice

A recombinant DNA construct containing a cDNA fragment encoding constitutively active mouse Nrf2 (caNrf2) was placed downstream of a mouse alpha-myosin heavy chain (α-MHC) promoter to facilitate cardiomyocyte specific expression. To prevent Keap1 cytosolic repression of nuclear translocation of Nrf2, the constitutively active Nrf2 construct (caNrf2) was developed by excising amino acids 1–89 of the Neh2 domain from the mouse WT Nrf2 cDNA. Therefore, a forward primer starting at the 90th amino acid (corresponding to 267th base pair position) was amplified and used for subsequent cloning. Restriction digestion and subsequent sequencing analysis confirmed orientation and integrity of the inserts. The plasmid backbone was then removed using *Bam*H1 and the α-MHC-caNrf2 fragment was gel purified. Transgenic mice were generated by pronuclear injection; founders were back-crossed onto the C57BL/6 background for at least 6 generations. Transgenic animals were genotyped and confirmed with the presence of a ~230 bp PCR product. Based on the expression of caNrf2 normalized to endogenous Nrf2 determined by quantitative RT-PCR using genotyping primers for caNrf2 and endogenous Nrf2 specific primers, transgenic low (TG-low) and transgenic high (TG-high) founders #9384 and #1531, respectively, were confirmed. TG-low and TG-high animals were separately bred and maintained. Mice housed under controlled temperature and humidity, a 12 h light/dark cycle and fed with a standard rodent diet and water ad libitum. The Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham and the University of Utah, Salt Lake City, Utah approved all animal experiments, in accordance with the standards established by the US Animal Welfare Act.

2.2. Reagents

RNeasy kit, reverse transcription kit, and QuantiTect SYBR Green PCR kit were purchased from Qiagen Inc., Valencia, CA. The antibodies for GCLM, GCLC, NQO1, GSR, GSTμ, SOD1, and SOD2 procured from Abcam, Cambridge, MA, USA, Catalase-ab from EMD Millipore, USA and rabbit anti-GAPDH from Cell Signaling, USA. The anti-rabbit or mouse secondary antibodies for immunoblots horse radish peroxidase conjugated with IgG were purchased from Vector Laboratories, Burlingame, CA, USA. Secondary antibodies for immunofluorescence conjugated with Alexa fluor 488 anti-rabbit and anti-mouse were obtained from Life Technologies Corporation, NY, USA. Primers for qPCR were designed using Harvard medical school PrimerBank website and purchased from Integrated DNA Technologies, Coralville, IA. Protein Assay reagent (#500–0006) was procured from Bio-Rad, Hercules, CA. All other chemicals including reduced and oxidized glutathione were purchased from Sigma-Aldrich unless otherwise stated.

2.3. Autopsy, tissue collection and storage

Mice were anesthetized using isoflurane and euthanized by cervical dislocation. Hearts were immediately excised, perfused with ice cold phosphate buffered saline and appropriately stored for RNA, protein and histological experiments. Tissues used for RNA isolation were stored in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) and for proteins; tissues were immediately flash frozen in liquid nitrogen. Middle region of heart tissues were fixed in 10% neutral buffered zinc formalin for immunofluorescence experiments. A portion of the heart tissue (~20 mg) was immediately processed for GSH and GSSG assays.

2.4. Myocardial glutathione and its redox ratio

Myocardial levels of reduced GSH and GSSG were assessed by a GSH detection kit from Cayman (Ann Arbor, MI, USA). In brief, MES buffer was used to prepare the cardiac homogenates and centrifuged at 2800*g* for 5 min at 4 °C. An aliquot of the supernatant was used for protein determination and equal amount of 10% MPA (meta- phosphoric acid, Cat. No. 239275, Sigma) was added to the remaining samples to precipitate the proteins. 100 μl of the MPA extracts were treated with triethanolamine (TEAM). After treating with TEAM, aliquots for GSSG analysis were incubated with 2-vinyl pyridine and then the enzymatic-recycling assay was performed as per the manufacture's instruction using a plate reader. Appropriate standards for GSH and GSSG were prepared and processed similarly to generate a standard curve.

2.5. Isolation of RNA and real-time qPCR analysis

Heart tissues preserved in RNA-later from NTg and caNrf2-TG mice (n = 6; at 10–12 weeks of age) were homogenized and extracted the total RNA using RNeasy mini kit (Qiagen, 74106). Then, the cDNA was synthesized using 1.25 μg RNA and QuantiTect reverse transcription kit (Qiagen, 205313). Quantitative RT-PCR was performed using 25–50 ng cDNA and 1 pmol primer of interest (Table 1) in a 10 μl SYBR green reaction mix (Qiagen, 204056) and amplified in a Roche Light Cycler 480 (Roche, Basel, Switzerland). Relative expression was quantified using Ct values, and expression fold-change was calculated by normalization to the Ct of housekeeping genes Gapdh or Arbp1 according to the 2-ΔΔCt methods [10,16]. Further, semi-quantitative RT-PCR reactions were carried out for using caNrf2 primers (20–25 cycles) (Table 1) and confirmed the product size by agarose gel electrophoresis (2.0%).

2.6. Protein isolation and immunoblotting

Heart tissues from NTg, caNrf2 TG-low and TG-high (n = 4–8) at 10–12 weeks of age were pulverized and homogenized in cytosolic extraction buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.5 mM MgCl2, with freshly prepared 0.1 mM phenyl methylsulfonyl fluoride (PMSF), 1 mM dithiothreitol and 1% Triton X-100, pH 7.9) and

Table 1
Complete list of real-time qPCR primer sequences.

Gene	Forward primer	Reverse primer
CaNrf2	ACTTTACATGGAGTCCTGGTGGGA	AGGCATCTTGTTTGGGAATGTGGG
Nrf2	CTGAACTCCTGGACGGGACTA	CGGTGGGTCTCCGTAAATGG
Gclc	GGACAAACCCCAACCATCC	GTTGAACTCAGACATCGTTCCT
Nqo1	AGGATGGGAGGTACTCGAATC	TGCTAGAGATGACTCGGAAGG
Gsr	CACGGCTATGCAACATTCGC	GTGTGGAGCGGTAAACTTTTTC
Cat	GGAGGCGGGAACCCAATAG	GTGTGCCATCTCGTCAGTGAA
Gst-m	CTGAAGGTGGAATACTTGGAGC	GCCCAGGAACTGTGAGAAGA
Gclm	CTTCGCCTCCGATTGAAGATG	AAAGGCAGTCAAATCTGGTGG
Gpx1	CCACCGTGTATGCCTTCTCC	AGAGAGACGCGACATTCTCAAT
Sod1	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
Sod2	TGGACAAACCTGAGCCCTAAG	CCCAAAGTCACGCTTGATAGC
Gapdh	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG
Arbp1	TGAGATTCGGGATATGCTGTTGG	CGGGTCCTAGACCAGTGTTCT

centrifuged at 2800*g* for 5–6 min. Nuclear fractions were obtained using nuclear extraction buffer (20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% glycerol with 0.5 mM PMSF and 1 mM dithiothei-tol, pH 7.9) and centrifuged at 8200 rpm for 10 min. Equal amount of cytosolic proteins were resolved on 10–12% SDS-PAGE and transferred to PVDF membranes and blocked in Tris Buffered Saline-Tween 20 (TBST) containing 5% non-fat dry milk. Individual blots were then incubated for overnight at 4 °C or 2 h at room temperature with the respective primary antibodies for GCLM (ab81445), GCLC (ab41463), NQO1 (ab34173), GSR (ab16801), GSTμ (ab178684), SOD1 (ab13498), SOD2 (ab13534) and catalase (219010) diluted with 2% bovine serum albumin in TBST. After 2×10 min washes with TBST, the blots were incubated with horseradish peroxidase IgG (Vector Laboratories, Burlingame, CA, USA) conjugated secondary antibodies (anti-rabbit or mouse) for 1 h. Blots were then treated with ECL (Pierce, Rockford, IL, USA) and imaged on autoradiography films or Amersham Imager 600 (GE Healthcare Life Sciences, Chicago, IL, USA). All membranes were stripped and reprobed with GAPDH (D16H11, Cell Signaling) to confirm the equal loading of proteins. The immune-reactive signals were quantified by densitometry using ImageJ software and density values were normalized to GAPDH [6,14,16].

2.7. Trans-AM DNA binding activity for Nrf2

Efficiency of Nrf2 DNA-binding activity was evaluated using a commercially available Trans-AM Nrf2 kit (50296; Active Motif, Carlsbad, CA, USA). Briefly, 10 μg of nuclear extract was incubated with immobilized wild-type or mutated competitor oligonucleotides bearing the antioxidant response element (ARE) consensus sequence. The bound Nrf2 was detected using an anti-Nrf2 primary antibody (100 μl of a 1:1000 dilution) and HRP-conjugated secondary antibody (100 μl of a 1:1000 dilution) prior to chromogenic reaction with TMB substrate and the absorbance was measured at 450 nm using a BioTek Epoch plate reader. Incubation with normal rabbit polyclonal IgG was also performed separately to confirm the specificity of the Nrf2 antibody [10,16].

2.8. Determination of reactive oxygen species (ROS) using DHE staining

The lipophilic, cell permeable fluorogenic dye, DHE (di-hydro ethidium) was used to measure the level of ROS. DHE yields a red fluorescence when it undergoes oxidation. Briefly, the frozen sections (10 μm) of the myocardium were incubated with 5 μg/ml of DHE in PBS in a light protected chamber maintained at 37 °C for 30 min. Slides were then fixed with fluoroshield mounting medium and the images were captured by randomly selecting 3–5 fields/section using a Nikon an Eclipse Ti-E inverted fluorescent microscope [19].

2.9. Total antioxidant capacity assay

The total antioxidant capacity (TAC) was determined in the myocardial homogenates of NTg and TG mice (STA-360, OxiSelect-Cell Biolabs Inc., USA) according to the manufacturer's instructions. The TAC assay measures the reducing capacity of the antioxidants present in the homogenate or extract by converting the copper (II) ion (Cu^{2+}) into copper (I) ion (Cu^{+}). Further, the reduced copper (I) ion reacts with chromogenic reagent to produce a color, which has the maximum absorbance at 490 nm. The limitation of TAC assay is that it excludes measuring the activities of enzymatic antioxidants [20]. In brief, equal amount of heart tissues (20–25 mg) were homogenized with 200 μl of ice-cold PBS, centrifuged and the supernatant was used for TAC assay. 20 μl of tissue lysates were mixed with 180 μl of reaction buffer and initial absorbance was measured at 490 nm. Copper ion reagent (50 μl) was then added to initiate the reaction and incubated for 5 min and the measured the absorbance at 490 nm. Antioxidant capacity was represented as fold change in relation to NTg [21].

2.10. Determination of myocardial lipid peroxidation

Lipid peroxidation levels were determined by measuring the tissue malondialdehyde (MDA) levels in the heart homogenates of NTg and TG mice [22]. Briefly, 500 μl of tissue homogenate was mixed with 2.0 ml of 20% TCA solution and centrifuged at 4000 rpm for 20 min. The supernatant (1.0 ml) was incubated with equal volume of 0.67% Thiobarbituric acid at 95 °C for 30 min. After cooling to room temperature, all samples were centrifuged at 2800*g* for 15 min. The supernatant was transferred to a 96-well microplate and the absorbance was measured at 532 nm. A standard curve was prepared using 1, 1, 3, 3-Tetramethoxypropane and concentrations of MDA were calculated as nmole/mg protein.

2.11. Statistical analysis

Data are presented as mean ± SEM. One-way ANOVA with post-hoc Tukey multiple comparison tests were performed using GraphPad Prism 7. Differences were considered significant at the values of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Design and construction of transgenic mice expressing constitutively active Nrf2 (ca-Nrf2) in mouse hearts, novel models representing a pro-reductive (PR) and hyper-reductive (HR) states

To design, the cardiac-specific reductive-state model, we used constitutively active Nrf2 [nuclear factor (erythroid-derived-2) – like 2 or NFE2L2] that was driven by mouse α-myosin heavy chain (αMHC)

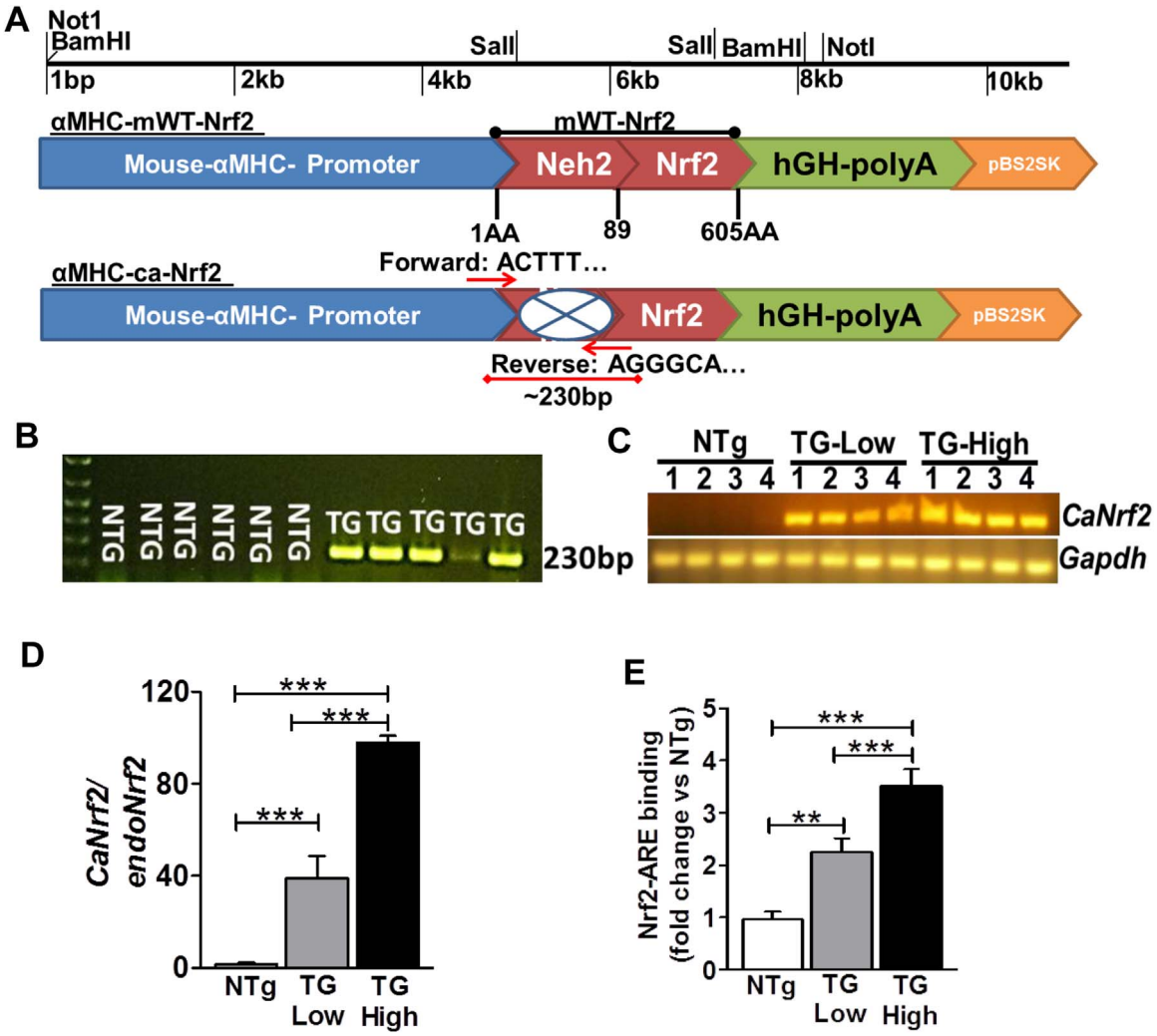

Fig. 1. Construction of αMHC-constitutively active Nrf2 plasmid and generation of Cardiac-Specific Nrf2 transgenic mice. (A) Cloning strategy for generating constitutively active Nrf2 (CA-Nrf2). A normal (WT) and truncated (ΔNeh2 domain – lack of AA1-89) was cloned in the αMHC promoter, a cardiac specific driver, (B) Genotyping for caNrf2 using specific primers for αMHC (forward) and caNrf2 (reverse) showing a positive product at ~230 bp, (C) Semi-quantitative RT-PCR using caNrf2 primers showing a dose-dependent expression for low vs. high caNrf2 transgene, (D) Quantitative RT-PCR analysis for caNrf2 transgene expression, in relation to endogenous levels of Nrf2, in the heart (n = 6–8 animals/group). (E) TransAM-Nrf2 activity assay in the nuclear extracts of NTg and TG mouse (n = 6 mice/group) at 10–12 weeks, Results were expressed as fold change of A 450 nm ± SE in each group (*p < 0.05, **p < 0.01, ***p < 0.001, ns-non significant).

promoter (Fig. 1A). Two different transgenic lines that express low and high levels of caNrf2 were generated. Transmission of the transgene from germline to progeny was confirmed by genotyping (Fig. 1B). The segregation of the transgene was found to be appropriately distributed to offspring of both genders indicating a normal Mendelian ratio in all filial generations. There is no significant difference in the body weight (BW), heart weight (HW) and HW/BW ratio (4.18 ± 0.06; 4.18 ± 0.15 and 4.49 ± 0.08 in NTg; TG-low and TG-high) at the age of 10–12 weeks. Semi-quantitative PCR analysis also revealed a dose dependent expression of transgene in TG-low and TG-high mice (Fig. 1C). When comparing the expression of caNrf2 gene in relation to the levels of endogenous Nrf2 mRNA expression, the mice that showed 40 fold changes were designated as TG-low and those that expressed nearly 100 fold were designated as TG-high (Fig. 1D). In this case, GAPDH-normalized caNrf2 expression and GAPDH-normalized endogenous Nrf2 mRNA expression were compared.

3.2. Cardiac-specific Nrf2 expression is functionally active

We assessed whether the caNrf2 is functionally active in the myocardium of TG animals by measuring Nrf2/antioxidant response element (ARE) DNA-binding ability using the Trans-AM-ELISA. A significant and dose dependent increase in the Nrf2-ARE (DNA) binding was noted in the nuclear fractions of 10–12 weeks old caNrf2-TG hearts compared to NTg with significantly increased Nrf2-DNA binding in the hearts of caNrf2-TG-high versus caNrf2-TG-low mice (Fig. 1E). Further, the specificity of binding was confirmed by incubating the nuclear extracts with either wild-type Nrf2 oligonucleotide or normal rabbit IgG or mutated Nrf2 oligonucleotide. These results indicate that the caNrf2 (lacking Neh2 domain) in the TG animals is functionally active.

3.3. Increased transactivation of antioxidant genes in caNrf2-TG mice

To confirm the Nrf2-induced transactivation of antioxidants genes, we determined gene expression of several antioxidant enzymes in the myocardial tissues of NTg, caNrf2-TG-low and caNrf2-TG-high lines. The caNrf2 transgenic lines exhibited significant trans-gene dose-dependent upregulation of the candidate genes (Fig. 2). At 10–12 weeks of age, Nrf2 (1.8 vs 2.3- fold), glutamate-cysteine ligase, catalytic subunit (Gclc, 1.25 vs. 1.8-fold), glutamate-cysteine ligase, modifier subunit (Gclm, 7.5 vs. 12.5-fold), glutathione reductase (Gsr, 1.35 vs. 2.5 fold), NAD(P)H dehydrogenase quinone 1 (Nqo1, 2 vs. 2.2 fold), glutathione S-transferase, mu (Gst-μ, 2 vs. 2 fold), and glutathione peroxidase 1 (Gpx1, 1.5 vs 1.2-fold) were significantly increased in TG-

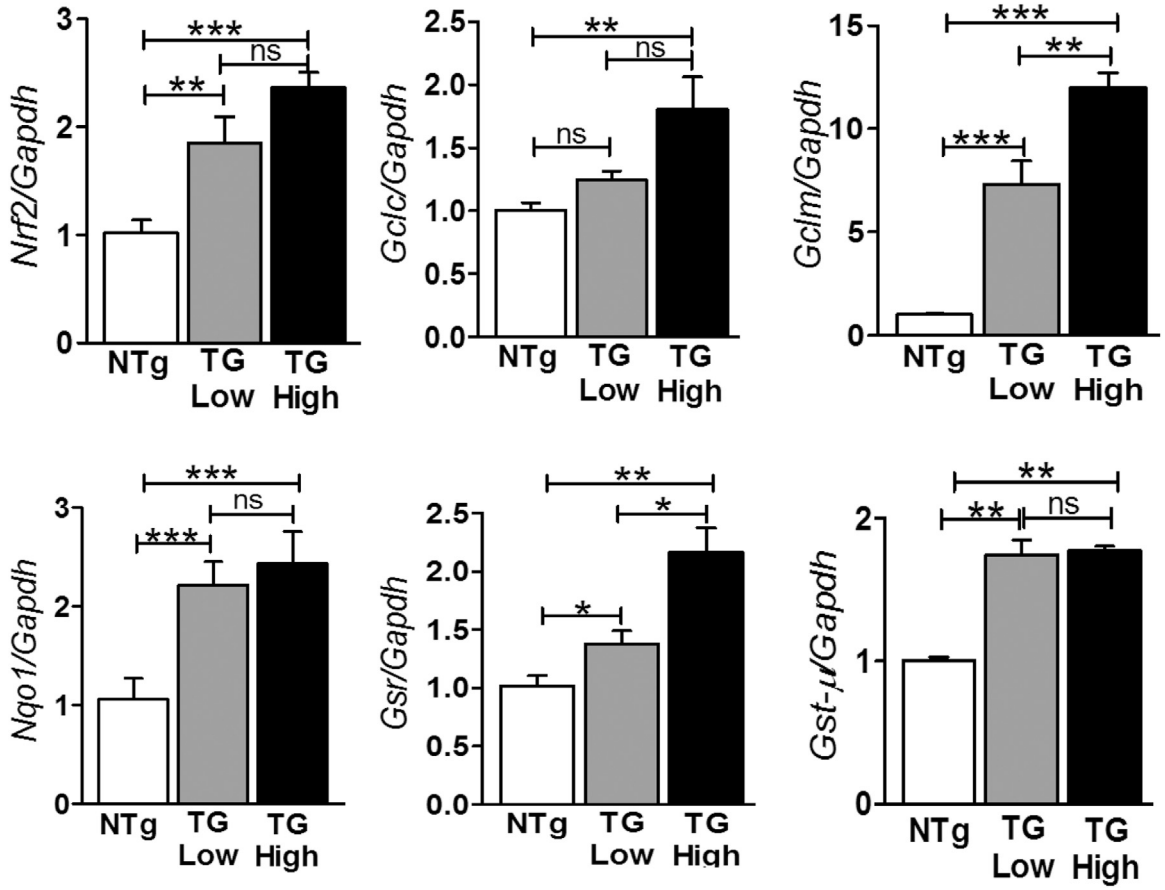

Fig. 2. Constitutively active Nrf2 increases antioxidant gene expression in the transgenic mouse heart. Quantitative real-time RT-PCR determinations of Nrf2 and its target genes in NTg and TG mice at 10–12 weeks of age were performed using gene specific mouse primer sets (n = 6 mice/group). Data were first normalized to *Arbp1* or *Gapdh* expression and then to the corresponding gene expression in the NTg group (Delta-delta-CT method). The messenger RNA (mRNA) expression for *Nrf2* and its major targets, *Gclc, Gclm, Nqo1, Gsr,* and Gst-μ genes were significantly and dose dependently upregulated in TG-low and TG-high versus NTg mice (*p < 0.05, **p < 0.01, ***p < 0.001, ns-non significant).

low and TG-high when compared to NTg mouse hearts. The expression of Sod1 and Gpx1 (Fig. S1) was significantly increased in TG-low hearts, but they were unchanged in TG-high, whereas Sod2 expression was unaltered in TG-low but significantly downregulated in TG-high compared to NTg mice. These results confirm induction of the key Nrf2-dependent antioxidant genes in response to transgene levels in a dose-dependent manner. However, some genes were selectively and/or differentially regulated.

3.4. Cardiac-restricted constitutive activation of Nrf2 in mice increased the expression of redox regulatory proteins

We tested whether the transcriptional changes induced by caNrf2 were reflected in protein expression in TG mouse hearts. We focused on the major Nrf2-antioxidant enzymes that regulate GSH metabolism. Immunoblotting analysis of cytosolic fractions demonstrated a marked increase in the antioxidant proteins paralleling the changes in antioxidant gene expression (Fig. 3A-F). The expression of GCLC (2.5 vs. 3.5-fold), GCLM (5 vs. 9-fold), GSR (4 vs. 6-fold), NQO1 (2.5 vs. 3.5-fold), GST-μ (4 vs. 8-fold), and CAT (2 vs. 3-fold) and SOD2 (1.2 vs 1.5-fold) were significantly increased in TG-low and TG-high respectively compared to NTg mouse hearts. Protein levels for SOD2 were moderately increased in TG-high hearts, whereas SOD1 expression was unaltered in both TGs. Further, immunohistochemistry analyses for GCLM, GSR, GST-μ and NQO1 also showed a dose-dependent increase in the myocardium of TG-low and TG-high versus NTg mice without a significant change in intracellular distribution (Figs. S2–S4). While, a strong correlation was observed between the majority of the antioxidant transcripts and protein abundance of the TG myocardium, the gene expression for SOD1 and SOD2, also targets of Nrf2 did not correlate with their protein levels, suggesting mechanisms beyond Nrf2, possibly post-transcriptional, are playing a role in the regulation of these genes.

3.5. Augmentation of the myocardial glutathione pool paralleled by decreased basal ROS levels in the hearts of caNrf2-TG mice

We next tested whether caNrf2 mediated increase in transcription and translation of antioxidant genes modulated the GSH level, its redox state, and basal ROS signaling. The levels of the reduced form of GSH, the ratio of GSH/GSSG (Fig. 4A-B) were significantly increased in the TG hearts when compared to NTg mice. Notably, GSH (1.5 and > 2.0 folds in TG-low and TG-high vs. NTg) and GSH/GSSG ratios were significantly increased in TG-high (> 2.5 fold vs. NTg) compared to TG-low (1.8 fold vs. NTg). Further, the ROS formation as measured by fluorescence detection of DHE demonstrated that the prominent DHE reactivity seen in NTg was substantially attenuated in caNrf2-TG hearts (Fig. 4C). Interestingly, the diminished DHE and high GSH levels observed in TG-high relative to TG-low and NTg hearts (Fig. 4A-C) were found to be transgene dose-dependent. These findings could be explained by a number of mechanisms including enhanced interactions of DHE reactive intermediates with GSH as well as decreased ROS levels. In either case it is consistent that the Nrf2-dependent change in the redox status is capable of changing either the rate of ROS production or their access to reactive target molecules. In addition, the myocardial total antioxidant activity was significantly increased (Fig. 4D) with a profound decrease in the lipid peroxidation levels (Fig. 4E) in the TG myocardium in a transgene dose-dependent manner

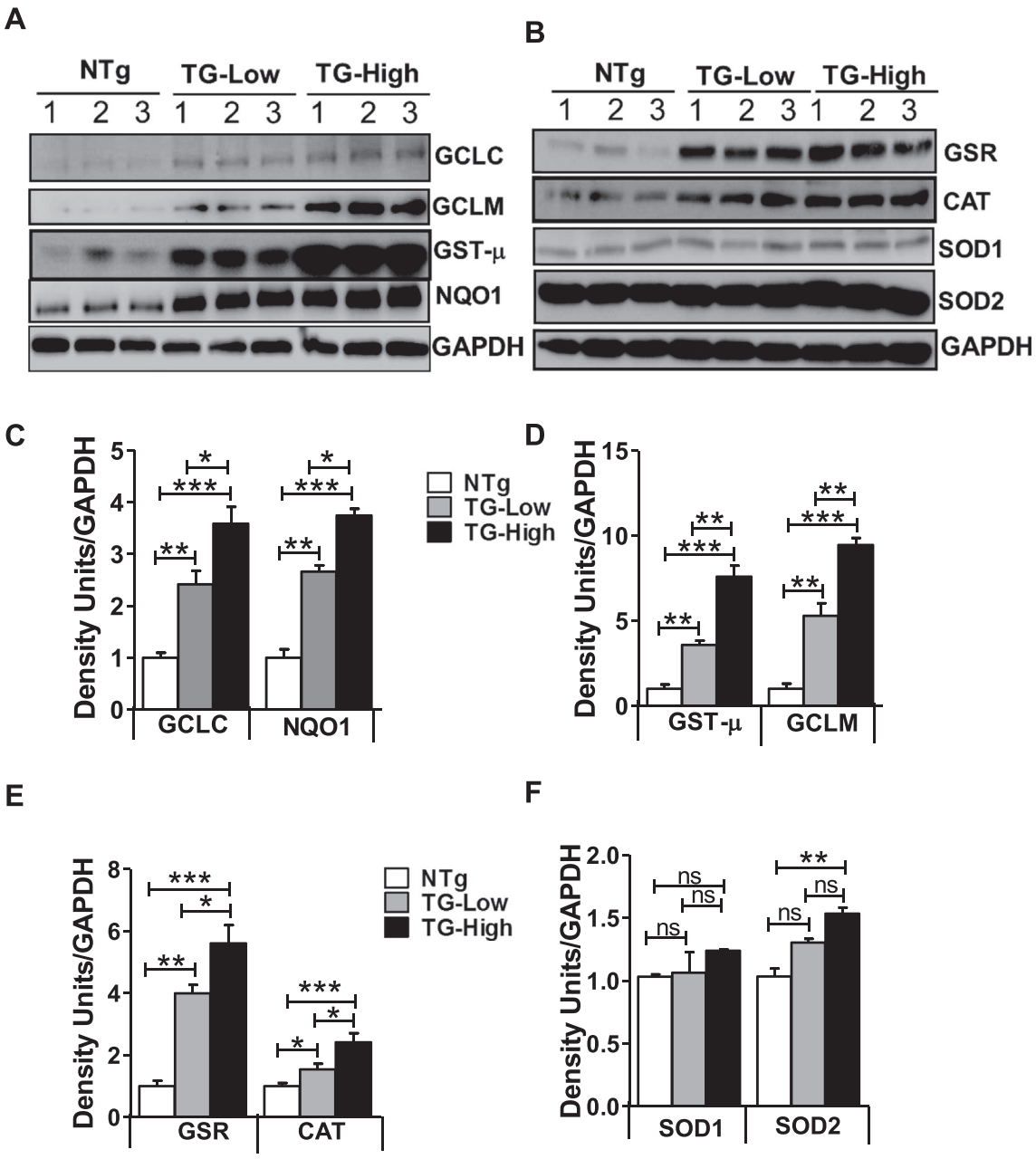

Fig. 3. Nrf2-dependent transcriptional upregulation of antioxidant genes resulted in increased translation of antioxidant enzymes in the caNrf2-TG mouse myocardium. Representative Immunoblots (IB) for antioxidant enzymes from cytosolic fractions in NTg and TG mice at 10–12 weeks of age (n = 6 mice/group). Increased transactivation of key antioxidant genes in the TG hearts induces their protein expression. Immunoblots were probed with appropriate antibodies for antioxidant proteins (A-B). Each lane indicates an individual mouse. Densitometry analysis were performed as relative intensity values calculated as mean arbitrary units obtained from the IB shown in (C), (D), (E) and (F), respectively (*p < 0.05, **p < 0.01, vs. NTg). Significant increases in the GSR, GST-μ, NQO1, GCLC, GCLM and CAT in the cytosol of TG mice were evident (*p < 0.05, **p < 0.01, ***p < 0.001, ns-non significant).

in relation to NTg. These results established the reliability of the model in tightly regulating the antioxidant networks and the ability of Nrf2 to establish a stable redox tone which is progressively more reductive depending on the level of its expression (Fig. 5).

4. Discussion

One of the most important issues in redox biology that has emerged over the last decade is how is a stable intracellular redox network is maintained in a cellular environment. The Keap1/Nrf2 system has received a lot of attention in this respect since it regulates the levels of the key enzymes which control the cell's redox tone in response to a changing extracellular environment. Redox homeostasis requires a more reductive activation when the extracellular environment becomes more oxidative and conversely suppression of the reductive network when it becomes more reduced. It is now clear that perturbation from the redox setpoint to either a more oxidative or reductive state (oxidative or reductive stress respectively) contributes to disease pathology [3,4,6–8]. To maintain the appropriate redox tone the relative expression of genes under the control of the ARE is critical. The primary sensor of perturbation from the stable redox tone is Keap1 which contains a large number of redox active thiol residues capable of direct reaction with electrophiles and antioxidants [9]. Keap1 controls the functionally active levels of the transcription factor Nrf2, which binds to the ARE. Typically, downstream of Nrf2 activation, ARE dependent transcription is also controlled by several factors [12]. The

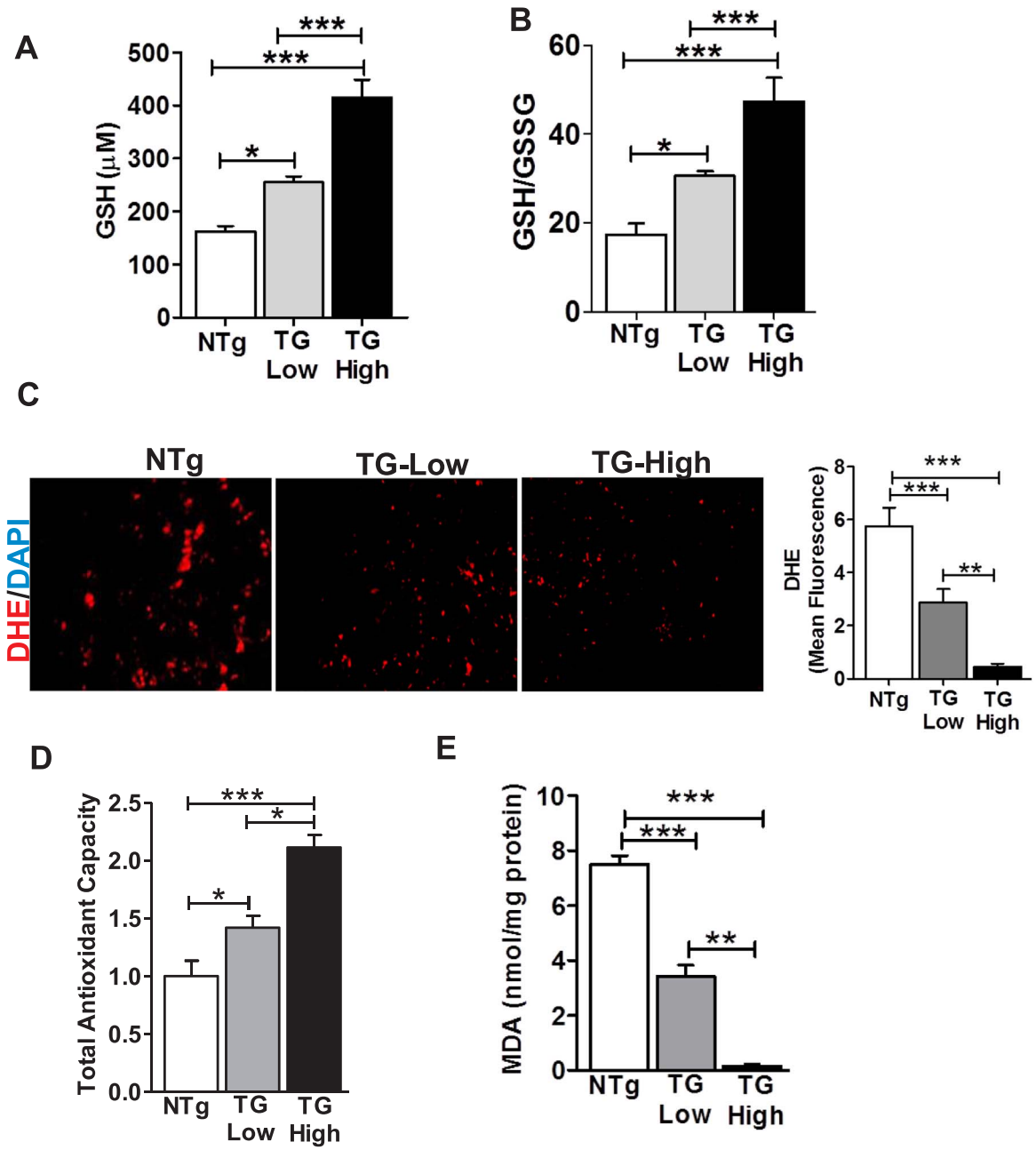

Fig. 4. Sustained transactivation and translation of antioxidants promotes myocardial glutathione, impairs ROS and cause reductive state in caNrf2-TG mouse myocardium. (A–B) Myocardial glutathione (GSH) and its redox ratio (GSH/GSSG) determined by enzyme-recycling assays (n = 6–8/group), (C) Redox sensitive di-hydroethidium (DHE) fluorescence staining for oxidative profiling show a dose-dependent decrease in ROS, (D) Total antioxidant activity was determined using Sigma-Aldrich TAC kit, and (E) Spectrophotometric determination of myocardial lipid peroxidation (malondialdehyde levels) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns-non significant).

basic leucine zipper (bZIP) family of transcription factors (i.e. Jun, Fos, and Maf), undergo dimerization (homo and hetero) to regulate gene transcription [15,23]. The MafK (musculo-aponeurotic fibrosarcoma oncogene homolog-K), lacking transactivation domain, interacts with other Mafs, bZIP proteins such as Nrf2 and BTB (Bric-a-Bac, Tramtrack, Broad-complex) and cap'n'collar homolog 1 (Bach1) for transcriptional regulation [24–26]. Upon stress Nrf2 is tethered and translocates to the nucleus, heterodimerizes with MafK, small maf transcription factor. Association of Nrf2 with MafK has been shown to activate target genes [27]. It is then possible that increased binding of Nrf2 would not necessarily result in increased expression of genes under the control of the ARE. Indeed, thus far it has not been possible to determine whether Nrf2 translocation to the nucleus is the master regulator of ARE gene regulation since the mechanisms needed to activate Keap1 involve exposing the cell to electrophiles or pro-oxidants, which have multiple effects on the cell including activation of Nrf2-independent pathways i.e. autophagy and perturbation of cell metabolism [13].

This newly developed heart-restricted constitutive Nrf2 activation provides a direct and sensitive antioxidant augmentation model. The myocardium exhibited dramatically increased transcription and protein synthesis of key antioxidant enzymes, and increased levels of reduced glutathione (Fig. 5). This is important because the post-translational modification of proteins known as glutathionylation is well-known to mediate a broad range of biological effects including changes in metabolism and cell signaling [28–31]. Our data suggest that these effects are intimately linked to the activation of Nrf2 as a transcription factor.

Importantly, we generated two different caNrf2 transgenic lines

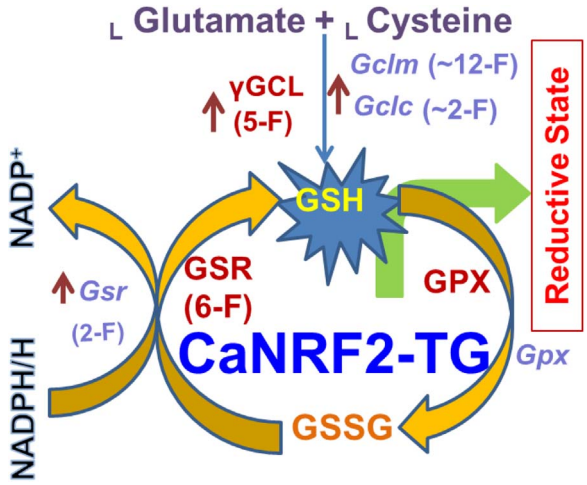

Fig. 5. Schematic representation of caNrf2 mediated trans-activation of genes, subsequent augmentation of antioxidant protein synthesis involve in glutathione metabolism leading to the development of reductive state.

with distinct penetrance to establish stable different reductive cellular conditions, namely pro-reductive and hyper-reductive. The chronic maintenance of a hyper-reductive state leads to reductive stress, which is detrimental to cardiomyocytes over time [6,14–16,32] and this can be assessed in these models without the confounding effects of indirect activation through proteotoxicity or exposure to oxidants or electrophiles. It is then important to determine whether chronic hyper-reductive models, might be detrimental. Here, we present a genetic mouse model with expression of Nrf2 that lacks binding to Keap1, which leads to hyper-reduction of the glutathione system, and the potential to modulate redox cellular signaling.

5. Conclusions

On the basis of these data we conclude that Nrf2 is the master regulator of cellular redox homeostasis and its activation leads to a reductive-state in the myocardium. We demonstrated that transgene dose dependent expression of constitutively active Nrf2 (caNrf2) is sufficient to develop a “pro-reductive” stable redox state that is not compensated for by modification of other signaling pathways or proteins associated with ARE transcription. In summary, these pro-reductive and reductive models will help clarifying the physiological and pathophysiological (acute vs. chronic) attributes of the reductive extreme (reductive stress; Fig. 5) of the redox balance in the myocardium.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.04.038.

References

[1] J.N. Cohn, R. Ferrari, N. Sharpe, Cardiac remodeling—concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling, J. Am. Coll. Cardiol. 35 (2000) 569–582.
[2] C. Mihl, W.R.M. Dassen, H. Kuipers, Cardiac remodelling: concentric versus eccentric hypertrophy in strength and endurance athletes, Neth. Heart J. 16 (2008) 129–133.
[3] N.S. Dhalla, R.M. Temsah, T. Netticadan, Role of oxidative stress in cardiovascular diseases, J. Hypertens. 18 (2000) 655–673.
[4] R. Schnabel, S. Blankenberg, Oxidative stress in cardiovascular disease: successful translation from bench to bedside? Circulation 116 (2007) 1338–1340.
[5] P.M. Kris-Etherton, A.H. Lichtenstein, B.V. Howard, D. Steinberg, J.L. Witztum, Antioxidant vitamin supplements and cardiovascular disease, Circulation 110 (2004) 637–641.
[6] N.S. Rajasekaran, P. Connell, E.S. Christians, L.J. Yan, R.P. Taylor, A. Orosz, X.Q. Zhang, T.J. Stevenson, R.M. Peshock, J.A. Leopold, W.H. Barry, J. Loscalzo, S.J. Odelberg, I.J. Benjamin, Human alpha B-crystallin mutation causes oxido-reductive stress and protein aggregation cardiomyopathy in mice, Cell 130 (2007) 427–439.
[7] F. Singh, A.-L. Charles, A.-I. Schlagowski, J. Bouitbir, A. Bonifacio, F. Piquard, S. Krähenbühl, B. Geny, J. Zoll, Reductive stress impairs myoblasts mitochondrial function and triggers mitochondrial hormesis, Biochim. Biophys. Acta (BBA) – Mol. Cell Res. 1853 (2015) 1574–1585.
[8] M.C. Badia, E. Giraldo, F. Dasi, D. Alonso, J.M. Lainez, A. Lloret, J. Vina, Reductive stress in young healthy individuals at risk of Alzheimer disease, Free Radic. Biol. Med. 63 (2013) 274–279.
[9] S. Magesh, Y. Chen, L. Hu, Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents, Med. Res. Rev. 32 (2012) 687–726.
[10] V.R. Muthusamy, S. Kannan, K. Sadhaasivam, S.S. Gounder, C.J. Davidson, C. Boeheme, J.R. Hoidal, L. Wang, N.S. Rajasekaran, Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium, Free Radic. Biol. Med. 52 (2012) 366–376.
[11] A. Uruno, Y. Furusawa, Y. Yagishita, T. Fukutomi, H. Muramatsu, T. Negishi, A. Sugawara, T.W. Kensler, M. Yamamoto, The Keap1-Nrf2 system prevents onset of diabetes mellitus, Mol. Cell. Biol. 33 (2013) 2996–3010.
[12] T.W. Kensler, N. Wakabayashi, Nrf2: friend or foe for chemoprevention? Carcinogenesis 31 (2010) 90–99.
[13] A.-L. Levonen, B.G. Hill, E. Kansanen, J. Zhang, V.M. Darley-Usmar, Redox regulation of antioxidants, autophagy, and the response to stress: implications for electrophile therapeutics, Free Radic. Biol. Med. 71 (2014) 196–207.
[14] S. Kannan, V.R. Muthusamy, K.J. Whitehead, L. Wang, A.V. Gomes, S.E. Litwin, T.W. Kensler, E.D. Abel, J.R. Hoidal, N.S. Rajasekaran, Nrf2 deficiency prevents reductive stress-induced hypertrophic cardiomyopathy, Cardiovasc. Res. 100 (2013) 63–73.
[15] M. Narasimhan, N.S. Rajasekaran, Reductive potential – a savior turns stressor in protein aggregation cardiomyopathy, Biochim. Biophys. Acta 1852 (2015) 53–60.
[16] N.S. Rajasekaran, S. Varadharaj, G.D. Khanderao, C.J. Davidson, S. Kannan, M.A. Firpo, J.L. Zweier, I.J. Benjamin, Sustained activation of nuclear erythroid 2-related factor 2/antioxidant response element signaling promotes reductive stress in the human mutant protein aggregation cardiomyopathy in mice, Antioxid. Redox Signal. 14 (2011) 957–971.
[17] J. Navarro-Yepes, M. Burns, A. Anandhan, O. Khalimonchuk, L.M. del Razo, B. Quintanilla-Vega, A. Pappa, M.I. Panayiotidis, R. Franco, Oxidative stress, redox signaling, and autophagy: cell death versus survival, Antioxid. Redox Signal. 21 (2014) 66–85.
[18] M. Dodson, M. Redmann, N.S. Rajasekaran, V. Darley-Usmar, J. Zhang, Correction: KEAP1-NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity, Biochem. J. 471 (2015) 431.
[19] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.J. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PloS One 7 (2012) e45697.
[20] G. Bartosz, Non-enzymatic antioxidant capacity assays: limitations of use in biomedicine, Free Radic. Res. 44 (2010) 711–720.
[21] T. Lao, Z. Jiang, J. Yun, W. Qiu, F. Guo, C. Huang, J.D. Mancini, K. Gupta, M.E. Laucho-Contreras, Z.Z.C. Naing, L. Zhang, M.A. Perrella, C.A. Owen, E.K. Silverman, X. Zhou, Hhip haploinsufficiency sensitizes mice to age-related emphysema, Proc. Natl. Acad. Sci. USA 113 (2016) E4681–E4687.
[22] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[23] J.R.S. Newman, A.E. Keating, Comprehensive identification of human bZIP interactions with coiled-coil arrays, Science 300 (2003) 2097–2101.
[24] A.L. Eggler, G. Liu, J.M. Pezzuto, R.B. van Breemen, A.D. Mesecar, Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2, Proc. Natl. Acad. Sci. USA 102 (2005) 10070–10075.
[25] V. Blank, Small Maf proteins in mammalian gene control: mere dimerization partners or dynamic transcriptional regulators? J. Mol. Biol. 376 (2008) 913–925.

[26] T. Oyake, K. Itoh, H. Motohashi, N. Hayashi, H. Hoshino, M. Nishizawa, M. Yamamoto, K. Igarashi, Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site, Mol. Cell. Biol. 16 (1996) 6083–6095.
[27] H. Motohashi, F. Katsuoka, J.D. Engel, M. Yamamoto, Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1–Nrf2 regulatory pathway, Proc. Natl. Acad. Sci. USA 101 (2004) 6379–6384.
[28] Y. Xiong, J.D. Uys, K.D. Tew, D.M. Townsend, S-glutathionylation: from molecular mechanisms to health outcomes, Antioxid. Redox Signal. 15 (2011) 233–270.
[29] C.L. Grek, J. Zhang, Y. Manevich, D.M. Townsend, K.D. Tew, Causes and consequences of cysteine S-glutathionylation, J. Biol. Chem. 288 (2013) 26497–26504.
[30] A.J. Cooper, J.T. Pinto, P.S. Callery, Reversible and irreversible protein glutathionylation: biological and clinical aspects, Expert Opin. Drug Metab. Toxicol. 7 (2011) 891–910.
[31] B.G. Hill, A.N. Higdon, B.P. Dranka, V.M. Darley-Usmar, Regulation of vascular smooth muscle cell bioenergetic function by protein glutathiolation, Biochim. Biophys. Acta 1797 (2010) 285–295.
[32] N.S. Rajasekaran, M.A. Firpo, B.A. Milash, R.B. Weiss, I.J. Benjamin, Global expression profiling identifies a novel biosignature for protein aggregation R120GCryAB cardiomyopathy in mice, Physiol. Genom. 35 (2008) 165–172.