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Original article

Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes

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ABSTRACT

Exposure to high levels of glucose induces the production of reactive oxygen species (ROS) in cardiomyocytes that may contribute to the development of cardiomyopathy in diabetes. Nuclear factor erythroid 2-related factor 2 (Nrf2) controls the antioxidant response element (ARE)-dependent gene regulation in response to oxidative stress. The role of Nrf2 in defense against high glucose-induced oxidative damage in cardiomyocytes was investigated. Glucose at high concentrations induced ROS production in both primary neonatal and adult cardiomyocytes from the Nrf2 wild type (WT) mouse heart, whereas, in Nrf2 knockout (KO) cells, ROS was significantly higher under basal conditions and high glucose markedly further increased ROS production in concentration and time-dependent manners. Concomitantly, high glucose induced significantly higher levels of apoptosis at lower concentrations and in shorter time in Nrf2 KO cells than in WT cells. Primary adult cardiomyocytes from control and diabetic mice also showed dependence on Nrf2 function for isoproterenol-stimulated contraction. Additionally, cardiomyocytes from Nrf2 KO mice exhibited increased sensitivity to 3-nitropropionic acid, an inhibitor of mitochondrial respiratory complex II, for both ROS production and apoptosis compared with Nrf2 WT cells, further emphasizing the role of Nrf2 in ROS defense in the cells. Mechanistically, Nrf2 was shown to mediate the basal expression and induction of ARE-controlled cytoprotective genes, *Nqo1* and *Ho1*, at both mRNA and protein levels in cardiomyocytes, as both the basal and inducible expressions of the genes were lost in Nrf2 KO cells or largely reduced by Nrf2 SiRNA. The findings, for the first time, established Nrf2 as a critical regulator of defense against ROS in normal and diabetic hearts.

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1. Introduction

Diabetic cardiomyopathy has been recognized as a major complication and cause of disability and mortality among diabetic patients [1–3]. Clinically, diabetic cardiomyopathy can occur without major vascular lesions, suggesting a primary role for direct effects of diabetes on cardiomyocytes [2]. Previous studies have demonstrated increased production of reactive oxygen species (ROS) in diabetic cardiomyocytes, implicating high levels of glucose induce ROS and oxidative damage in the heart that directly contribute to the development of diabetic cardiomyopathy [4]. On the other hand, cumulative evidence has shown that antioxidant responses that combat against oxidative stress play critical roles in the manifestation of the lesions induced by oxidative stimuli in various tissues and cells [5,6].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the cap "n" collar basic region leucine zipper (cnc bZip) group of transcription factors [6,7]. Nrf2 is broadly expressed in tissues but is only activated in response to a range of oxidative and electrophilic stimuli including ROS, some antioxidants, heavy metals, and certain disease processes [5,6]. Quiescent Nrf2 localizes in the cytoplasm and is rapidly turned over through a specific ubiquitin–26S proteasome pathway controlled by the Keap1/Cul3-dependent ubiquitin ligase (E3) [8–10]. Interaction between the inducers and the Keap1 protein through chemical–protein thiol interactions initiates the signal transduction leading to the stabilization and activation of the Nrf2 protein [11]. Upon activation, Nrf2 mediates the induction of a spectrum of cytoprotective proteins including phase II enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1), and antioxidant proteins, such as heme oxygenase 1 (HO1), through the antioxidant response element (ARE)-dependent pathway [9,10,12,13]. Both genetic and biochemical studies have implicated the Nrf2/Keap1/ARE system

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DHE, dihydroethium; HO1, heme oxygenase 1; AMVM, adult mouse ventricular myocyte; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, Nuclear factor erythroid 2-related factor 2; 3NP, 3-nitropropionic acid; STZ, streptozotocin.

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in the defense against chemical toxicity, cancer, and chronic diseases, such as autoimmune, neurodegenerative, and inflammatory illnesses, many of which involve oxidative stress [5,6,14–21]. It has been shown that Nrf2 appeared to mediate the induction of cytoprotective genes by 3H-1,2-dithiole-3-thione, an Nrf2 activator, in cardiomyocytes [22]. However, the potential function of Nrf2 in protection against glucose-induced oxidative damage in diabetic cardiomyopathy has not been investigated.

In the present study, we analyzed the role and mechanism of action of Nrf2 in ROS-defense in primary cardiac myocytes exposed to high glucose *in vitro* as well as in adult ventricular myocytes from diabetic mice. The findings demonstrate that loss of Nrf2 function in cardiomyocytes markedly increases high glucose-induced oxidative stress and apoptosis with decreased contractility. Mechanistically, Nrf2 regulates the basal expression and induction of ARE-controlled cytoprotective genes in primary cardiomyocytes, *in vivo* heart tissues, and a cardiomyoblast cell line, H9C2, as both the basal and inducible expression of the genes are lost in Nrf2 knockout (Nrf2 KO) cells or largely reduced by Nrf2 SiRNA. The study demonstrated, for the first time, that Nrf2 is required for protection against glucose-induced oxidative stress and cardiomyopathy in the heart.

2. Materials and methods

2.1. Mice

Nrf2 knockout (KO) mice in a 129SVJ genetic background were originally provided by Dr. Y.W. Kan (University of California, San Francisco, CA). The mice were converted to the C57BL/6 background as described previously [21]. C57BL/6 mice from Jackson Laboratory were used as the wild type control (WT). The mice were housed in the environmentally controlled National Institute for Occupational Safety and Health barrier facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The mice were provided with water and an irradiated diet *ad libitum*. The mice were housed in microisolator cages on sterile Beta chip bedding (Northeastern Products, Warrensburg, NY) and received positive pressure HEPA-filtered air to each individual cage using a Maxi-Miser System (Thoren Caging Systems, Hazelton, PA).

2.2. Diabetes model

Nrf2 WT and KO mice (male, 8 weeks old) in the C57BL/6 background were given a single dose of streptozotocin (STZ dissolved in sodium citrate buffer, 0.1 M, pH4.5; 150 mg/kg body weight i.p.; Sigma, St. Louise, MO); control mice received a single injection of the sodium citrate buffer (0.1 M, pH 4.5). Whole blood glucose (WBG) from the mouse tail vein was measured using the OneTouch Ultra Blood Glucose Monitoring System (Lifescan, Milpitas, CA) 3 days after STZ injection. Mice with WBG levels of 250 mg/dl or higher were considered diabetic. Diabetic mice were observed for signs of illness daily.

2.3. Isolation and culture of primary cardiomyocytes

Neonatal cardiomyocytes were isolated as described elsewhere [23]. Hearts were collected from one-day-old Nrf2 WT or Nrf2 KO mice and were maintained in a cold Hank's balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (HBSS/-Ca-Mg) (HyClone, Logan, UT). The hearts were washed with HBSS/-Ca-Mg and minced to small fragments. Cardiomyocytes were dissociated from each other with trypsin digestion (0.05% Trypsin-EDTA) for 15 min; digestion was continued for an additional 30 min after an equal volume of cold HBSS containing Ca^{++} and Mg^{++} was added. The resulting mixture was centrifuged for 8 min at 200 ×*g* and cell pellets were resuspended in Minimal Essential Medium (MEM) with 20% fetal bovine serum (FBS) for 2 h. Suspended cells (cardiomyocytes) were then transferred to a new dish and were cultured in MEM with 10% FBS for further experiments.

Adult mouse ventricular myocytes (AMVM) were isolated as described elsewhere [24]. Young adult mice (~8–10 week old) were euthanized with sodium pentobarbital and the heart was removed rapidly. The heart was perfused with Krebs Hensleit Bicarbonate (KHB) at a constant rate of 2 ml/min using a peristaltic pump. The heart was perfused with KHB for 5 min, followed by changing to a low Ca^{++} KHB for an additional 10 min. The heart was then immersed in recirculating KHB with low Ca^{++} containing collagenase B for 30 min. The ventricle was minced and placed into a 50-ml centrifuge tube, adjusted to 25 ml with low Ca^{++} KHB and centrifuged at 50 ×*g* for 2 min, and the supernatant aspirated. The concentration of Ca^{++} in KHB was increased in 3 increments (0.08, 0.6, 1.2 mM). Finally, the mixture was passed through a 225-μm nylon mesh and centrifuged at 50 ×*g* for 2 min. The centrifuge procedure was repeated until the preparation was composed of 80% viable left ventricular myocytes. Only those myocytes that were rod shaped, with striations, no blebs and not spontaneously contracting were included for analysis. Myocytes typically retained their baseline fractional shortening for 4 h and were viable for biochemical and molecular analyses for 24–48 h after harvest. Only freshly isolated cells were used for physiological experiments.

2.4. ROS detection

Intracellular ROS production was detected by following a method by Carter, et al. [25]. Cells were cultured in 4-well chamber slides and were treated as indicated. 30 min prior to the end of treatment, dihydroethium (hydroethidine or DHE, Invitrogen, Carlsbad, CA) was added at 5 μM as a fluorescent indicator of ROS generation. Cells were washed with ice-cold phosphate buffered saline (PBS) 3 times, fixed with 4% paraformaldehyde, and mounted with a mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) (Vectorshield, Vector Laboratories, Burlingame, CA) to counter stain the nucleus. Fluorescent images of cells with elevated ROS levels (in bright red) and cell nuclei (in blue) were obtained from a Zeiss LSM510 confocal microscope using a Rhodamin-DAPI setting with laser lines of 543 nm and 364 nm. Fluorescent micrographs were taken with equal exposure times and settings.

2.5. TUNEL assay

Apoptosis of cardiomyocytes was detected by the TUNEL assay using the DeadEnd Fluoremetric TUNEL system (Promega, Madison, WI) following the manufacturer's instructions. Briefly, the cells were cultured and treated as indicated in 4-well chamber slides. The slides were labeled with the TdT reaction mixture (Promega) for 90 min and were mounted with Vectorshild containing DAPI. Fluorescent images of apoptotic cells (green) and cell nuclei (blue) were obtained from a confocal microscope under a fluorescein isothiocyanate (FITC)-DAPI setting with laser lines of 488 nm and 364 nm.

2.6. Measurement of cardiomyocyte contractile function

Measurements of the amplitude and velocity of unloaded single AMVM shortening and relengthening were made on the stage of an inverted phase-contrast microscope (Olympus, IX70-S1F2, Olympus Optical Co., LTD., Japan) using the Myocyte Calcium Imaging/Cell Length System in which the analog motion signal was digitized and analyzed by the EDGACQ edge detection software (Ionoptix Cor., Milton, MA). Electrical field stimulation was applied at 1 Hz and about 20 V to achieve threshold depolarization and experiments were performed at 20% above threshold. Each cell serves as its own control by continuous superfusion of buffers and drugs [24]. Data represent

the mean±S.E.M. of 12–15 different determinations derived from 12–15 different individual myocytes from seven to nine separate myocyte preparations from seven to nine different mice.

2.7. Real-time PCR

Total RNA was purified from cardiomyocytes using a total RNA isolation kit (Qiagen, Valencia, CA) and was reverse transcribed into single strand cDNAs, which were subsequently analyzed by real-time PCR using the SYBR GREEN PCR master mix (Applied Biosystems, Foster City, CA) following standard procedures. Briefly, for each reaction, DNA template, forward and reverse primers (10 μM each), PCR master mix, and water were added to make a final volume of 50 μl. Thermal cycling was carried out as follows: 95 °C for 3 min as initial denaturing, followed by 45 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 2 min. Threshold cycles (C_T values) were determined. Real-time PCR results were normalized using 1% of input as an internal control. Relative DNA amounts were calculated from C_T values for each sample by interpolating into the standard curve obtained using a series of dilutions of standard DNA samples that were run under the same conditions. The sequences of the primer sets used for real-time PCR are available upon request. Representative data from three separate experiments were presented.

2.8. Immunoblotting

Total cell extracts were prepared from cardiomyocytes using the radioimmune precipitation assay (RIPA) buffer with protease inhibitors. Extracts of 20 to 30 μg each were separated on a 4–20% SDS-PAGE gradient gel (Bio-Rad, Hercules, CA). Proteins separated were

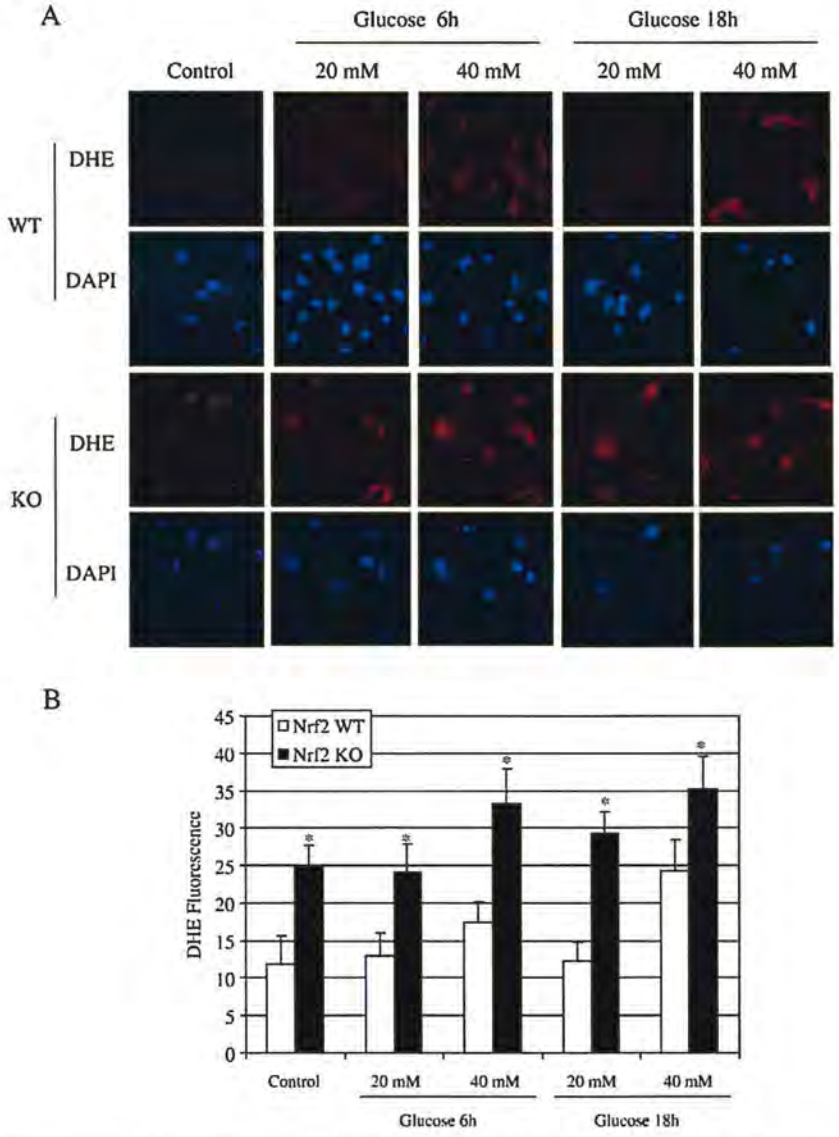

Fig. 1. Induction of oxidative stress in neonatal primary cardiomyocytes by glucose. Primary cardiomyocytes from one-day-old Nrf2 WT and KO mice were cultured in four-well chamber slides and were treated with glucose at concentrations of 0, 20, and 40 mM for 6 or 18 h, respectively. DHE, a fluorescent dye specific for the superoxide anion radical, was added 30 min prior to the end of the treatments. DHE oxidized by ROS intercalates with DNA and stains the nucleus in bright red. DAPI was used to visualize nuclei of all cells (blue). Fluorescence was examined under a fluorescent confocal microscope (A). Elevated level of ROS was seen in untreated and markedly increased ROS production in glucose-treated Nrf2 null cardiomyocytes compared with WT cells. Magnification=40×. Fluorescent signals were quantified and statistical analysis was performed as described under Materials and Methods. *, $p<0.05$.

transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and blotted with specific antibodies for overnight at 4 °C with gentle shaking. After incubating with appropriate horseradish peroxidase-conjugated secondary antibodies, the protein bands were visualized using ECL (Pierce). Antibodies against Nrf2, HO1, and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.9. SiRNA knockdown of Nrf2 in H9C2

H9C2 cells (rat cardiomyoblasts, ATCC, Manassas, VA) were cultured in the DMEM medium with 10% fetal bovine serum. The cells were transfected with either the control SiRNA or rNrf2 SiRNA that is specific for the rat Nrf2 (synthesized at Invitrogen; sequences are available upon request) according to manufacturer's instructions for 36 h. Knockdown of the Nrf2 protein expression in the cells were confirmed by immunoblotting of the Nrf2 protein in the cells.

2.10. Northern blotting

Total RNA was prepared from cardiomyocytes using a Total RNA isolation kit (Qiagen, Valencia, CA). Northern blotting was performed as described previously [26]. Total RNA of 3 μg was fractionated in a 1.2% agarose-formaldehyde gel, transferred to a Nytran membrane, and probed with specific digoxigenin (DIG) -labeled riboprobes prepared with the Dig-labeling kit (Roche Applied Science, Indianapolis, IN). Chemiluminescent signals were visualized using DIG RNA detection reagents with CDP Star as a substrate (Roche Applied Sciences). Parallel blots of the same samples were probed with a mouse actin riboprobe to ensure equal loading.

2.11. Data quantification and statistical analysis

Fluorescent intensity was quantified using the Optimums Version 6.51 software (Media Cybernetics, Silver Spring, MD). Quantitative data represent means and standard deviations from 100 cells from

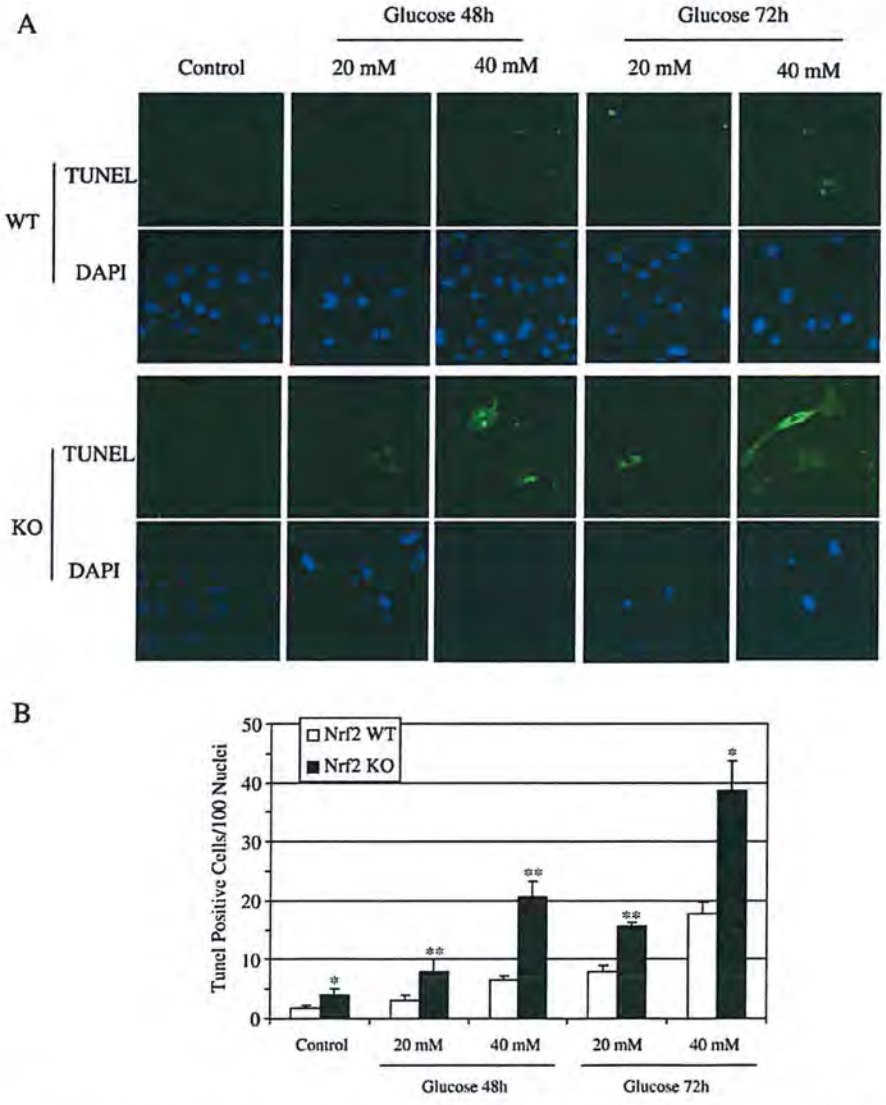

Fig. 2. Induction of apoptosis in neonatal primary cardiomyocytes by high glucose. Neonatal primary cardiomyocytes from Nrf2 WT or KO mice were cultured and treated with glucose as indicated. Apoptotic myocytes were stained with the TUNEL reagents (green) and the nuclei of all cells were stained with DAPI (blue). Fluorescent photographs were shown (A). Magnification = 40×. Quantification and statistical analysis (B) were as described in Materials and Methods. *, $p<0.05$; **, $p<0.01$.

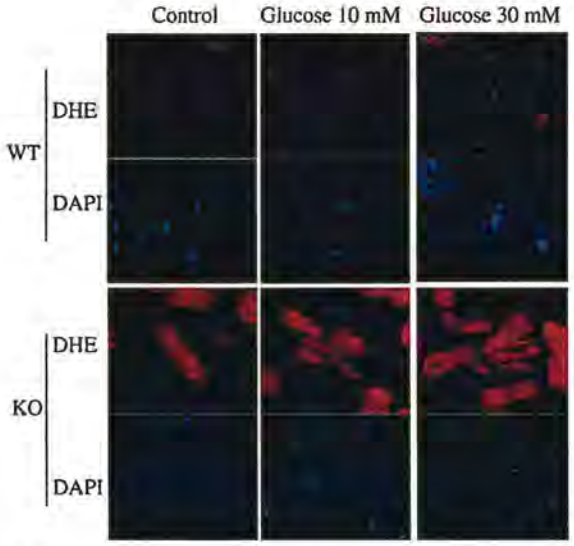

Fig. 3. Elevated ROS production in adult cardiomyocytes from Nrf2 KO mice. Adult mouse ventricular myocytes were isolated from the hearts of 8-week-old Nrf2 WT and KO mice. The cells were treated with glucose for 18 h and were stained with DHE (red) and DAPI (blue) to indicate intracellular ROS levels and cell nuclei, respectively. Magnification = 40×.

three different experiments. Statistical analysis was performed with one-way ANOVA followed by *t*-test using the Microsoft Excel program. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Glucose aggravates oxidative stress, increases apoptosis, and impairs contractility in Nrf2 KO cardiomyocytes in vitro and in vivo

To examine the function of Nrf2 in protection against diabetic cardiomyopathy, we tested if Nrf2 is involved in the defense against glucose-induced ROS production in mouse cardiomyocytes. The protective effect of Nrf2 on oxidative damage was first examined *in vitro*. Neonatal cardiomyocytes were derived from Nrf2 WT and KO mice and were treated with high levels of glucose in culture. In WT cells, glucose at 20 mM for 6 h induced ROS production at a low but detectable level (Fig. 1). Induction was, however, significantly increased at a higher glucose concentration (40 mM) and increased exposure time (18 h), demonstrating concentration and time-dependent induction of ROS by glucose in mouse cardiomyocytes. On the other hand, the Nrf2 KO cells exhibited a markedly elevated level of ROS even in the absence of high glucose. Moreover, ROS production was further increased by exposure to glucose in a concentration and time-dependent manner to significantly higher levels in KO than WT cells (Fig. 1). The results indicate that glucose promotes ROS production in cardiomyocytes and Nrf2 was required for the control of ROS production in the cells under basal conditions and for high glucose-stimulated oxidative stress.

Elevated ROS production impairs cell survival. Glucose at 20 and 40 mM induced low but detectable levels of apoptosis (Figs. 2A and B). However, in Nrf2 KO cells, apoptosis was detected in the absence of high glucose treatment and glucose induced concentration and time-dependent increases in apoptosis, which corresponded with elevated ROS production in the cells. The findings implicate that exposure to high glucose induces marked cell death in Nrf2 KO cardiomyocytes as a consequence of exacerbated oxidative damage in the cells.

Adult cardiomyocytes differ from neonatal cardiomyocytes both metabolically and functionally and retain major characteristics of myocytes of the heart *in vivo*, such as contraction. AMVM were isolated from the hearts of adult Nrf2 WT and KO mice and were treated with high levels of glucose to examine the protective role of Nrf2 in mature cardiomyocytes. Nrf2 WT AMVM showed low levels of ROS under a basal condition or in the presence of 10 mM glucose; the level of ROS production was increased at 30 mM of glucose (Fig. 3, upper panels). On the other hand, Nrf2 KO AMVM displayed a high level of ROS production in the absence of glucose treatment; glucose at 10 and 30 mM further increased the ROS production (Fig. 3, lower panels). The differences in the ROS levels between WT and KO cells under both basal and high glucose-stimulated conditions were significantly more striking in the AMVM cells than in the neonatal cardiomyocytes, suggesting that adult cardiomyocytes are more prone to oxidative damage than neonatal cardiomyocytes.

The marked increase in sensitivity of both neonatal and adult Nrf2 KO cardiomyocytes to high glucose-induced ROS *in vitro* implicates Nrf2 in maintaining the function of cardiomyocytes under diabetic conditions *in vivo*, under which cardiomyocytes are likely to be exposed to high levels of glucose carried in the blood. To test this postulate, diabetes was induced in Nrf2 WT and KO mice by a single injection of STZ. Peripheral blood glucose levels were monitored and mice with glucose levels of ≥250 mg/dl and clinical signs of diabetes were diagnosed as diabetic. Fourteen days after STZ injection, the mice were sacrificed and AMVM were isolated. Contractile activities of the cells were measured. Wild type control AMVM cells (WT control) exhibited increased contraction in the presence of a β-adrenergic agonist, isoproterenol, in a concentration-dependent manner; whereas, wild type AMVM from diabetic mice (WT diabetic) displayed significantly reduced contractile activities (Fig. 4), indicating functional damage of AMVM by high levels of blood glucose *in vivo*. AMVM cells from Nrf2 KO control mice (KO control) responded similarly to WT cells at a low concentration of isoproterenol (10^{-9} M); but the response was significantly reduced compared with that of WT at a higher concentration of the agonist (10^{-7} M). On the other hand, AMVM cells from diabetic KO mice (KO diabetic) showed a total lack of response to isoproterenol stimulation at all concentrations tested, revealing severely impaired contractile function of cardiomyocytes in diabetic Nrf2 KO mice. Taken together, the findings demonstrate that Nrf2 is critical in protecting cardiomyocytes from high glucose and diabetes-induced oxidative damage both *in vitro* and *in vivo*.

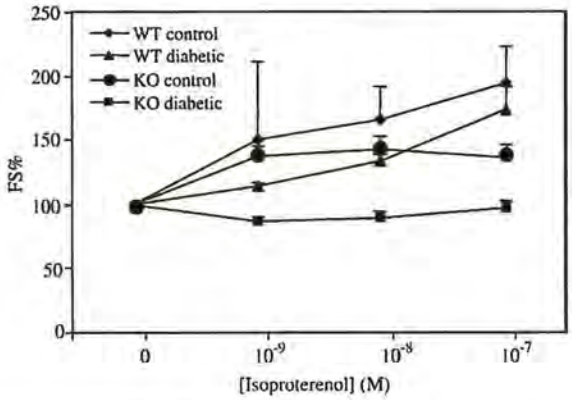

Fig. 4. Impaired contractility in diabetic cardiomyocytes. Diabetes was induced in young adult Nrf2 WT and KO mice for two weeks with a single injection of STZ. AMVM were isolated and the contractility of the cells was measured as concentration–response curves of β-adrenergic responsiveness. Contractility was shown as percent of cell shortening (%FS) by AMVM in response to increasing concentrations of β-adrenergic agonist, isoproterenol. WT control, WT diabetic, and KO control groups all showed significantly higher contractility in response to isoproterenol in comparison with the KO diabetic group ($p<0.05$), except for the WT control group at 10^{-9} M of isoproterenol, which showed a large standard deviation. WT diabetic is significantly lower than WT control at 10^{-7} M of isoproterenol ($p<0.05$).

3.2. Cardiomyocytes lacking Nrf2 are sensitive to inhibition of the mitochondrial respiratory complex II by 3-nitropropionic acid (3NP)

Mitochondrial respiration is a major source of ROS during many physiological and disease processes. High levels of glucose in diabetes damage mitochondrial respiratory electron transfer leading to increased production of ROS [27–32]. The findings that Nrf2 protects cardiomyocytes from glucose-induced oxidative stress suggest cardiomyocyte mitochondrial respiration as a target of Nrf2. We directly tested this postulate by examining whether cardiomyocytes lacking Nrf2 are vulnerable to inhibition of mitochondrial respiration complex II by 3NP. 3NP at 1 mM increased the ROS level in Nrf2 WT cardiomyocytes, consistent with the notion that inhibition of complex II by 3NP induces mitochondrial oxidative stress (Figs. 5A and B). Nrf2 KO cardiomyocytes showed a higher level of ROS production compared with WT cells in the absence of 3NP, whereas treatment with 3NP at 1 mM increased ROS production to a much higher level than that of WT treated with 3NP (Figs. 5A and B). Parallel to ROS

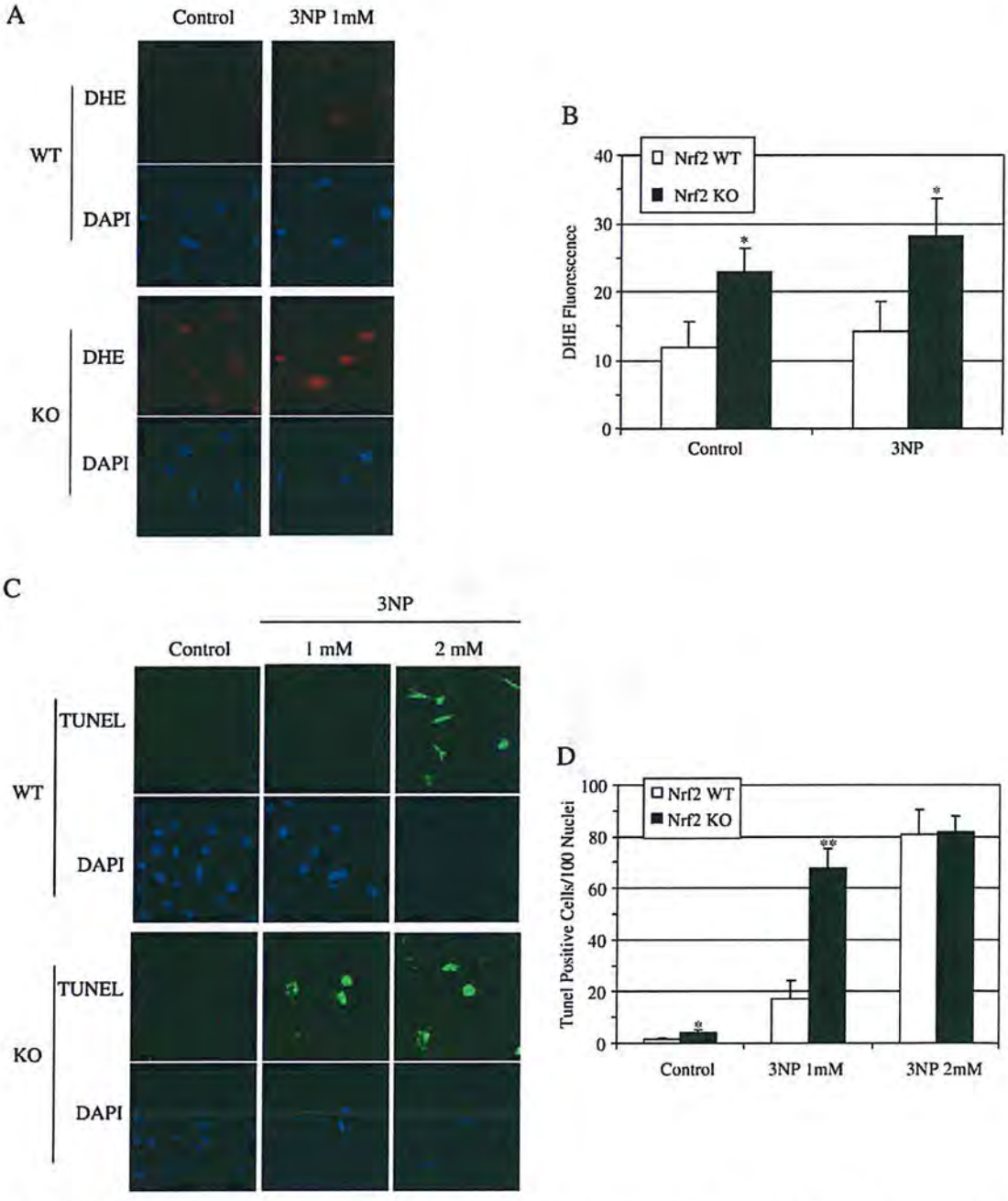

Fig. 5. Nrf2 null cardiomyocytes are sensitive to inhibition of mitochondrial complex II by 3NP. Neonatal cardiomyocytes isolated from Nrf2 WT and KO mice were treated with 3NP as indicated for 12 h. (A) Oxidative stress. Intracellular ROS production was detected with DHE and cell nuclei were shown with DAPI as described for Fig. 1. (B) Quantification of fluorescent signals in (A). (C) TUNEL assay. Apoptosis was examined with the TUNEL assay. Apoptotic cells were stained green and cell nuclei were stained blue (DAPI). (D) Quantification of TUNEL assay. Magnification = 40×. *, $p<0.05$; **, $p<0.01$.

production, 3NP induced concentration-dependent apoptosis in WT cells, but stronger apoptotic effects by 3NP were observed in Nrf2 KO cells (Figs. 5C and D). The findings are consistent with the notion that Nrf2 indeed protects against mitochondrial damage-induced oxidative lesions in cardiomyocytes.

3.3. Aberrant expression and induction of cytoprotective genes in *Nrf2* KO cardiomyocytes in vitro and in vivo

Nrf2 controls the basal transcription and induction of ARE-regulated antioxidant and detoxification genes. Therefore, we examined the regulation of the genes by Nrf2 in response to high glucose-induced oxidative stress in cardiomyocytes. As expected, *Nrf2* mRNA was detected in WT neonatal cardiomyocytes but not in Nrf2 KO cells (Fig. 6), because a major portion of the Nrf2 gene was deleted in the KO mice. Treatment with glucose at 20 and 40 mM for 24 h increased the expression of *Nrf2* mRNA to less than 2-fold (Fig. 6A); however, treatment with 3NP at 2 and 5 mM for 24 h induced *Nrf2* mRNA expression in WT cells to 3 and 5-fold higher, respectively (Fig. 6B).

Nqo1, a prototype of Nrf2-regulated chemical-detoxification gene, was shown to be expressed in WT cardiomyocytes (Fig. 7). Expression of the gene was induced by glucose at 40 mM (Fig. 7A) and by 3NP at 2 an 5 mM (Fig. 7B); longer treatment further enhanced induction (Fig. 7B, compare induction at 8 h and 24 h). The basal expression and induction of *Nqo1* in the presence of high glucose or 3NP were lost in Nrf2 KO cardiomyocytes, confirming that Nrf2 mediates *Nqo1* expression and induction in cardiomyocytes. Expression and induction of *Ho1*, a ROS-inducible gene important in heme catabolism, were shown in Fig. 8. Similarly to *Nqo1*, *Ho1* mRNA was expressed in WT cells and was induced by both high glucose and 3NP in concentration and time-dependent manners (Figs. 8A and B). However, the basal expression of *Ho1* mRNA was markedly reduced and induction by high glucose and 3NP was totally lost in Nrf2 KO cardiomyocytes, demonstrating Nrf2 is required for the expression and induction of *Ho1* in heart muscle cells.

Immunoblotting was performed to verify the protein expression and induction of Nrf2 and HO1 in cardiomyocytes. Glucose at 20 mM increased the protein levels of Nrf2 and HO1 in WT cells in comparison with mannitol, which was used as an osmotic control for glucose (Fig. 9A, upper panel). Induction by glucose was concentration-dependent (Fig. 9B, upper panel). In Nrf2 KO cells, the Nrf2 protein was not detected in either the absence or presence of high glucose (Figs. 9A and B, lower panel); induction of HO1 by glucose was also lost (Fig. 9B, lower panel). Similarly, treatment with 3NP increased the protein levels of both Nrf2 and HO1 in a concentration-dependent manner (Fig. 9C, upper panels), but the expression and induction of both proteins were not observed in Nrf2 KO cells (Fig. 9C, lower panels).

Activation of Nrf2 involves stabilization of the Nrf2 protein and translocation of the protein into the nucleus. To directly examine Nrf2 activation by glucose, H9C2, a rat cardiomyoblast cell line, was treated with glucose (40 mM) for 48 h or tBHQ (as a positive control) at 30 μM for 5 h (Fig. 10). Immunofluorescent confocal microscopic examination of the cells revealed that, as expected, tBHQ strongly increased both the total fluorescent intensity in cells and the nuclear staining of Nrf2. High glucose treatment significantly increased the nuclear and total cell staining of Nrf2 in comparison with the control cells, indicating that glucose indeed increased the protein level and nuclear accumulation of Nrf2.

To examine if Nrf2 is activated by high glucose *in vivo*, induction of cytoprotective genes were examined in the hearts of diabetic mice

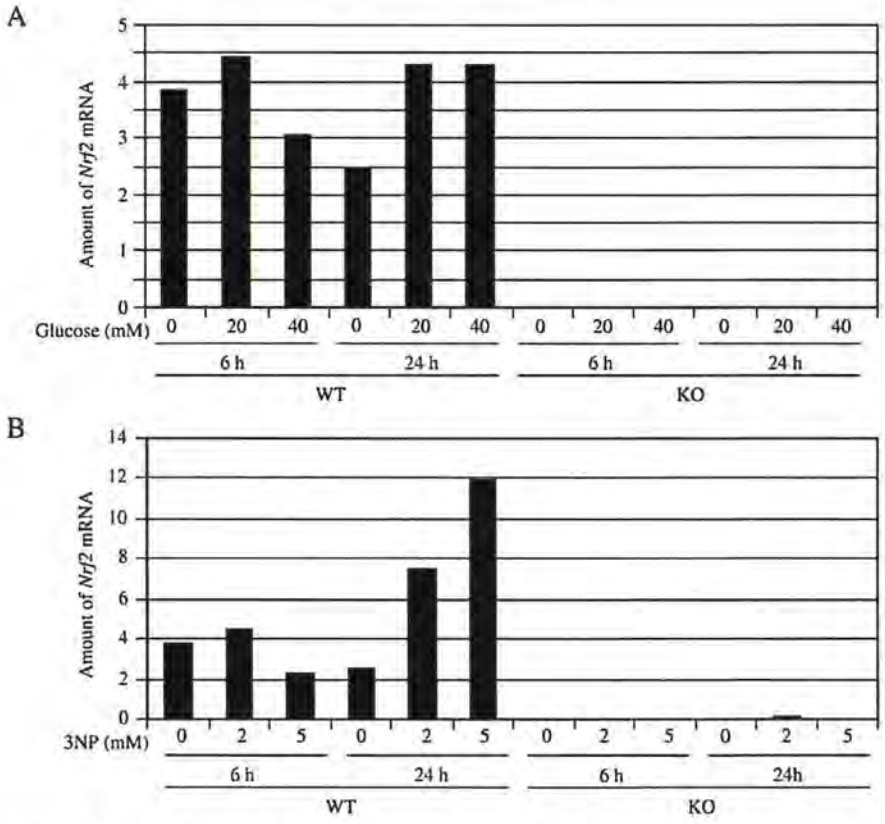

Fig. 6. Expression and induction of *Nrf2* mRNA in cardiomyocytes. Nrf2 WT and KO neonatal cardiomyocytes were isolated and were treated with glucose (A) or 3NP (B) as indicated. Nrf2 mRNA expression was measured by real time-PCR.

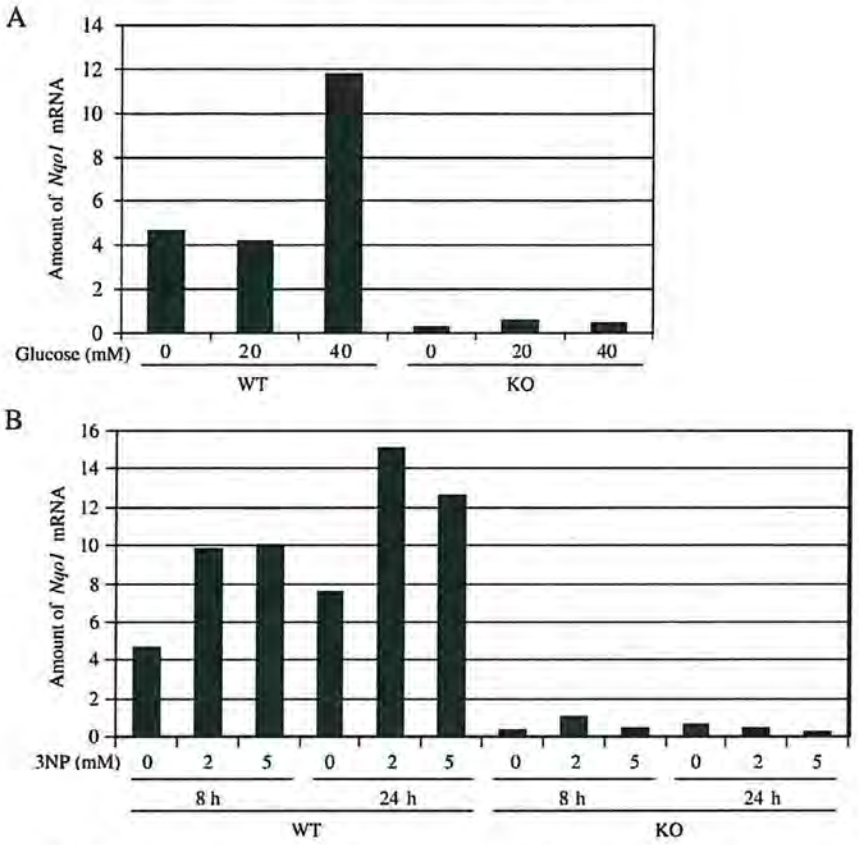

Fig. 7. Expression and induction of *Nqo1* mRNA in cardiomyocytes. Neonatal cardiomyocytes from Nrf2 WT or KO mice were treated with glucose (A) or 3NP (B) as indicated. *Nqo1* mRNA expression and induction were detected by real time-PCR.

(Fig. 11). As expected, *Nrf2* mRNA was expressed in both control and diabetic wild type mice and STZ treatment did not change the levels, whereas in Nrf2 KO hearts, *Nrf2* mRNA was not detected. *Nqo1* mRNA was constitutively expressed in the control WT mice and the mRNA was induced by STZ. However, *Nqo1* mRNA was largely diminished in the hearts from both control and STZ treated mice, showing a total dependence of *Nqo1* expression on Nrf2 in heart tissues.

We further tested the role of Nrf2 in ROS defense in cardiomyocytes by using SiRNA knockdown. H9C2 was transfected with control SiRNA or rat Nrf2 (rNrf2) SiRNA, followed by treatment with glucose or 3NP (Fig. 12). Nrf2 protein was largely reduced in rNrf2 SiRNA transfected cells compared with the control (Fig. 12A). HO1 protein was induced by high glucose and 3NP in control SiRNA transfected cells but induction was totally blocked in Nrf2 SiRNA transfected cells (Fig. 10A). Similarly, *Nqo1* mRNA was expressed at high levels in H9C2 cells but the expression was largely decreased in the Nrf2 knockdown cells (Fig. 10B). Consistent with reduced expression of ARE-controlled genes, knockdown of Nrf2 in H9C2 increased oxidative stress in the cells (data not shown).

Taken together, the mRNA and protein expression data supported the notion that high levels of glucose activate Nrf2 to induce ARE-dependent antioxidant proteins and enzymes in cardiomyocytes both *in vitro* and *in vivo*, which mediate the protection against high glucose-induced oxidative damage in the cells.

4. Discussion

The continuous and repetitive contraction and high energy demand in the heart may expose cardiomyocytes to excessive amounts of ROS produced endogenously, making the cardiac muscle vulnerable to oxidative damage. Oxidative stress in the heart can also be produced from ROS-promoting chemicals carried in the blood. High levels of blood glucose in diabetes promote ROS production in many tissues that contributes to the pathogenesis of multi-tissue damage in diabetes, such as insulin resistance in peripheral muscle and fat tissues, blood vessel hardening, and cardiomyopathy—the leading cause of disability and mortality among diabetic patients [4,33,34]. Because diabetic cardiomyopathy can occur clinically without major vascular lesions, a direct toxic effect of hyperglycemia on heart muscle cells via oxidative damage in diabetes has been suggested.

Parallel to oxidative damage, the body evokes defensive responses to oxidative stimuli to counteract ROS and oxidative damage, which include increased production of small antioxidant molecules and the enzymes/proteins involved in ROS catabolism and chemical detoxification. We and others have shown that Nrf2 plays a critical role in oxidative defense; in this context, Nrf2 functions as a xenobiotic-activated receptor to integrate a wide range of chemical signals, in particular oxidative and electrophilic stimuli, and coordinately regulates the expression and induction of a battery of cytoprotective proteins and enzymes through ARE-dependent gene transcription [5,6,8,12,14]. Consistent with this notion, loss of Nrf2 function is associated with increased production of ROS in a range of chemical toxicity, cancer, and chronic diseases, whereas boosting Nrf2 function by antioxidants and certain natural products provides chemoprotection against the lesions by reducing oxidative damage [5–7,10,15–21]. In light of the prominent role of Nrf2 in oxidative defense, we postulate that Nrf2 plays a critical role in the development of diabetic cardiomyopathy.

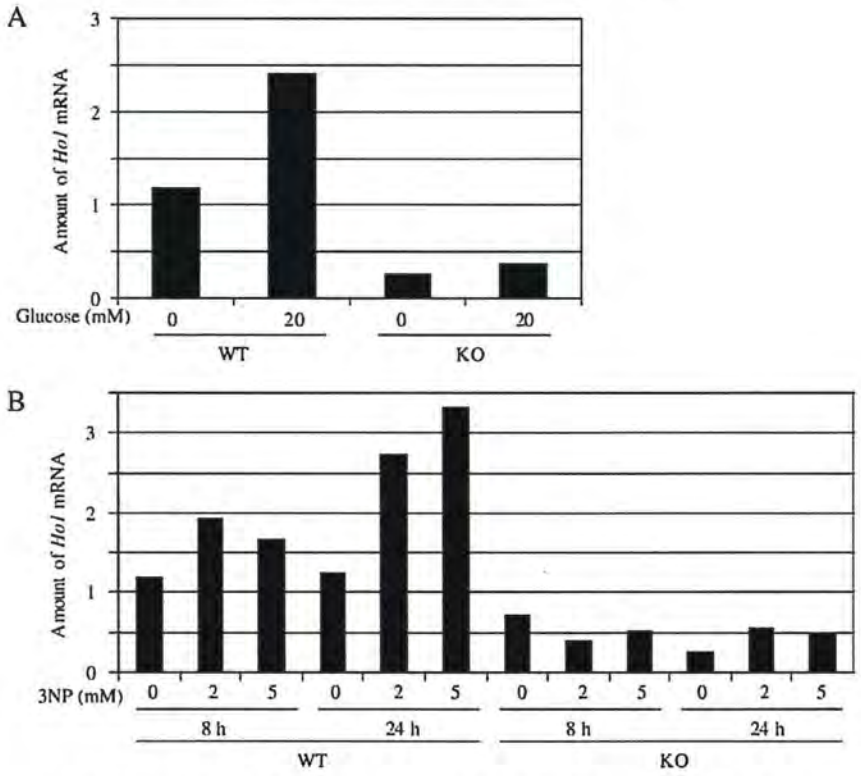

Fig. 8. Expression and induction of *Ho1* mRNA in cardiomyocytes. Neonatal cardiomyocytes of Nrf2 WT or KO genotypes were treated with glucose (A) or 3NP (B). *Ho1* mRNA expression and induction were examined by real-time PCR.

The results obtained from this study demonstrate that Nrf2 is essential in repressing high glucose-induced ROS production in cardiomyocytes and is required for the cells to cope with high glucose-induced oxidative damage for survival. Treatment with high glucose in cultured primary neonatal and adult cardiomyocytes induced ROS production, which is in agreement with the notion that high levels of glucose directly stimulate the muscle cells to induce ROS production in the heart. In cells lacking of functional Nrf2, ROS production was significantly elevated even in the absence of high glucose, whereas high glucose treatment further increased ROS levels in the cells in concentration and time-dependent manners. Consistent with heightened production of ROS, glucose induced significant apoptosis in the Nrf2 KO cells in comparison with the Nrf2 WT cells in which high levels of glucose were required to produce a smaller

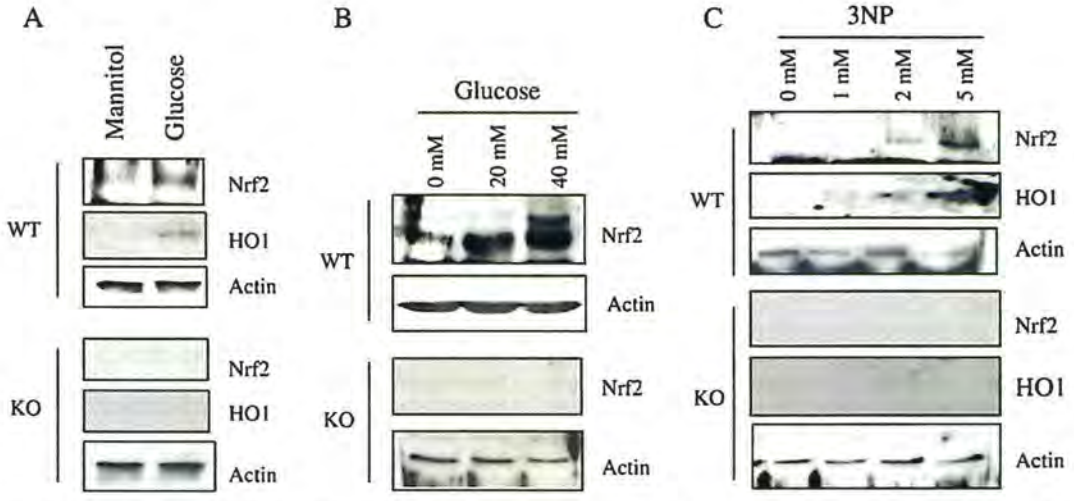

Fig. 9. Immunoblotting of Nrf2 and target proteins in cardiomyocytes. Neonatal cardiomyocytes from Nrf2 WT and KO mice were treated with (A): glucose and mannitol (as an osmotic control) (6 h); (B): varying concentrations of glucose (18 h), or (C): 3NP (12 h). Total cell lysates were analyzed by immunoblotting for protein expression. Actin was used as a loading control.

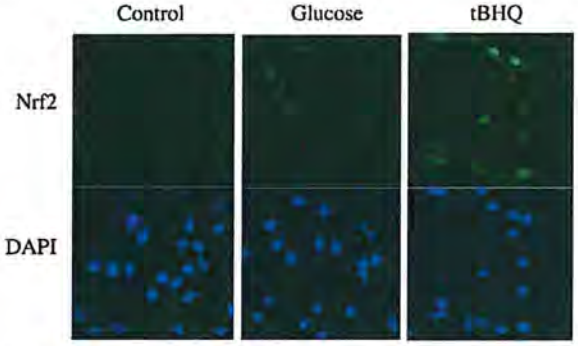

Fig. 10. Glucose induces Nrf2 nuclear enrichment. H9C2 cells were treated with glucose at 40 mM for 48 h, or tBHQ at 30 μM for 5 h. The cells were stained with anti-Nrf2 (green) and DAPI (blue). Nuclear accumulation of Nrf2 was visualized with fluorescent confocal microscopy.

increase in apoptosis. Furthermore, adult cardiomyocytes from diabetic Nrf2 KO mice exhibited dramatically elevated ROS levels and severely damaged contractility compared with WT controls, demonstrating a critical *in vivo* function of Nrf2 in defense against diabetic cardiomyocyte damage by high glucose in adult hearts.

The sources of ROS under different physiological and disease conditions may vary. Inhibition of mitochondrial respiratory electron transfer by chemicals or during mitochondrial degeneration can lead to increased production of ROS that has been associated with neurodegenerative diseases, cancer, and metabolic syndromes in animal models and in humans [35,36]. It has been observed that diabetic hyperglycemia damages mitochondrial respiration and thereby, increases ROS production [27–32]. The observed protection of cardiomyocytes from diabetic oxidative damage by Nrf2 thus suggests mitochondrial respiration as an important target of Nrf2 action under diabetic conditions. Indeed, we found that inhibition of mitochondrial respiratory complex II by 3NP induced the production of ROS in cardiomyocytes and knockout of Nrf2 significantly increased the ROS production and apoptosis by 3NP. The results indicate that Nrf2 is required for protection from mitochondrial ROS in cardiomyocytes, supporting a critical role of Nrf2 in the control of mitochondrial ROS homeostasis in diabetic hearts.

Molecular analyses of Nrf2 action in cardiomyocytes reveal that both high glucose and 3NP induce *Nqo1* and *Ho1*, two ARE-controlled cytoprotective genes. Importantly, the results demonstrate that high

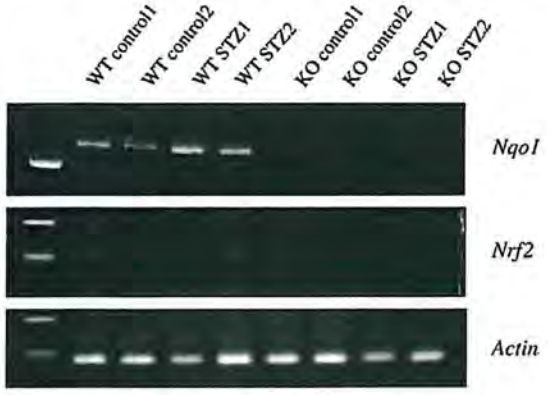

Fig. 11. Induction of *Nqo1* and *Ho1* mRNAs *in vivo*. Nrf2 WT and KO mice were made diabetic by STZ injection as described under Materials and methods. Total RNA was prepared from the hearts. *Nqo1* and *Nrf2* mRNAs were amplified by RT-PCR. Actin was used as loading controls.

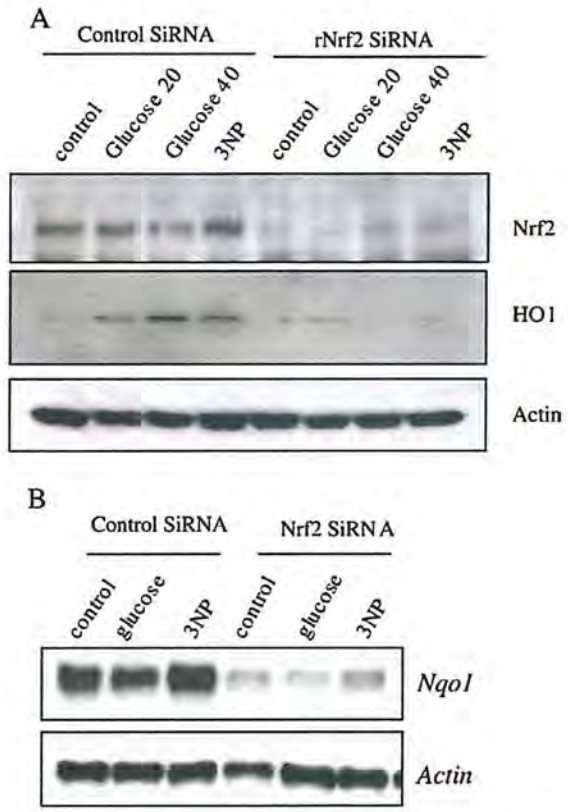

Fig. 12. Knockdown of Nrf2 reduced expression of antioxidant genes and increased oxidative damage in H9C2 cells. H9C2 cells were transfected with control SiRNA or rNrf2 SiRNA and were treated with glucose or 3NP for 18 h. (A): Knockdown of Nrf2 with siRNA. Total cell lysate was immunoblotted with anti-Nrf2 or anti-HO1 antibodies. Actin was blotted as a loading control. (B) Northern blotting. Total RNA of 3 μg each was analyzed by Northern blotting for mRNA expression of *Nqo1* and *Actin*.

levels of glucose stabilizes the Nrf2 protein, induces the nuclear translocation of Nrf2, and mediates the basal and inducible expression of cytoprotective genes at both the mRNA and protein levels in cardiomyocytes *in vitro* and *in vivo*, as both the basal expression and induction of the genes were totally blocked in Nrf2 KO cells or largely reduced in Nrf2 SiRNA transfected cells. HO1 and NQO1 have been implicated as protective factors in a range of disease processes, including diabetes, oxidative damage, inflammatory lesions, and cardiovascular diseases [19,37,38]. The findings provide the first evidence supporting the regulation of ARE-dependent gene transcription as a major mechanism of protection by Nrf2 in cardiomyocytes under both physiological and diabetic conditions.

Inducers of ARE-controlled genes may activate Nrf2 by directly interacting with the thiol groups of critical cysteine residues in Keap1. In this framework, Keap1 serves as a component of the cytoplasmic Nrf2 complex and represses Nrf2 via two mechanisms: (a) anchoring the complex in the cytoplasm and, (b) promoting the ubiquitination and proteasomal degradation of Nrf2 through the Cul3-dependent ubiquitin ligase (E3) [8,11]. Binding of inducers (direct-acting inducers, such as the phenolic antioxidant, tBHQ, and the toxic metal, arsenic) to critical cysteine thiol groups of Keap1 stabilizes and activates Nrf2. On the other hand, some inducers, such as glucose, do not appear to be reactive toward protein thiols, and thus, may activate Nrf2 indirectly. In this scenario, glucose-increased ROS or other secondary oxidants/electrophilic products may mediate the activation of Nrf2. It is known that diabetic

hyperglycemia induces the formation of advanced glycation end products (AGE) that binds to AGE receptors (RAGE) [39–41]. AGE–RAGE interaction can lead to the production of ROS, which activates NF-κB to induce pro-inflammatory cytokines [39]. Whether glucose activates Nrf2 via AGE and RAGE similarly to the activation of NF-κB remains to be studied. Alternatively, diabetic hyperglycemia may modulate the activities of kinases, such as protein kinase C isoforms [39], that in turn results in the activation of Nrf2. Thirdly, hyperglycemia increases the production of *N*-acetylglucosamine (GlcNAc) that can bind to the serine and threonine residues of transcription factors, such as Sp1, to modulate gene transcription [39]. By analogy with Sp1, glucose may activate Nrf2 via the GlcNAc pathway. In aggregates, induction of Nrf2 target genes in cardiomyocytes can serve as a molecular model for the analysis of receptor activation and gene regulation induced by diabetic hyperglycemia.

3NP is capable of covalent binding with proteins. 3NP forms covalent adducts with the side chain of Arg297 in the active site of the mitochondrial respiratory complex II (succinate ubiquinone oxidoreductase), and thereby, inhibits mitochondrial respiratory electron transfer [42]. Whether 3NP acts as a direct inducer to activate Nrf2 requires further study. Additionally, 3NP appears to stimulate the expression of *Nrf2* mRNA, whereas other inducers, such as tBHQ, and arsenic do not [10]. The molecular mechanisms account for the differential effects of the inducers on *Nrf2* mRNA expression is currently unclear. Auto-regulation of *Nrf2* mRNA by Nrf2 itself has been reported previously [43]. Induction of *Nrf2* mRNA by 3NP requires Nrf2 (Fig. 6B), suggesting that 3NP may stimulate this loop of positive auto-regulation of Nrf2.

Diabetic cardiomyopathy represents a chronic complication in diabetic patients. Preventive and therapeutic measures targeting the underlying pathophysiology of cardiac muscle damage during diabetes are lacking at present. The demonstration of critical roles of Nrf2 and ARE-regulated cytoprotective enzymes/proteins in defense against oxidative stress by high glucose and 3NP in this study provides insights into the antioxidative response in heart muscle cells and raises the possibility of developing Nrf2 activators as anti-diabetic cardiomyopathy agents in the future.

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References

[1] Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001, Dec 13;414(6865):782–7.
[2] Trost S, LeWinter M. Diabetic cardiomyopathy. Curr Treatm Opt Cardiovasc Med 2001;3:481–92.
[3] Francis GS. Diabetic cardiomyopathy: fact or fiction. Heart 2001, Mar;85(3):247–8.
[4] Cai L, Kang YJ. Oxidative stress and diabetic cardiomyopathy. Cardiovasc Toxicol 2001;1:181–93.
[5] Ma Q. Xenobiotic-activated receptors: from transcription to drug metabolism to disease. Chem Res Toxicol 2008, Sep;21(9):1651–71.
[6] Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1–Nrf2–ARE pathway. Annu Rev Pharmacol Toxicol 2007;47:89–116.
[7] Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. Proc Natl Acad Sci U S A 1999, Oct 26;96(22):12731–6.
[8] Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 2004, Aug;24(16):7130–9.
[9] Ma Q, Kinneer K, Bi Y, Chan JY, Kan YW. Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. Biochem J 2004, Jan 1;377(Pt 1):205–13.
[10] He X, Chen MG, Lin GX, Ma Q. Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting the Nrf2·Keap1·Cul3 complex and recruiting Nrf2·Maf to the antioxidant response element enhancer. J Biol Chem 2006, Aug 18;281 (33):23620–31.
[11] Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci U S A 2002, Sep 3;99(18):11908–13.
[12] Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 2003;43:233–60.
[13] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997, Jul 18;236 (2):313–22.
[14] Leung L, Kwong M, Hou S, Lee C, Chan JY. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. J Biol Chem 2003, Nov 28;278(48):48021–9.
[15] Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci U S A 2001, Apr 10;98 (8):4611–6.
[16] Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci U S A 2001, Mar 13;98(6):3410–5.
[17] Hu X, Roberts JR, Apopa PL, Kan YW, Ma Q. Accelerated ovarian failure induced by 4-vinyl cyclohexene diepoxide in Nrf2 null mice. Mol Cell Biol 2006, Feb;26 (3):940–54.
[18] Hubbs AF, Benkovic SA, Miller DB, O'Callaghan JP, Battelli L, Schwegler-Berry D, et al. Vacuolar leukoencephalopathy with widespread astrogliosis in mice lacking transcription factor Nrf2. Am J Pathol 2007, Jun;170(6):2068–76.
[19] He X, Lin GX, Chen MG, Zhang JX, Ma Q. Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association. Toxicol Sci 2007, Jul;98(1):298–309.
[20] He X, Chen MG, Ma Q. Activation of Nrf2 in defense against cadmium-induced oxidative stress. Chem Res Toxicol 2008, Jul;21(7):1375–83.
[21] Ma Q, Battelli L, Hubbs AF. Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. Am J Pathol 2006, Jun;168(6):1960–74.
[22] Zhu H, Jia Z, Misra BR, Zhang L, Cao Z, Yamamoto M, et al. Nuclear factor E2-related factor 2-dependent myocardiac cytoprotection against oxidative and electrophilic stress. Cardiovasc Toxicol 2008, Summer;8(2):71–85.
[23] Wang GW, Kang YJ. Inhibition of doxorubicin toxicity in cultured neonatal mouse cardiomyocytes with elevated metallothionein levels. J Pharmacol Exp Ther 1999, Mar;288(3):938–44.
[24] Kan H, Xie Z, Finkel MS. iPLA2 inhibitor blocks negative inotropic effect of HIV gp120 on cardiac myocytes. J Mol Cell Cardiol 2006, Jan;40(1):131–7.
[25] Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J Leukoc Biol 1994, Feb;55 (2):253–8.
[26] Ma Q, Renzelli AJ, Baldwin KT, Antonini JM. Superinduction of CYP1A1 gene expression. Regulation of 2,3,7, 8-tetrachlorodibenzo-p-dioxin-induced degradation of Ah receptor by cycloheximide. J Biol Chem 2000 Apr, 28;275(17):12676–83.
[27] Amaral S, Oliveira PJ, Ramalho-Santos J. Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. Curr Diabetes Rev 2008, Feb;4(1):46–54.
[28] Maasen JA. Mitochondria, body fat and type 2 diabetes: what is the connection. Minerva Med 2008, Jun;99(3):241–51.
[29] Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, et al. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. J Physiol 2007, Aug 15;583(Pt 1):9–24.
[30] Nishikawa T, Kukidome D, Sonoda K, Fujisawa K, Matsuhisa T, Motoshima H, et al. Impact of mitochondrial ROS production in the pathogenesis of insulin resistance. Diabetes Res Clin Pract 2007, Sep;77 Suppl(1):S161–4.
[31] Nishikawa T, Kukidome D, Sonoda K, Fujisawa K, Matsuhisa T, Motoshima H, et al. Impact of mitochondrial ROS production on diabetic vascular complications. Diabetes Res Clin Pract 2007, Sep;77 Suppl(1):S41–5.
[32] Song Y, Du Y, Prabhu SD, Epstein PN. Diabetic cardiomyopathy in OVE26 mice shows mitochondrial ROS production and divergence between in vivo and in vitro contractility. Rev Diabet Stud 2007, Fall;4(3):159–68.
[33] Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006, Apr 13;440(7086):944–8.
[34] Meigs JB, Larson MG, Fox CS, Keaney Jr JF, Vasan RS, Benjamin EJ. Association of oxidative stress, insulin resistance, and diabetes risk phenotypes: the Framingham offspring study. Diabetes Care 2007, Oct;30(10):2529–35.
[35] Schapira AH. Oxidative stress and mitochondrial dysfunction in neurodegeneration. Curr Opin Neurol 1996, Aug;9(4):260–4.
[36] Brouillet E, Conde F, Beal MF, Hantraye P. Replicating Huntington's disease phenotype in experimental animals. Prog Neurobiol 1999, Dec;59(5):427–68.
[37] Liu X, Wei J, Peng DH, Layne MD, Yet SF. Absence of heme oxygenase-1 exacerbates myocardial ischemia/reperfusion injury in diabetic mice. Diabetes 2005, Mar;54 (3):778–84.
[38] Chen K, Gunter K, Maines MD. Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J Neurochem 2000, Jul;75(1):304–13.
[39] Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001, Dec 13;414(6865):813–20.
[40] Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med 2005, Nov;83(11):876–86.

[41] Ramasamy R, Yan SF, Herold K, Clynes R, Schmidt AM. Receptor for advanced glycation end products: fundamental roles in the inflammatory response: winding the way to the pathogenesis of endothelial dysfunction and atherosclerosis. Ann NY Acad Sci 2008, Apr;1126:7–13.
[42] Huang LS, Sun G, Cobessi D, Wang AC, Shen JT, Tung EY, et al. 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. J Biol Chem 2006, Mar 3;281(9):5965–72.
[43] Kwak MK, Itoh K, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol 2002, May;22 (9):2883–92.