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Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: Protection against reactive oxygen and nitrogen species-induced cell injury

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Abstract **Understanding the molecular pathway(s) of antioxidant gene regulation is of crucial importance for developing antioxidant-inducing agents for the intervention of oxidative cardiac disorders. Accordingly, this study was undertaken to determine the role of Nrf2 signaling in the basal expression as well as the chemical inducibility of endogenous antioxidants and phase 2 enzymes in cardiac fibroblasts. The basal expression of a scope of key cellular antioxidants and phase 2 enzymes was significantly lower in cardiac fibroblasts derived from Nrf2$^{-/-}$ mice than those from wild type control. These include catalase, reduced glutathione (GSH), glutathione reductase (GR), GSH *S*-transferase (GST), and NAD(P)H:quinone oxidoreductase-1 (NQO1). Incubation of Nrf2$^{+/+}$ cardiac fibroblasts with 3*H*-1,2-dithiole-3-thione (D3T) led to a significant induction of superoxide dismutase (SOD), catalase, GSH, GR, glutathione peroxidase (GPx), GST, and NQO1. The inducibility of SOD, catalase, GSH, GR, GST, and NQO1, but not GPx by D3T was completely abolished in Nrf2$^{-/-}$ cells. The Nrf2$^{-/-}$ cardiac fibroblasts were much more sensitive to reactive oxygen and nitrogen species-mediated cytotoxicity. Upregulation of antioxidants and phase 2 enzymes by D3T in Nrf2$^{+/+}$ cardiac fibroblasts resulted in a dramatically increased resistance to the above species-induced cytotoxicity. In contrast, D3T-treatment of the Nrf2$^{-/-}$ cells only provided a slight cytoprotection. Taken together, this study demonstrates for the first time that Nrf2 is critically involved in the regulation of the basal expression and chemical induction of a number of antioxidants and phase 2 enzymes in cardiac fibroblasts, and is an important factor in controlling cardiac cellular susceptibility to reactive oxygen and nitrogen species-induced cytotoxicity.**
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Keywords: Cardiac cell; Antioxidant; Nrf2 signaling; 3*H*-1, 2-Dithiole-3-thione; Reactive oxygen species; Reactive nitrogen species; Cytotoxicity

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Abbreviations: SOD, superoxide dismutase; GSH, glutathione; GST, glutathione *S*-transferase; GR, glutathione reductase; GPx, glutathione peroxidase; NQO1, NAD(P)H:quinone oxidoreductase-1; D3T, 3*H*-1,2-dithiole-3-thione; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; XO, xanthine oxidase; CDNB, 1-chloro-2,4-dinitrobenzene; DCIP, 2,6-dichloroindophenol; FBS, fetal bovine serum; PBS, phosphate buffered saline; PCR, polymerase chain reaction

1. Introduction

Substantial evidence supports a critical role for reactive oxygen and nitrogen species in the development of various forms of cardiac disorders [1–3]. Accordingly, exogenous antioxidative compounds have been used for the preventive and/or therapeutic intervention of oxidative cardiac disorders [1,3,4]. Another strategy for protecting against oxidative cardiac injury may be via chemically mediated upregulation of endogenous antioxidants and phase 2 enzymes (enzymes involved in detoxification of electrophilic xenobiotics) in cardiac tissue/cells. Such a strategy relies on a profound understanding of the chemical inducibility of cardiac antioxidants and phase 2 enzymes, as well as the underlying signaling mechanisms.

Recently, Nrf2 has been demonstrated to be a critical transcription factor that binds to the antioxidant response element in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes in several types of cells and tissues [5–7]. However, whether Nrf2 signaling also controls the expression of antioxidants and phase 2 enzymes in cardiac cells has not been previously reported in the literature. Cardiac tissue is primarily made of two major types of cells: cardiomyocytes and fibroblasts. In fact, the number of fibroblasts in cardiac tissue exceeds that of cardiomyocytes. Cardiac fibroblasts play important roles in both cardiac physiology and pathophysiology [8]. These cells are critically involved in cardiac tissue remodeling [8]. Recently, several studies have suggested that injury and/or death of fibroblasts may be involved in cardiac disorders, such as dilated cardiomyopathy [9–11]. While there are studies on the antioxidative and phase 2 enzymes, and their chemical regulation in cardiomyocytes, studies on the regulation of antioxidative and phase 2 defenses in cardiac fibroblasts are lacking. Accordingly, this study was undertaken to determine the role of Nrf2 signaling in the basal expression as well as the chemical induction of endogenous antioxidants and phase 2 enzymes in neonatal cardiac fibroblasts derived from Nrf2-null and wild type mice. Our results demonstrate for the first time that Nrf2 is critically involved in the regulation of both basal expression and chemical inducibility of a number of endogenous antioxidants and phase 2 enzymes in cardiac fibroblasts, and is an important factor in controlling cardiac cellular susceptibility to reactive oxygen and nitrogen species-induced cytotoxicity.

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2. Materials and methods

2.1. Materials

3*H*-1,2-Dithiole-3-thione (D3T) with a purity of 99.8% was generously provided by Dr. Mary Tanga at SRI International (Menlo Park, CA) and Dr. Linda Brady at National Institute of Mental Health (Bethesda, MD). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate buffered saline (PBS) were from Gibco-Invitrogen (Carlsbad, CA). All other chemicals and reagents were from Sigma Chemical (St. Louis, MO). Tissue culture flasks and 24-well tissue culture plates were from Corning (Corning, NY).

2.2. Animals and genotyping

Breeding pairs of $Nrf2^{+/-}$ (ICR/Sv129) mice were obtained from a colony at Tsukuba University and maintained in the animal facility at The Ohio State University Medical Center. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were generated following the breeding procedures described previously [12]. Purina laboratory animal chow (Richmond, IN) and water were available ad libitum. Genotypes ($Nrf2^{+/+}$, $Nrf2^{-/-}$, and $Nrf2^{+/-}$) of the animals were determined by polymerase chain reaction (PCR) amplification of genomic DNA from tails. PCR amplification was carried out using three different primers, 5′-TGGACGGGACTATTGAAGGCTG-3′ (sense for $Nrf2^{+/+}$ and $Nrf2^{-/-}$), 5′-CGCCTTTTCAGTAGATGGAGG-3′ (antisense for $Nrf2^{+/+}$), and 5′-GCGGATTGACCGTAATGGGATAGG-3′ (antisense for LacZ). All of the animal procedures were approved by the Institutional Animal Care and Use Committee at The Ohio State University Medical Center.

2.3. Isolation and culture of neonatal cardiac fibroblasts

Neonatal mice at the age of 1–3 days were euthanized via cervical dislocation. The hearts were removed aseptically and rinsed extensively with ice-cold PBS. The ventricles were then minced into small pieces. The cardiac cells were dissociated at 37 °C for 15 min with an enzyme solution (0.25% trypsin in PBS). The cells released from the first digestion were discarded, whereas the cells from subsequent digestion were added to an equal volume of ice-cold DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium). The cells were pelleted by centrifugation at $200 \times g$ for 10 min. The resulting cell pellets were resuspended in the above culture medium, and plated into 150 cm^2 tissue culture flasks. The cells were incubated at 37 °C for 2 h in a humidified atmosphere of 5% CO_2 to allow the cardiac fibroblasts to attach to the flasks. After this incubation, the non-attached cells were discarded, and the flasks were rinsed twice with culture medium to remove any residual unattached cells. The attached fibroblasts were continuously cultured in fresh culture medium, and used for experiments within 2 weeks after isolation.

2.4. Preparation of cell extract

Cardiac fibroblasts were collected and resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at $13\,000 \times g$ for 10 min at 4 °C. The resulting supernatants were collected and the protein concentrations were quantified with Bio-Rad protein assay dye (Hercules, CA) using bovine serum albumin as the standard. The samples were kept on ice for measurement of the antioxidants and phase 2 enzymes within 2–3 h, as described below.

2.5. Assay for cellular superoxide dismutase activity

Total cellular superoxide dismutase (SOD) activity was determined by the method of Spitz and Oberley [13] with slight modifications. In brief, the reaction mix (prepared freshly) contained in 50 mM potassium phosphate buffer, pH 7.8, 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/ml catalase, 70 μM nitroblue tetrazolium, 0.2 mM xanthine, 50 μM mM bathocuproinedisulfonic acid, and 0.13 mg/ml bovine serum albumin. 0.8 ml of the reaction mix was added to each cuvette, followed by addition of 100 μl of sample. The cuvettes were incubated at 37 °C for 3 min. The reaction was then initiated by adding 100 μl of xanthine oxidase (XO) (0.1 U/ml). The formation of formazan blue was monitored at 560 nm, 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma Chemical) standard curve, and expressed as units per mg of cellular protein.

2.6. Assay for cellular catalase activity

The method of Aebi [14] was used to measure the catalase activity. In brief, to a quartz cuvette, 0.65 ml of 50 mM potassium phosphate buffer (pH 7.0) and 50 μl of sample were added. The reaction was started by adding 0.3 ml of 30 mM H_2O_2. The decomposition of H_2O_2 was monitored at 240 nm, 25 °C for 2 min. The catalase activity was expressed as μmol of H_2O_2 consumed per min per mg of cellular protein.

2.7. Assay for cellular glutathione content

The cellular glutathione (GSH) content was measured according to the procedures described previously by Cao and Li [15]. Briefly, 10 μl of the cell extract sample was incubated with 12.5 μl of 25% HPO_3, and 37 μl of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0 at 4 °C for 10 min. The samples were centrifuged at $13\,000 \times g$ for 5 min at 4 °C. The resulting supernatant (10 μl) was incubated with 0.1 ml of *o*-phthalaldehyde solution (0.1% in methanol) and 1.89 ml of the above phosphate buffer for 15 min at room temperature. Fluorescence was then read using a Perkin–Elmer luminescence spectrometer (LS50B) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Cellular GSH content was calculated using a concurrently run GSH (Sigma Chemical) standard curve and expressed as nmol of GSH per mg of cellular protein.

2.8. Assay for cellular glutathione reductase activity

Cellular glutathione reductase (GR) activity was measured by the method of Wheeler et al. [16] according to the procedures previously described [15]. GR activity was calculated using the extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

2.9. Assay for cellular GSH peroxidase activity

Cellular glutathione peroxidase (GPx) activity was measured by the method of Flohe and Gunzler [17]. Briefly, to an assay cuvette containing 0.5 ml of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 2 mM sodium azide, 100 μl of sample, 100 μl of 10 mM GSH, 100 μl of glutathione reductase (2.4 U/ml), and 100 μl of 1.5 mM NADPH were added. The cuvette was incubated at 37 °C for 3 min. After addition of 100 μl of 2 mM H_2O_2, the rate of NADPH consumption was monitored at 340 nm, 37 °C for 5 min. This was designated as the total rate of NADPH consumption. The non-enzyme-dependent consumption of NADPH was also measured as above except that the 100 μl of sample was replaced by 100 μl of sample buffer. The rate of enzyme-dependent NADPH consumption was obtained by subtracting the non-enzyme-dependent NADPH consumption rate from the total NADPH consumption rate. GPx activity was calculated using the extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

2.10. Assay for cellular glutathione S-transferase activity

Cellular glutathione *S*-transferase (GST) activity was measured by the method of Habig et al. [18] according to the procedures described previously [15]. 1-Chloro-2,3-dinitrobenzene (CDNB) was used as the substrate for GST. GST activity was calculated using the extinction coefficient of 9.6 $mM^{-1}\,cm^{-1}$, and expressed as nmol of CDNB–GSH conjugate formed per min per mg of cellular protein.

2.11. Assay for cellular NAD(P)H: Quinone oxidoreductase 1 activity

Cellular NAD(P)H:quinone oxidoreductase 1 (NQO1) activity was determined according to the procedures described previously [15]. Briefly, the reaction mix (prepared freshly) contained 50 mM Tris–HCl, pH 7.5, 0.08% Triton X-100, 0.25 mM NADPH, 80 μM 2,6-dichloroindophenol (DCIP) in the presence or absence of 60 μM dicumarol. To an assay cuvette, 0.695 ml of reaction mix was added. The reaction was started by adding 5 μl of sample, and the 2-electron reduction of DCIP was monitored at 600 nm, 25 °C for 3 min. The dicumarol-inhibitable NQO1 activity was calculated using the extinction coefficient of 21.0 $mM^{-1}\,cm^{-1}$, and expressed as nmol of DCIP reduced per min per mg of cellular protein.

2.12. RT-PCR analysis

Total RNA from cardiac fibroblasts was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. cDNA synthesis and subsequent PCR reaction were performed using Superscript II One-Step system (Invitrogen) in a volume of 25 μl according to manufacturer's instruction. The cycling conditions for RT-PCR were as following: 50 °C for 30 min (reverse transcription), 94 °C for 2 min (pre-denaturation), followed by 25 cycles of PCR amplification process including denaturing at 94 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 45 s, and by 1 cycle of final extension at 72 °C for 10 min. The sequences of the PCR primers for NOQ1 are: 5′-CCATTCTGAAAGGCTGGTTTG-3′ (sense), 5′-CTAGCTTTGATCTGGTTGTC-3′ (antisense). PCR products were separated by 1% agarose gel electrophoresis. Gels were stained with 0.5 μg/ml solution of ethidium bromide for 30 min followed by another 30 min destaining in water. The gels were then analyzed under ultraviolet light using Alpha Innotech Imaging system. In this study, a standard curve using 6.25–200 ng of total RNA was included in each assay so as to reliably estimate changes in NOQ1 mRNA levels, as described before [19].

2.13. MTT reduction assay

Cell viability was determined by a slightly modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously [15]. In brief, cells were plated into 24-well tissue culture plates. After incubation of the cells with chemicals in DMEM supplemented with 0.5% FBS at 37 °C for 24 h, 50 μl of MTT (2 mg/ml PBS) was added to each well. The plates were incubated at 37 °C for another 2 h. Media were removed and wells were rinsed with PBS. To each well 0.6 ml of mix of dimethyl sulfoxide, isopropanol and deionized water (1:4:5) was added at room temperature to solubilize the formazan crystals. The dissolved formazan was then transferred into semi-micro cuvettes, and the absorbance was measured at 570 nm.

2.14. Statistical analyses

All data are expressed as means ± S.E.M. from at least three independent experiments. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) or Student *t* test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Basal levels of antioxidants and phase 2 enzymes in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts

Table 1 summaries the basal levels/activities of a scope of key cellular antioxidants and phase 2 enzymes in cardiac fibroblasts derived from Nrf2-null and wild type mice. Except for SOD and GPx, the levels/activities of all other antioxidants and phase 2 enzymes examined, including catalase, GSH, GR, GST, and NQO1 were significantly lower in $Nrf2^{-/-}$ cardiac fibroblasts than $Nrf2^{+/+}$ cells. Notably, the activity for GST and NQO1 in $Nrf2^{-/-}$ cells was about 2 and 6 times lower than that in $Nrf2^{+/+}$ cells, respectively (Table 1).

Table 1
Basal levels of antioxidants and phase 2 enzymes in cardiac fibroblasts isolated from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice

Antioxidants/phase 2 enzymes	$Nrf2^{+/+}$ cells	$Nrf2^{-/-}$ cells
SOD (units/mg protein)	5.28 ± 0.92	5.29 ± 0.93
Catalase (μmol/min/mg protein)	13.39 ± 1.07	9.90 ± 0.62*
GSH (nmol/mg protein)	77.15 ± 5.15	55.7 ± 7.9*
GR (nmol/min/mg protein)	62.84 ± 6.27	50.47 ± 2.74*
GPx (nmol/min/mg protein)	26.63 ± 5.35	22.23 ± 4.46
GST (nmol/min/mg protein)	152.19 ± 8.80	80.58 ± 7.39*
NQO1 (nmol/min/mg protein)	85.98 ± 18.41	15.73 ± 1.77*

Data represent means ± S.E.M. from 4 to 7 separate experiemnts.

3.2. Inducibility of SOD and catalase by D3T in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts

SOD and catalase are two important antioxidants in mammalian cells, which work coordinately in the detoxification of superoxide and H_2O_2 to eventually form water and molecular oxygen [20]. As shown in Fig. 1A, incubation of $Nrf2^{+/+}$ cells with 50 and 100 μM D3T for 48 h resulted in a significant 40–45% increase in SOD activity. A 20–40% elevation in catalase activity was seen in $Nrf2^{+/+}$ cells after incubation with 25–100 μM D3T (Fig. 1B). The D3T inducibility of both SOD and catalase was completely abolished in $Nrf2^{-/-}$ cardiac fibroblasts (Fig. 1).

3.3. Inducibility of GSH, GR, and GPx by D3T in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts

GSH and GSH-linked enzymes, including GR and GPx are critical enzymes involved in the detoxication of both reactive oxygen and nitrogen species, and have been shown to be protective against various forms of cardiac disorders [21,22]. As shown in Fig. 2, both GSH and GR in $Nrf2^{+/+}$ cardiac fibroblasts were markedly induced by all three concentrations of D3T in a concentration-dependent manner. A significant 55% increase in GSH level was seen with 25 μM D3T; incubation of $Nrf2^{+/+}$ cells with 50 and 100 μM D3T led to a 65% and 140% increase of GSH level, respectively (Fig. 2A). Similarly, D3T treatment

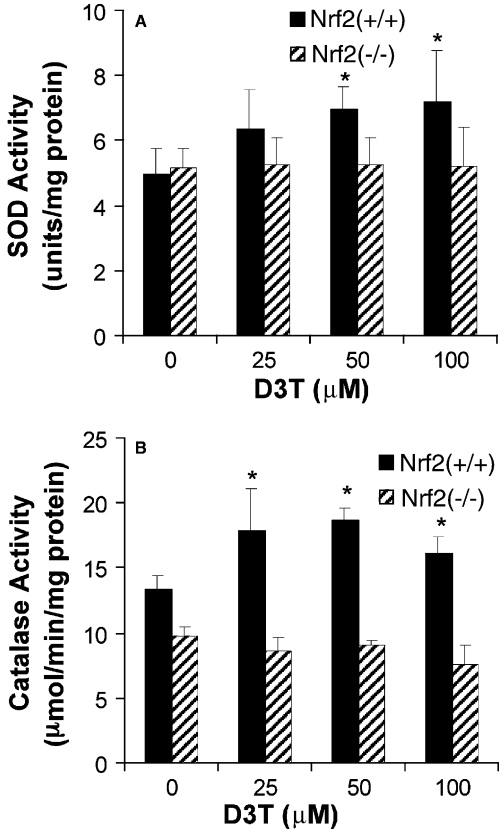

Fig. 1. Effects of D3T treatment on SOD (panel A) and catalase (panel B) activities in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts. Cells were incubated with the indicated concentrations of D3T for 48 h. Cellular SOD and catalase activities were measured as described in Section 2. Values represent means±S.E.M. from 4 to 6 independent experiments. *Significantly different from 0 μM D3T.

also caused a significant concentration-dependent elevation of cellular GR activity in $Nrf2^{+/+}$ fibroblasts; a maximum 70% increase in GR activity was seen with 100 μM D3T (Fig. 2B). In contrast, incubation of $Nrf2^{-/-}$ cells with 25–100 μM D3T did not lead to any significant increase in the level/activity of GSH and GR (Fig. 2A and B). Incubation of either $Nrf2^{+/+}$ or $Nrf2^{-/-}$ cardiac fibroblasts with 25–100 μM D3T resulted in a similar marked induction (70–150% increase) of GPx in a D3T concentration-dependent fashion (Fig. 2C).

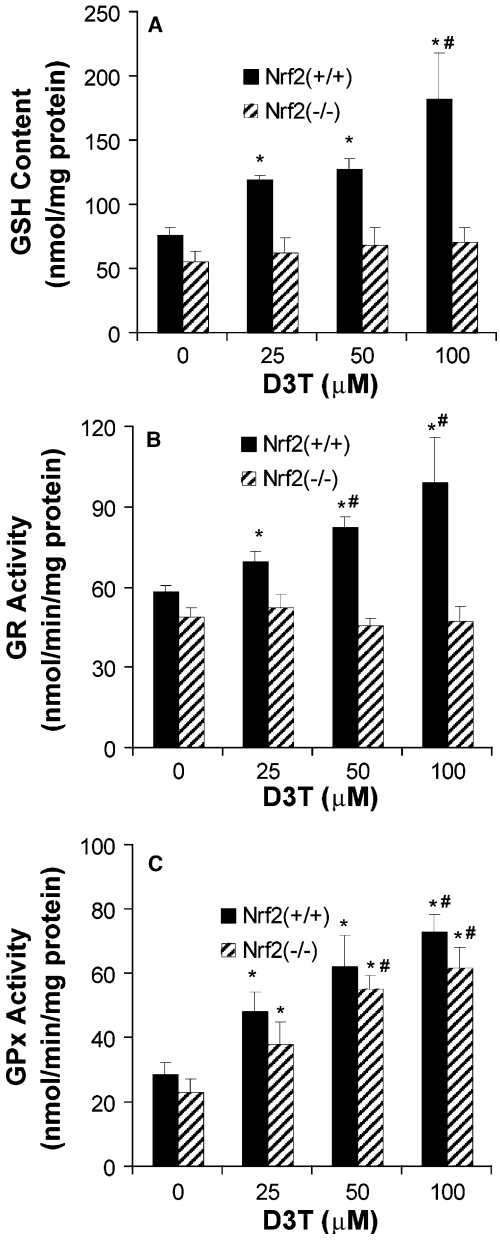

Fig. 2. Effects of D3T treatment on GSH content (panel A), and GR (panel B) and GPx (panel C) activities in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts. Cells were incubated with the indicated concentrations of D3T for 48 h. Cellular GSH content, and GR and GPx activities were measured as described in Section 2. Values represent means ± S.E.M. from 4 to 6 independent experiments. *Significantly different from 0 μM D3T; #significantly different from 25 and 50 μM D3T (panel A) or 25 μM D3T (panels B and C).

3.4. Inducibility of GST and NQO1 by D3T in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts

GST and NQO1 are two important phase 2 enzymes, involved in the detoxification of electrophilic chemicals, including carcinogens and cardiovascular toxicants [23–26]. Recently, these two enzymes have been demonstrated to protect against oxidative cell injury via acting as antioxidative enzymes [24–28]. As shown in Fig. 3A, incubation of $Nrf2^{+/+}$ cells with 25, 50, and 100 μM D3T for 48 h resulted in a significant 25%, 40%, and 70% increase in GST activity, respectively. Notably, a 5.5-, 8-, and 12-fold induction of NQO1 was observed in $Nrf2^{+/+}$ cells after incubation with 25, 50, and 100 μM D3T, respectively (Fig. 3B). In $Nrf2^{+/+}$ cardiac fibroblasts, NQO1 was the most inducible protein among the 7 D3T-inducible antioxidants and phase 2 enzymes examined (Figs. 1–3). In contrast to what were observed with $Nrf2^{+/+}$ cells, neither GST nor NQO1 in $Nrf2^{-/-}$ cells was induced by incubation with 25–100 μM D3T for 48 h (Fig. 3A and B). Because of the dramatic inducibility of NQO1 enzyme by D3T in an Nrf2-dependent manner, we also examined the induction of the mRNA for this phase 2 protein by D3T in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts. As shown in Fig. 4, incubation of $Nrf2^{+/+}$ cells with 100 μM D3T for 3–48 h led to a remarkable 3–20-fold induction of the NQO1

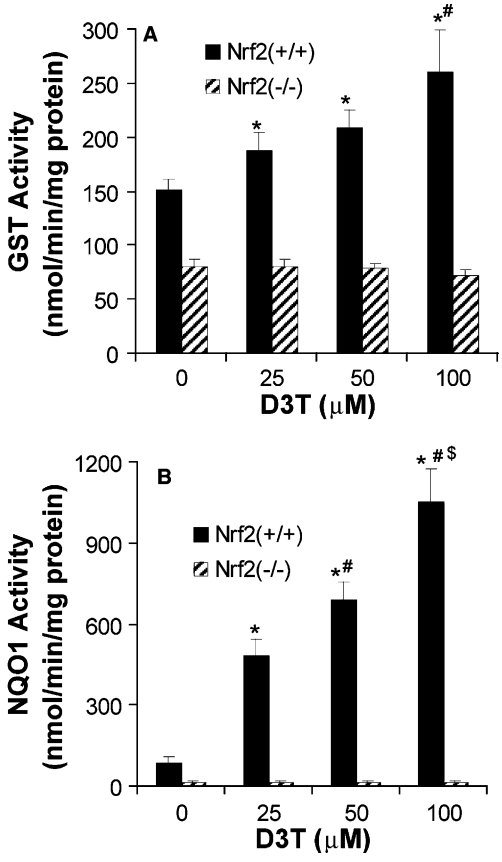

Fig. 3. Effects D3T treatment on GST (panel A) and NQO1 (panel B) activities in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts. Cells were incubated with the indicated concentrations of D3T for 48 h. Cellular GST and NQO1 activities were measured as described in Section 2. Values represent means ± S.E.M. from 4 to 6 independent experiments. *Significantly different from 0 μM D3T; #significantly different from 25 μM D3T; $significantly different from 50 μM D3T.

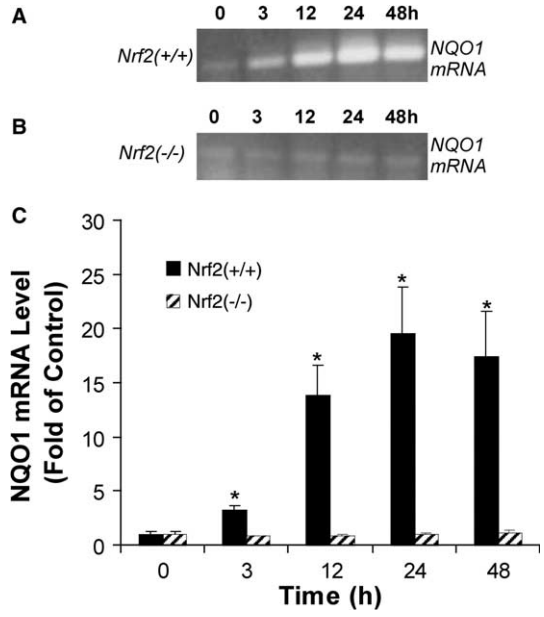

Fig. 4. Time-dependent induction of NQO1 mRNA expression by D3T in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts. Pictures in panels A and B are representative gels showing the mRNA expression of NQO1 at the indicated time points after treatment with 100 μM D3T in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts, respectively. Panel C, quantitative analysis of NQO1 mRNA expression at the indicated time points after treatment with 100 μM D3T. Values in panel C represent means ± S.E.M. from four independent experiments. *Significantly different from 0 h.

mRNA. Notably, a 17-fold increase in NQO1 mRNA level was still seen at 48 h after incubation of the $Nrf2^{+/+}$ cells with D3T, indicating that the upregulation of this phase 2 gene expression by D3T is long lasting. The D3T-mediated elevation of NQO1 mRNA was completely ablated in $Nrf2^{-/-}$ cells. In this experiment, 4 times more RNA from $Nrf2^{-/-}$ cells was used for the RT-PCR analysis due to the lower basal expression of NQO1 mRNA in $Nrf2^{-/-}$ cells as compared with wild type cells (data not shown).

3.5. XO/xanthine-induced cytotoxicity in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts and the protective effects of D3T pretreatment

Since both the basal expression and D3T-inducibility of the antioxidative and phase 2 defenses were diminished in cardiac fibroblasts derived from $Nrf2^{-/-}$ mice, we examined if these cells were more sensitive to oxidative injury than wild type cells. As shown in Fig. 5, $Nrf2^{-/-}$ cardiac fibroblasts exhibited a significantly increased sensitivity to XO/xanthine-induced cytotoxicity. The LC50 in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cells was estimated to be 7.8 and 4.5 mU/ml of XO, respectively. Pretreatment of $Nrf2^{+/+}$ cardiac fibroblasts with D3T led to a marked protection against XO/xanthine-induced cytotoxicity, as indicated by a marked increase of LC50 from 7.8 to 17.8 mU/ml of XO (Fig. 5). In contrast, D3T pretreatment of $Nrf2^{-/-}$ cardiac fibroblasts only afforded a slight protection on XO/xanthine-elicited cytotoxicity, as reflected by a slight increase of LC50 from 4.5 to 6.4 mU/ml of XO (Fig. 4). Since sensitivity to oxidative stress depends on the cell density, we examined if $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts proliferated differently in culture. We observed that the $Nrf2^{-/-}$ cells

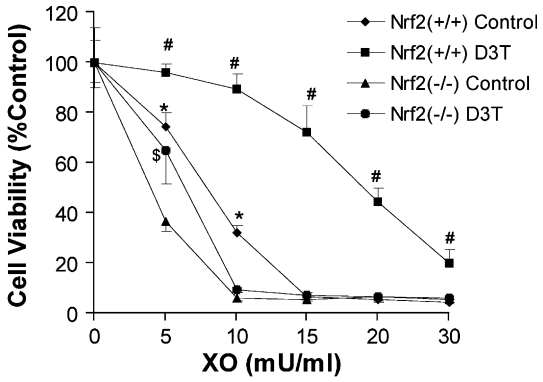

Fig. 5. XO/xanthine-induced cytotoxicity in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts and the protective effects of D3T pretreatment. Cells were incubated with or without 100 μM D3T for 48 h, followed by incubation with various concentrations of XO in the presence of 0.5 mM xanthine for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent means ± S.E.M. from three independent experiments. *Significantly different from Nrf2(−/−) control; #significantly different from Nrf2(+/+) control; $significantly different from Nrf2(−/−) control.

proliferated similarly as the wild type cells, and the cell density at the time of exposure to the oxidative insult did not differ between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cells (data not shown).

3.6. SIN-1-induced cytotoxicity in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac cells and the protective effects of D3T pretreatment

SIN-1 is commonly used to generate the reactive nitrogen species, peroxynitrite, which is a potent oxidant and electrophile [19,29–31]. As shown in Fig. 6, the $Nrf2^{-/-}$ cardiac fibroblasts showed a markedly increased susceptibility to SIN-1-induced cytotoxicity as compared with $Nrf2^{+/+}$ cells. The LC50 for SIN-1 in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cells was 335 and 168 μM, respectively. Pretreatment of $Nrf2^{+/+}$ cells with D3T resulted in a dramatically increased resistance to SIN-1-induced cytotoxicity, as indicated by a remarkable increase of LC50 from 335 to >800 μM. However, the cytotoxicity of SIN-1 in $Nrf2^{-/-}$ cells was only marginally protected by

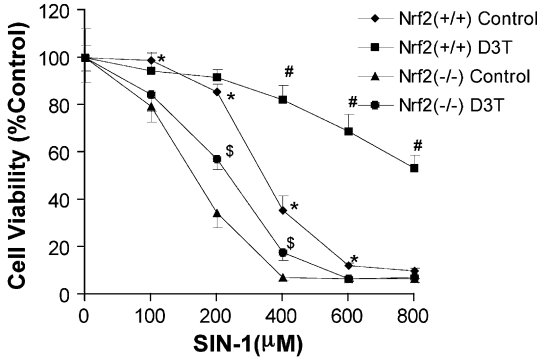

Fig. 6. SIN-1-induced cytotoxicity in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cardiac fibroblasts and the protective effects of D3T pretreatment. Cells were incubated with or without 100 μM D3T for 48 h, followed by incubation with the indicated concentrations of SIN-1 for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent means ± S.E.M. from three independent experiments. *Significantly different from Nrf2(−/−) control; #significantly different from Nrf2(+/+) control; $significantly different from Nrf2(−/−) control.

D3T pretreatment, as reflected by a slight increase of LC50 from 168 to 210 μM (Fig. 6).

4. Discussion

A number of recent studies have demonstrated that Nrf2 is a key transcription factor in regulating the expression of a variety of cytoprotective genes in various types of cells/tissues [5–7]. However, the role of this signaling mechanism in regulating the expression of antioxidants and phase 2 enzymes in cardiac cells has not been previously investigated. The results of this study clearly demonstrated that the basal levels of several key antioxidants and phase 2 enzymes, including catalase, GSH, GR, GST, and NQO1 were significantly lower in $Nrf2^{-/-}$ cardiac fibroblasts as compared with those in $Nrf2^{+/+}$ cells. Most notably, the basal expression of both GST and NQO1 was dramatically reduced in $Nrf2^{-/-}$ cells, which is in line with previous observations that the constitutive expression of these 2 genes are highly regulated by Nrf2 signaling [32,33]. In contrast, the basal levels of SOD and GPx did not differ between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cells, suggesting that Nrf2 signaling was not involved in the regulation of the basal expression of these two antioxidative enzymes in cardiac fibroblasts. The varying degree of decrease in the levels/activities of the antioxidants and phase 2 enzymes in $Nrf2^{-/-}$ fibroblasts suggested that the dependence of basal expression of the cellular defenses on Nrf2 signaling varied with different antioxidative and phase 2 genes.

Incubation of $Nrf2^{+/+}$ cells with the chemoprotective agent, D3T led to a significant induction of all the 7 antioxidants and phase 2 enzymes examined in this study (Figs. 1–3). Although the basal level of total SOD did not differ between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ fibroblasts, D3T significantly elevated the SOD activity in $Nrf2^{+/+}$ cells, whereas the D3T inducibility of SOD was completely diminished in $Nrf2^{-/-}$ cells. This result indicated that Nrf2 signaling played an essential role in the induction of SOD by D3T in cardiac fibroblasts. This observation is in agreement with previous reports that MnSOD gene expression in hepatic cells was induced by D3T in an Nrf2-dependent manner [32,33]. The complete abolishment of the D3T-mediated induction of catalase in $Nrf2^{-/-}$ cells (Fig. 1B) pointed to an essential role for Nrf2 in chemical induction of catalase in cardiac fibroblasts. The Nrf2-dependent induction of catalase by D3T in cultured cells or animals had not been previously reported in the literature.

Nrf2 was also reported to critically regulate the chemical induction of gene expression of GR and γ-glutamylcysteine ligase, a key enzyme in GSH biosynthesis [32,33]. In consistent with this notion, the induction of both GSH and GR by D3T was completely ablated in $Nrf2^{-/-}$ cells (Fig. 2A and B), confirming a critical role for Nrf2 signaling in the chemical inducibility of both GSH and GR in cardiac fibroblasts. Incubation of $Nrf2^{+/+}$ cardiac fibroblasts with D3T also led to a marked increase (70–150%) of cellular GPx activity (Fig. 2C). Previous studies showed that as compared with other antioxidative enzymes, GPx was the least inducible enzyme by D3T in a number of cell types, including cardiomyocytes [33,34, unpublished observations]. The mechanisms underlying the high D3T-inducibility of GPx in cardiac fibroblasts warrant further investigation. In contrast to other antioxidants, the inducibility of GPx by D3T was not altered in $Nrf2^{-/-}$ cardiac fibroblasts. This observation suggested that the D3T-mediated upregulation of GPx in cardiac fibroblasts occurred via Nrf2-independent mechanism(s).

GST and NQO1 have recently been extensively investigated regarding the regulatory role of Nrf2 signaling in their gene expression [5–7,32,33]. In both cell cultures and animal studies, these two enzymes were shown to be highly inducible by various chemoprotective agents, including D3T [32–34]. Consistently, we observed that incubation of $Nrf2^{+/+}$ cardiac fibroblasts with D3T led to a marked induction of these two enzymes in a concentration-dependent fashion (Fig. 3). The ability of D3T to induce both GST and NQO1 was completely abolished in $Nrf2^{-/-}$ cells, pointing to an indispensable role for Nrf2 signaling in the chemical induction of these two enzymes in cardiac fibroblasts. The remarkable 5.5–12-fold induction of NQO1 activity seen in $Nrf2^{+/+}$ cardiac fibroblasts after incubation with 25–100 μM D3T (Fig. 3B) was unexpected. This high degree induction of NQO1 activity by D3T was not observed in any previous studies with other types of cells, including cardiomyocytes, where usually a 2-3-fold induction of this enzyme was seen after incubation of the cells with 25–100 μM D3T for 24–48 h [34–36]. In agreement with the increased NQO1 activity induced by D3T, the mRNA level of NOQ1 in $Nrf2^{+/+}$ cardiac fibroblasts was also dramatically elevated by D3T treatment, whereas such inducibility of the NQO1 mRNA by D3T was completely abolished in $Nrf2^{-/-}$ cells (Fig. 4). The high inducibility of NQO1 gene expression by D3T via Nrf2 signaling in cardiac fibroblasts may have important implications in view of the recent observation that NQO1 is capable of scavenging superoxide [28].

The profile of suppressed induction of endogenous antioxidants and phase 2 enzymes in $Nrf2^{-/-}$ cardiac fibroblasts was not specific to D3T. In data not shown, the induction of antioxidants and phase 2 enzymes by resveratrol and α-lipoic acid was also diminished in the $Nrf2^{-/-}$ cardiac fibroblasts. These results suggested that Nrf2 signaling might be a common molecular mechanism governing the inducibility of antioxidants and phase 2 enzymes by various chemoprotective agents in cardiac cells.

Since $Nrf2^{-/-}$ cardiac fibroblasts exhibited reduced basal levels as well as D3T-inducibility of antioxidants and phase 2 enzymes, we determined if these cells were more susceptible to reactive oxygen and nitrogen species-induced injury. In this regard, cytotoxicity induced by XO/xanthine and SIN-1 was examined. XO in the presence of xanthine is able to generate both superoxide and H_2O_2, and has been implicated in various forms of oxidative cardiac injury [37,38]. SIN-1 undergoes autooxidation at physiological pH to generate both superoxide and nitric oxide, leading to the formation of peroxynitrite, a potent oxidant and electrophile [39]. As such, SIN-1 is commonly used as a peroxynitrite generator for studying the pathophysiological effects of peroxynitrite in various biological systems [19,29–31]. As expected, the cytotoxicity induced by XO/xanthine or SIN-1 was significantly augmented in $Nrf2^{-/-}$ cardiac fibroblasts as compared with wild type cells (Figs. 5 and 6). The marked induction of cellular antioxidants and phase 2 enzymes by D3T in $Nrf2^{+/+}$ fibroblasts was also accompanied by a dramatically increased resistance of these cells to the cytotoxicity elicited by XO/xanthine or SIN-1. The increased resistance of the D3T-pretreated $Nrf2^{+/+}$ cardiac fibroblasts to XO/xanthine- or SIN-1-induced cytotoxicity was apparently due to the augmented cellular defenses in those

D3T-pretreated cells. The cytoprotective effects of D3T pretreatment on XO/xanthine- or SIN-1-induced toxicity were largely ablated in the Nrf2$^{-/-}$ cardiac fibroblasts, where the induction by D3T of all the antioxidants and phase 2 enzymes examined in this study except for GPx was completely abolished. The above results strongly indicated that disruption of the Nrf2 signaling sensitized cardiac fibroblasts to reactive oxygen and nitrogen species-induced toxicity, and diminished the cytoprotective effects of D3T on cytotoxicity elicited by the above insults. Although, the above results suggested that the diminished induction of antioxidants and phase 2 enzymes by D3T in Nrf2$^{-/-}$ cardiac fibroblasts most likely accounted for the increased sensitivity of these cells to reactive oxygen and nitrogen species-induced cytotoxicity, the possible involvement of other Nrf2-regulated cell surviving factor(s) could not be excluded.

In conclusion, the results of this study demonstrate conclusively that Nrf2 is critically involved in the regulation of both basal expression and chemical induction of a number of endogenous antioxidants and phase 2 enzymes in cardiac fibroblasts. Nrf2 signaling appears to be an important mechanism in controlling cardiac fibroblast susceptibility to reactive oxygen and nitrogen species-induced cytotoxicty. Since cardiac fibroblasts represent an important cell population in cardiac tissue, and play critical roles in cardiac pathophysiology [8–11], Nrf2-mediated regulation of antioxidants and phase 2 enzymes in these cells is of importance for protection against oxidative cardiac disorders.

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