Antioxidants and Phase 2 Enzymes in Cardiomyocytes: Chemical Inducibility and Chemoprotection Against Oxidant and Simulated Ischemia-Reperfusion Injury

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The increasing recognition of the role for oxidative stress in cardiac disorders has led to extensive investigation on the protection by exogenous antioxidants against oxidative cardiac injury. On the other hand, another strategy for protecting against oxidative cardiac injury may be through upregulation of the endogenous antioxidants and phase 2 enzymes in the myocardium by chemical inducers. However, our current understanding of the chemical inducibility of cardiac cellular antioxidants and phase 2 enzymes is very limited. In this study, using rat cardiac H9c2 cells we have characterized the concentration- and time-dependent induction of cellular antioxidants and phase 2 enzymes by 3*H*-1,2-dithiole-3-thione (D3T), and the resultant chemoprotective effects on oxidative cardiac cell injury. Incubation of H9c2 cells with D3T resulted in a marked concentration- and time-dependent induction of a number of cellular antioxidants and phase 2 enzymes, including catalase, reduced glutathione (GSH), GSH peroxidase, glutathione reductase (GR), GSH *S*-transferase (GST), and NAD(P)H:quinone oxidoreductase-1 (NQO1). D3T treatment of H9c2 cells also caused an increase in mRNA expression of catalase, γ-glutamylcysteine ligase catalytic subunit, GR, GSTA1, M1 and P1, and NQO1. Moreover, both mRNA and protein expression of Nrf2 were induced in D3T-treated cells. D3T pretreatment led to a marked protection against H9c2 cell injury elicited by various oxidants and simulated ischemia-reperfusion. D3T pretreatment also resulted in decreased intracellular accumulation of reactive oxygen in H9c2 cells after exposure to the oxidants as well as simulated ischemia-reperfusion. This study demonstrates that a series of endogenous antioxidants and phase 2 enzymes in H9c2 cells can be induced by D3T in a concentration- and time-dependent fashion, and that the D3T-upregulated cellular defenses are accompanied by a markedly increased resistance to oxidative cardiac cell injury. Exp Biol Med 231:1353–1364, 2006

Key words: cardiac cells; 3*H*-1,2-dithiole-3-thione; antioxidants; oxidants; simulated ischemia-reperfusion; cytotoxicity

Introduction

Cardiovascular disease remains a leading cause of death worldwide. More than 60 million Americans have cardiovascular disease, which accounts for approximately 40% of all deaths in the United States (1). Substantial evidence points to a critical role for oxidative stress in the development of various forms of cardiovascular disorders, including myocardial ischemia-reperfusion injury, congestive heart failure, coronary arterial atherosclerosis, and chemical-induced cardiotoxicity (2–6). In this context, administration of some exogenous antioxidative compounds has been shown to exert protection against oxidative cardiovascular disorders in animal models (6–9). However, use of exogenous antioxidative compounds in protecting against cardiovascular injury also has produced conflicting results in both animal and human clinical trials, pointing to the limitations associated with the use of exogenous antioxidants (6, 8, 10, 11). In fact, the use of exogenous antioxidants in protecting against oxidative cardiovascular injury suffers several potential drawbacks, including limited cell permeability and bioavailability, as well as untoward effects of these compounds, which may contribute to the inconsistence in their protection against cardiovascular injury (6, 8, 10, 11). In this regard, another promising

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strategy for protecting against oxidative cardiac injury may be through the upregulation of endogenous antioxidants and phase 2 enzymes in cardiac cells by chemical inducers.

While induction of antioxidants and phase 2 proteins by chemoprotective agents, including 3*H*-1,2-dithiole-3-thione (D3T), has been demonstrated to be an effective approach to protecting against carcinogenesis in animal models and/or human clinical trials (12, 13), whether the cardiac protection also can be achieved through chemical induction of the endogenous antioxidants and phase 2 enzymes in myocardium has not been carefully investigated. In this study, we have characterized both the induction of a series of endogenous antioxidants and phase 2 enzymes and their gene expression by D3T in rat H9c2 cardiomyocytes, a cell line widely used for studying cardiac cell biology and pathophysiology (14–16). We also have investigated the protective effects of the D3T-induced cellular defenses on cardiac cell injury elicited by various oxidants and simulated ischemia-reperfusion. Our results demonstrate that incubation of H9c2 cells with low micromolar D3T results in significant increases in the levels of a number of key cellular antioxidants and phase 2 enzymes, as well as their gene expression. Furthermore, upregulation of the above cardiac cellular defenses by D3T is accompanied by a markedly increased resistance to oxidative cell injury.

Materials and Methods

Chemicals and Materials. 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, and fetal bovine serum (FBS) were from Gibco-Invitrogen (Carlsbad, CA). Authentic peroxynitrite was from Calbiochem (San Diego, CA). All other chemicals and reagents were from Sigma Chemical (St. Louis, MO).

Cell Culture and Treatment. Rat cardiac H9c2 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete culture medium) in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were fed every 2 to 3 days and were subcultured once they reached 70% to 80% confluence. For experiments on induction of antioxidants and phase 2 enzymes, H9c2 cells were incubated with D3T (25–100 μ*M*) for various times in complete culture medium. For cytoprotection studies H9c2 cells were pretreated with D3T for 24 hrs. The D3T-containing medium then was removed, and the cells were washed twice with fresh medium. The cells were then exposed to various oxidative insults, followed by determination of changes in cell viability.

Preparation of Cell Extract. Cardiac H9c2 cells were collected and resuspended in ice-cold 50-m*M* potassium phosphate buffer (pH 7.4) containing 2 m*M* EDTA. The cells were sonicated, followed by centrifugation at 13,000 *g* for 10 mins 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 to 3 hrs, as described below.

Measurement of Cellular Superoxide Dismutase Activity. Total cellular superoxide dismutase (SOD) activity was determined by the method of Spitz and Oberley (17) with slight modifications, as described previously (18). The sample total SOD activity was calculated using a concurrently run SOD (Sigma Chemical) standard curve and was expressed as units per milligram of cellular protein.

Measurement of Cellular Catalase Activity. The method of Aebi (19) was used to measure cellular catalase activity, which was expressed as micromoles of H_2O_2 consumed per minute per milligram of cellular protein.

Measurement of Cellular Reduced Glutathione (GSH) Content. Cellular GSH content was measured according to the fluorometric assay described previously (20). Cellular GSH content was calculated using a concurrently run GSH (Sigma Chemical) standard curve and was expressed as nanomoles of GSH per milligram of cellular protein.

Measurement of Cellular Glutathione Reductase Activity. Cellular glutathione reductase (GR) activity was measured according to the method of Wheeler et al. (21) with modifications, as described previously (18). GR activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1} and was expressed as nanomoles of NADPH consumed per minute per milligram of cellular protein.

Measurement of Cellular GSH Peroxidase Activity. Cellular glutathione peroxidase (GPx) activity was measured by the method of Flohe and Gunzler (22) with slight modifications, as described previously (18). GPx activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1} and was expressed as nanomoles of NADPH consumed per minute per milligram of cellular protein.

Measurement of Cellular Glutathione *S*-transferase (GST) Activity. 1-Chloro-2,3-dinitrobenzene (CDNB) was used as the substrate for measuring GST activity. GST activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1} and was expressed as nanomoles of CDNB/GSH conjugate formed per minute per milligram of cellular protein (20).

Measurement of Cellular NAD(P)H:quinone Oxidoreductase 1 Activity. Cellular NAD(P)H:quinone oxidoreductase 1 (NQO1) activity was determined using dichloroindophenol (DCIP) as the 2-electron acceptor (23). In brief, the reaction mix contained 50 m*M* Tris-HCl (pH 7.5), 0.08% Triton X-100, 0.25 m*M* NADPH, and 80 μ*M* DCIP in the presence or absence of 60 μ*M* dicumarol. A total of 0.695 ml reaction mix was added to an assay

Table 1. Oligonucleotide Sequences for RT-PCR Analysis of Gene Expression of Antioxidants and Phase 2 Enzymes

Enzymes	Primer sequence
Catalase	
Sense	GACATGGTCTGGGACTTCTG
Antisense	GTAGGGACAGTTCACAGGTA
γGCL	
Sense	CCTTCTGGCACAGCACGTTG
Antisense	TAAGACGGCATCTCGCTCCT
GR	
Sense	TGCCTGCTCTGGGCCATT
Antisense	CTCCTCTGAAGAGGTAGGAT
GST-A1	
Sense	GAAGCCAGTCCTTCACTACT
Antisense	CAGCTCTTCCACATGGTAGA
GST-M1	
Sense	CTGCTCCTGGAATACACAGA
Antisense	CAGGAAGTCCTTCAGGTTTG
GST-P1	
Sense	CCATTGTGTACTTCCCAGTTC
Antisense	GAGGACAGAAAGGCCTTGAT
NQO1	
Sense	CCATTCTGAAAGGCTGGTTTG
Antisense	CTAGCTTTGATCTGGTTGTC
Nrf2	
Sense	CAGCAGGACATGGATTTGAT
Antisense	CCTGCCAAACTTGCTCCAT

cuvette. The reaction was started by adding 5 μl of sample, and reduction of DCIP was monitored at 600 nm, 25°C for 3 mins. The dicumarol-inhibitable NQO1 activity was calculated using the extinction coefficient of 21.0 mM^{-1} cm^{-1} and was expressed as nanomoles of DCIP reduced per minute per milligram of cellular protein.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis of mRNA Expression. Total RNA from cardiac cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. 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 instructions. The cycling conditions for RT-PCR were: 50°C for 30 mins (reverse transcription) and 94°C for 2 mins (pre-denaturation); followed by 25 cycles of PCR amplification process, including denaturing at 94°C for 15 secs, annealing at 57°C for 30 secs, and extension at 72°C for 45 secs; and then one cycle of final extension at 72°C for 10 mins. The sequences of the PCR primers were shown in Table 1. PCR products were separated by 1% agarose gel electrophoresis. Gels were stained with 0.5-μg/ml solution of ethidium bromide for 30 mins, followed by another 30-min destaining in water. The gels were then analyzed under ultraviolet light using an Alpha Innotech Imaging system (San Leandro, CA). In this study a standard curve for each of the antioxidant mRNAs using various amounts of total RNA was included in each assay so as to reliably estimate changes in mRNA levels, as described previously (24).

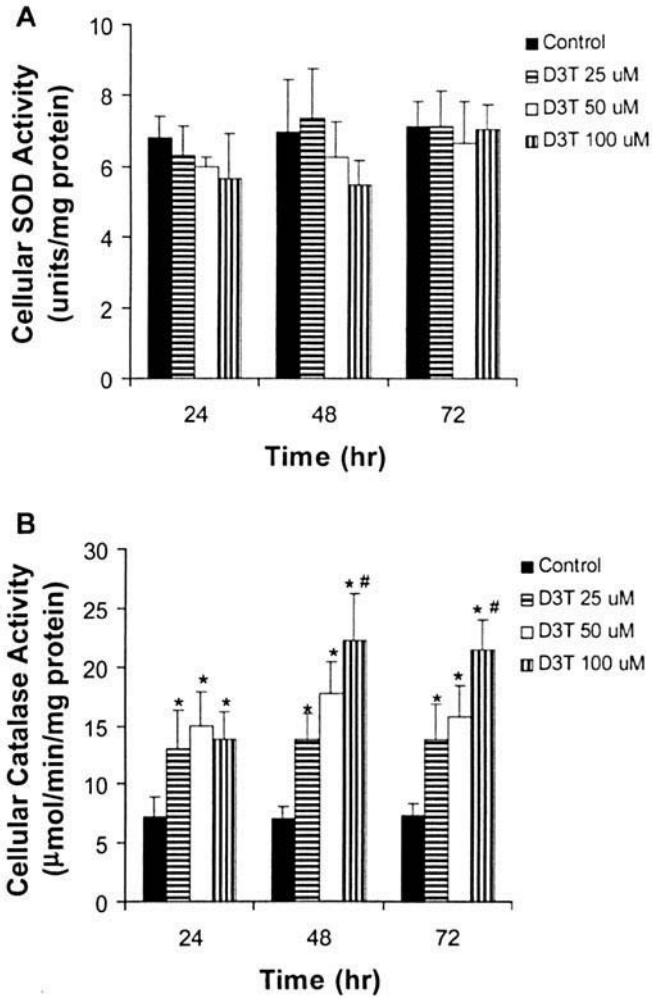

Figure 1. Effects of D3T treatment on SOD (A) and catalase (B) activities in H9c2 cells. Cells were incubated with the indicated concentrations of D3T for 24 to 72 hrs. Cellular SOD and catalase activities were measured as described in Materials and Methods. Values represent means ± SEM from 3 to 5 independent experiments. * Significantly different from control. # Significantly different from 24 hrs.

Immunoblot Analysis. The procedures described previously (24) were followed to detect Nrf2 protein expression by immunoblot analysis. Briefly, cells were lysed by sonication, followed by centrifugation to yield the supernatant samples. Equal amounts of protein from each of the samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred electrophoretically to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% nonfat dried milk in Tris-Tween buffered saline at room temperature for 1.5 hrs. The membrane was then incubated with primary polyclonal anti-Nrf2 antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C, followed by incubation with horseradish peroxidase–labeled secondary antibody (Santa Cruz Bio-

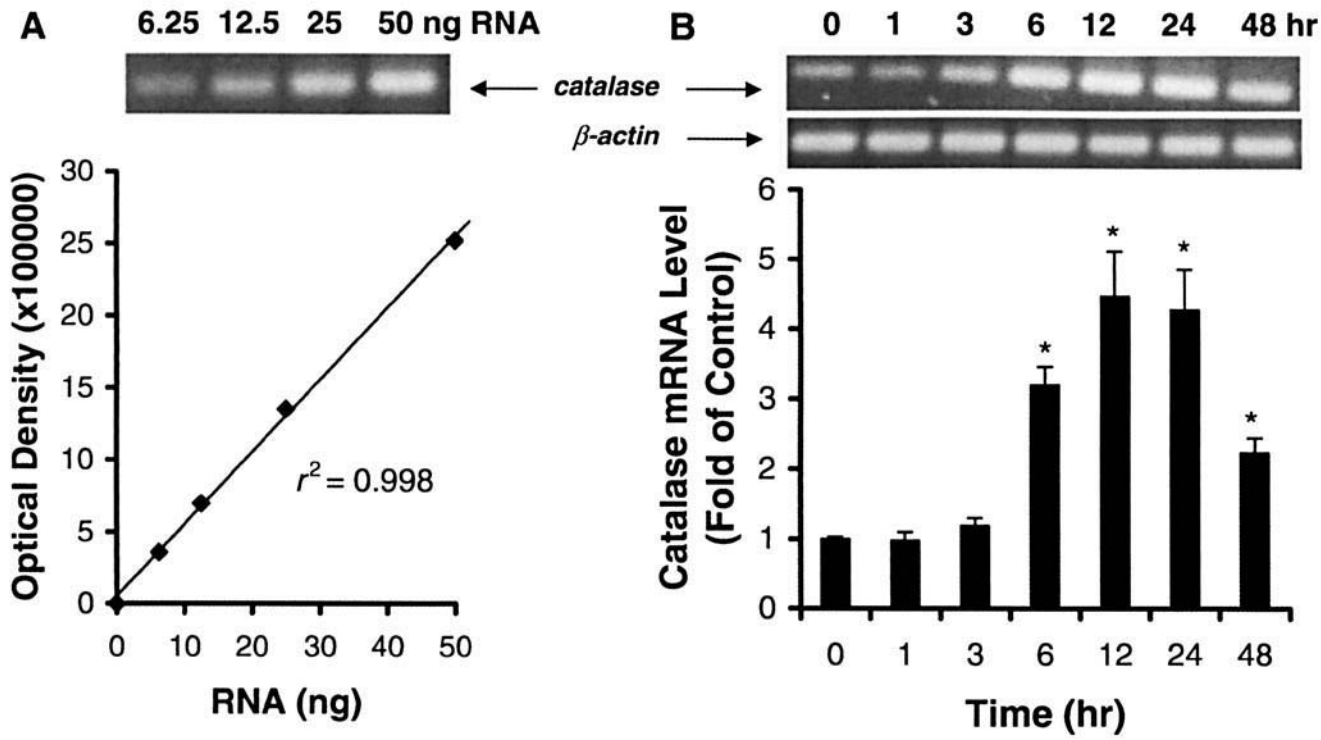

Figure 2. Standard curve for quantification of catalase mRNA levels (A) and time-dependent induction of catalase mRNA expression by D3T (B) in H9c2 cells. In panel A, representative gel picture and line graph show the linear amplification of the PCR product derived from indicated amounts of total RNA. In panel B, top panels display representative gel pictures showing the mRNA expression of catalase and β-actin at the indicated times after treatment of H9c2 cells with 100 μ*M* D3T; bottom panel shows quantitative analysis of catalase mRNA expression. Values in panel B represent means ± SEM from 4 independent experiments. * Significantly different from 0 hrs.

tech) at room temperature for another 1.5 hrs. The membrane was visualized using an enhanced chemiluminescence system (Amersham Biosciences), and blots were quantified by the Alpha Innotech Imaging system.

Assay for Cell Viability. Cell viability was determined by a slightly modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) reduction assay, as described by Cao et al. (20).

Detection of Intracellular Reactive Oxygen Species (ROS). 2′,7′-Dichlorodihydrofluorescein (DCF) assay was used to detect intracellular ROS levels in cardiac cells according to the procedures described previously by Cao et al. (18).

Simulated Ischemia-Reperfusion. The simulated ischemia-reperfusion protocol described previously (25) was followed. Briefly, ischemia was simulated by replacing the culture medium with serum-free DMEM without glucose. The culture plates were then incubated at 37°C for 10 hrs in an anaerobic jar containing a Pack-Anaero oxygen-absorbing and CO_2-generating paper sachet (AnaeroPack System; Mitsubishi Gas Chemical America Inc., New York, NY). Once the paper sachet was placed in the jar oxygen level was reduced to <1% in 30 mins and CO_2 level was maintained at approximately 18%. For simulated reperfusion the culture plates were removed from the anaerobic jar after 10 hrs and incubated with original culture medium (DMEM supplemented with 10% FBS) under normoxic conditions at 37°C for another 16 hrs, followed by detection of cell viability by MTT reduction assay. To determine the ROS formation 10 μ*M* DCF was added to the cell cultures immediately upon simulated reperfusion (return to normoxic conditions). The cells were incubated with DCF for 1 hr under the normoxic conditions, followed by measurement of DCF fluorescence as described previously in this paper.

Statistical Analyses. All data are expressed as means ± SEM from at least 3 separate experiments. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. Differences between 2 groups were analyzed by Student's *t* test. Statistical significance was considered at $P < 0.05$.

Results

Effects of D3T Treatment on Cellular SOD and Catalase. SOD and catalase are two key enzymes in detoxifying intracellular $O_2^{\cdot-}$ and H_2O_2. As shown Figure 1A, treatment of H9c2 cells with 25 to 100 μ*M* D3T for 24 to 72 hrs did not result in any significant changes in SOD activity. In contrast, the same D3T treatment caused a marked increase in cellular catalase activity in a concentration- and time-dependent manner (Fig. 1B). Overall, a 2- to 3-fold induction in the catalase activity was observed in D3T-treated cells. Incubation of H9c2 cells with 100 μ*M* D3T also led to a significant increase in the level of catalase mRNA (Fig. 2). A

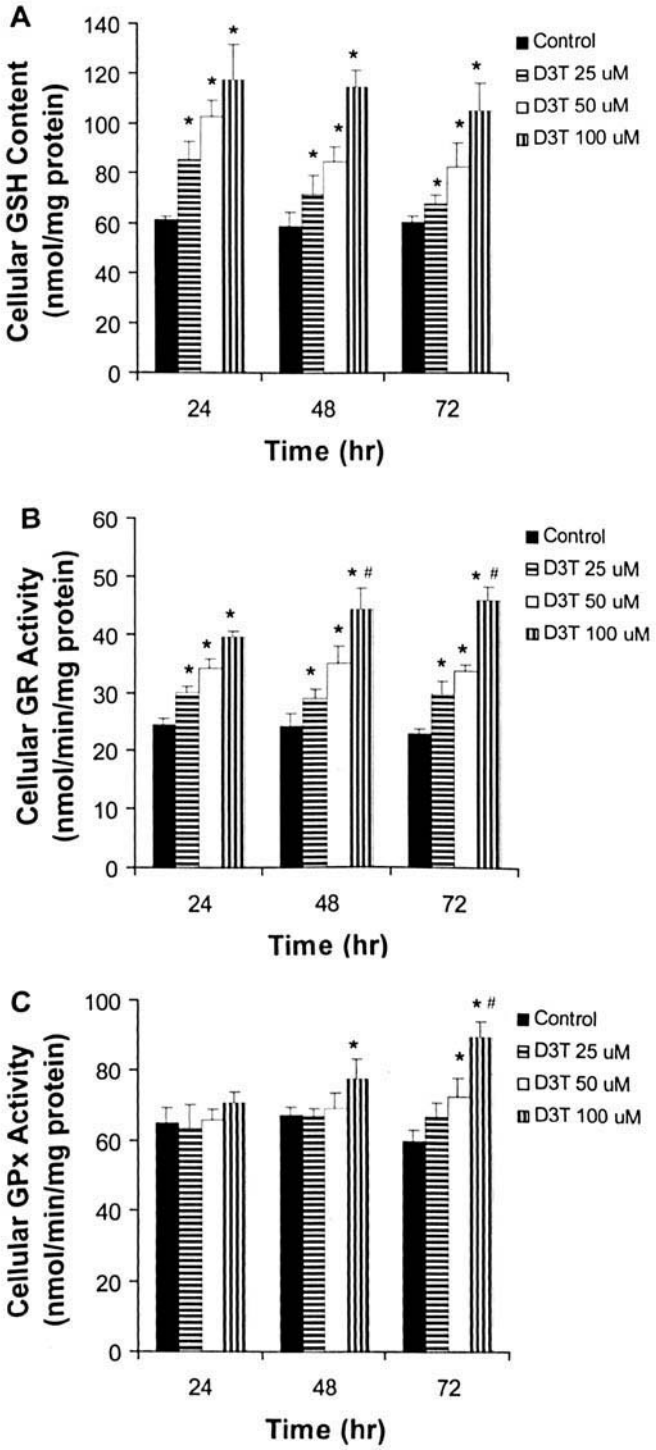

Fig 3. Effects of D3T treatment on GSH content (A) and GR (B) and GPx (C) activities in H9c2 cells. Cells were incubated with the indicated concentrations of D3T for 24 to 72 hrs. Cellular GSH content, and GR and GPx activities were measured as described in Materials and Methods. Values represent means ± SEM from 3 to 5 independent experiments. * Significantly different from control; # significantly different from 24 hrs (B) or 48 hrs (C).

3.3- to 4.5-fold increase in catalase mRNA level was observed at 6 to 24 hrs after incubation with D3T. A greater than 2-fold elevation of catalase mRNA was still seen at 48 hrs after incubation of H9c2 cells with D3T (Fig. 2).

Effects of D3T Treatment on Cellular GSH, GR, and GPx. GSH and GSH-linked antioxidants, including GR and GPx, are critically involved in the detoxification of ROS, and it has been suggested that they are protective against various forms of oxidative cardiovascular injury (8, 26, 27). Therefore, we investigated the inducibility of these antioxidants by D3T in H9c2. As shown in Figure 3A, incubation of H9c2 with 25 to 100 μM D3T resulted in a significant elevation of cellular GSH content at all of the three time points (24, 48, and 72 hrs) in a D3T concentration–dependent manner. A 2-fold induction of cellular GSH was observed with 100 μM D3T at 24 and 48 hrs. Figure 3B showed that incubation of H9c2 cells with 25 to 100 μM D3T for 24 to 72 hrs led to a concentration- and time-dependent increase in GR activity. A concentration of 100 μM D3T caused a 2-fold induction of GR at 48 and 72 hrs. In contrast to the induction of GSH and GR, incubation of H9c2 cells with 25 to 100 μM D3T for 24 hrs did not result in any significant increase in cellular GPx activity (Fig. 3C). Cellular GPx activity increases of 20% and 50% were seen with 100 μM D3T at 48 and 72 hrs, respectively. At 72 hrs, 50 μM D3T also was shown to elevate cellular GPx activity by 20%. Since both GSH and GR activities were markedly increased in D3T-treated cells, we examined the gene expression for γ-glutamylcysteine ligase (γGCL)–catalytic subunit and GR. As shown in Figure 4A, incubation of H9c2 cells with 100 μM D3T for 1 to 48 hrs led to a significant 2- to 3-fold induction of the mRNA for γGCL-catalytic subunit. Similarly, D3T treatment also caused a significant induction of GR mRNA. A 25% increase in GR mRNA was seen at 1 hr, and a 2.5- to 3.5-fold induction of GR mRNA was observed at 3 to 48 hrs after incubation of cardiac cells with D3T (Fig. 4B).

Effects of D3T Treatment on Cellular GST and NQO1. GST and NQO1 are phase 2 enzymes that are critically involved in the detoxification of xenobiotics as well as ROS (28–33). Incubation of H9c2 cells with D3T led to marked increases in cellular GST and NQO1 activities (Fig. 5). The induction of GST and NQO1 by D3T exhibited a concentration- and time-dependent manner. Significant increases in both GST and NQO1 activities were observed with all of the 3 concentrations of D3T at 24, 48, and 72 hrs. Notably, incubation of H9c2 cells with 100 μM D3T for 48 to 72 hrs resulted in a remarkable 3- and 5-fold induction of cellular GST and NQO1, respectively (Fig. 5). Figure 6 showed the changes of the mRNA levels for GSTA1, M1, P1, and NQO1 after D3T treatment. A 2-fold induction of GSTA1 mRNA was seen at 6 hrs, and incubation of H9c2 cells with D3T for 12 to 48 hrs led to a 6- to 9-fold induction of GSTA1 mRNA (Fig. 6A). Surprisingly, as shown in Figure 6B, a 40% decrease in GSTM1 mRNA was observed at 1 and 3 hrs after D3T treatment. A greater than 3-fold induction of GSTM1 mRNA was seen at 12 and 24 hrs. At 48 hrs, the GSTM1 mRNA level was still 30% higher than the basal level. In contrast to GSTM1, significant increases in GSTP1 mRNA levels were observed at 3 and 6 hrs; inductions of 4- and 2-

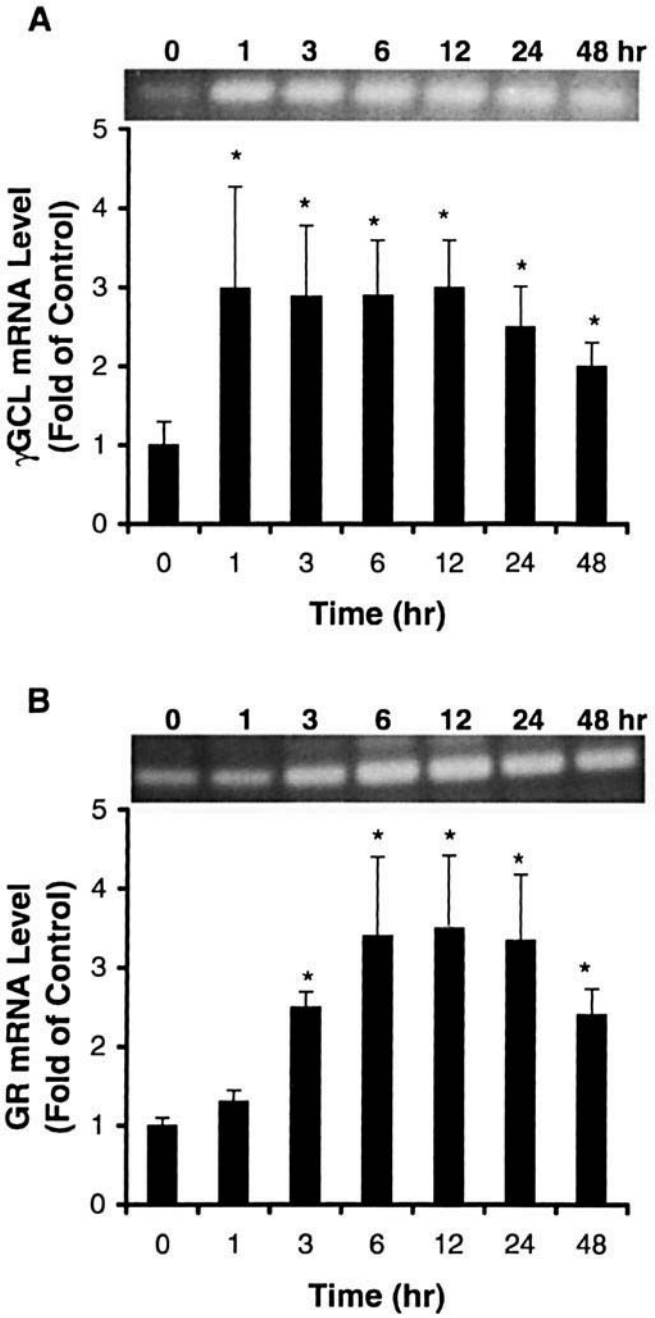

Figure 4. Time course of induction of mRNA expression of γGCL catalytic subunit (A) and GR (B) by D3T in H9c2 cells. In panels A and B, the top panel shows a representative gel picture of the mRNA expression at the indicated times after treatment of H9c2 cells with 100 μ*M* D3T; the bottom panel shows a quantitative analysis of mRNA expression. Values represent means ± SEM from 3 to 4 independent experiments. * Significantly different from 0 hrs.

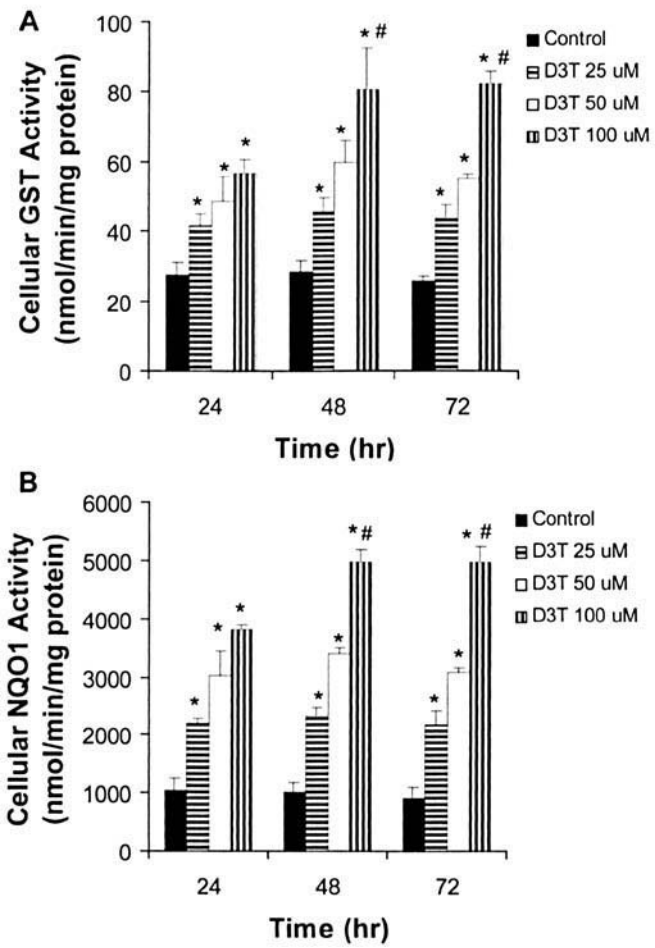

Figure 5. Effects D3T treatment on GST (A) and NQO1 (B) activities in H9c2 cells. Cells were incubated with the indicated concentrations of D3T for 24 to 72 hrs. Cellular GST and NQO1 activities were measured as described in Materials and Methods. Values represent means ± SEM from 3 to 4 independent experiments. * Significantly different from control. #Significantly different from 24 hrs.

fold were seen at 12/24 and 48 hrs, respectively (Fig. 6C). As shown in Figure 6D, the mRNA level of NQO1 began to increase significantly at 1 hr and was elevated by 7- to 8-fold at 12 and 24 hrs. A greater than 3-fold induction of NQO1 mRNA was still observed at 48 hrs after D3T treatment.

Effects of D3T Treatment on Cellular Nrf2 mRNA and Protein Expression. As shown in Figure 7, incubation of H9c2 cells with 100 μ*M* D3T led to a remarkable elevation of both mRNA and protein expression of Nrf2, a central regulator of antioxidant and phase 2 genes (34). An overall 2- to 3-fold induction of Nrf2 mRNA expression was observed from 1 to 48 hrs after D3T treatment of H9c2 cells (Fig. 7A). Similarly, a 2- to 3.5-fold elevation of Nrf2 protein expression occurred from 3 to 72 hrs after D3T treatment (Fig. 7B).

Inhibitory Effects of D3T Pretreatment on Oxidant-Induced Cell Injury. To examine whether the D3T-elevated cellular defenses could lead to chemoprotection against oxidant injury, H9c2 cells were pretreated with D3T and then exposed to xanthine oxidase (XO)/xanthine, H_2O_2, 3-morpholinosydnonimine (SIN-1, a peroxynitrite generator), or authentic peroxynitrite. As shown in Figures 8–10, incubation of H9c2 cells with various concentrations of the above oxidants for 24 hrs led to significant decreases in cell viability as detected by MTT reduction assay. The decreases in cell viability were dependent on the concen-

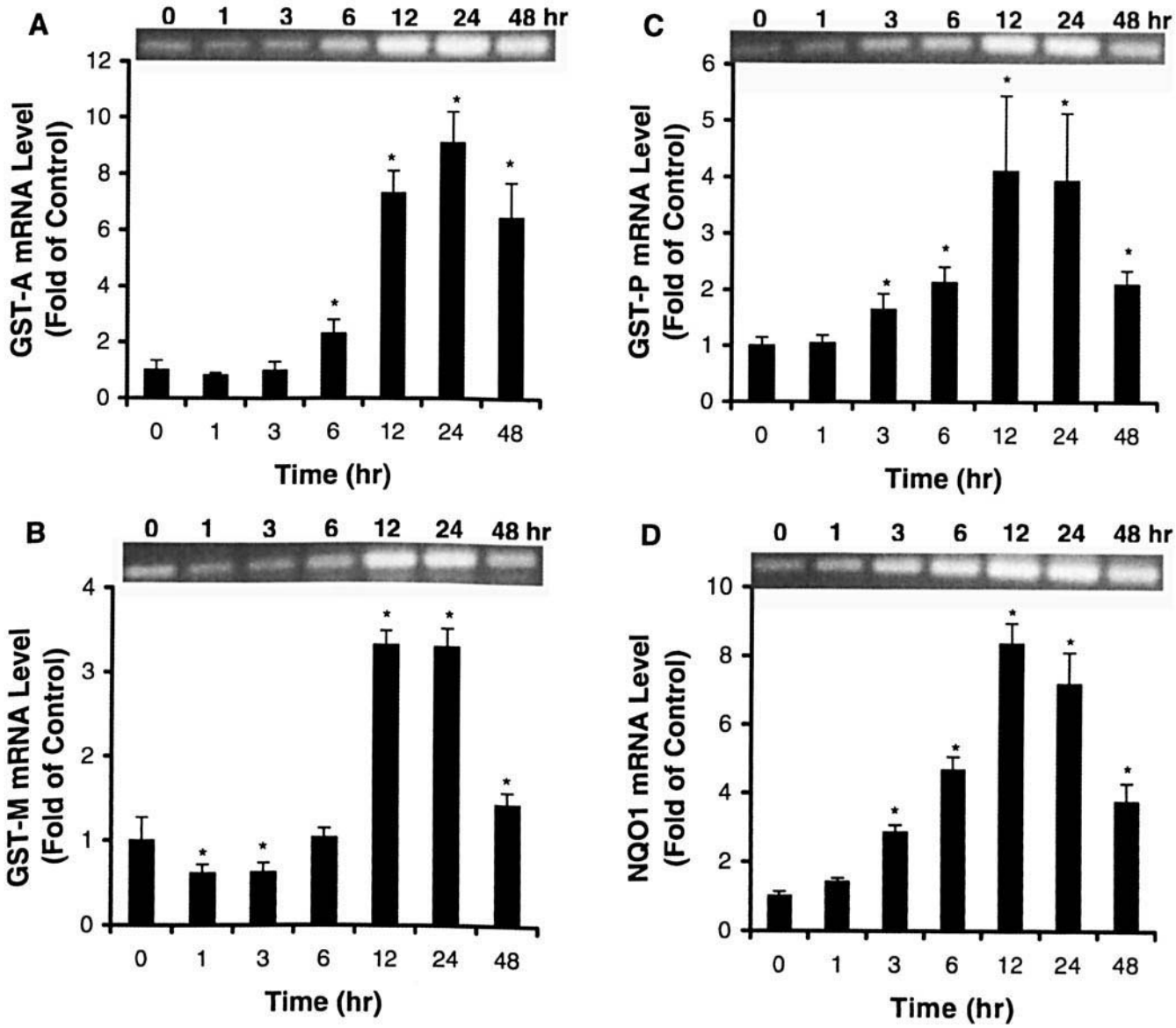

Figure 6. Time course of induction of mRNA expression of GSTA1 (A), GSTM1 (B), GSTP1 (C), and NQO1 (D) by D3T in H9c2 cells. In panels A, B, C, and D, the top panel shows a representative gel picture of the mRNA expression at the indicated times after treatment of H9c2 cells with 100 μ*M* D3T; the bottom panel shows quantitative analysis of mRNA expression. Values represent means ± SEM from 3 to 5 independent experiments. * Significantly different from 0 hr.

trations of the oxidants. Pretreatment of H9c2 cells with D3T for 24 hrs afforded a marked protection against the above oxidant-induced cytotoxicity. The cytoprotection resulting from D3T pretreatment exhibited a D3T concentration–dependent manner; the protection was most dramatic with 100 μ*M* D3T, followed by 50 and 25 μ*M* D3T (Figs. 8 and 9). The effects of D3T pretreatment on XO/xanthine- or H_2O_2-mediated intracellular ROS accumulation were also determined by DCF assay. As shown in Figures 8B and 9B, incubation of control cells with 10 mU/ml XO plus 0.5 m*M* xanthine, or 75 μ*M* H_2O2 for 30 mins resulted in significantly increased intracellular levels of ROS as indicated by ~2.2- and 8-fold increases in DCF-derived fluorescence, respectively. Compared with control cells, the XO/xanthine- or H_2O_2-induced intracellular accumulation of ROS was markedly reduced in the D3T-pretreated cells. Notably, the basal level of intracellular ROS also was much lower in D3T-pretreated cells than in control cells, which is in line with the higher levels of antioxidants and phase 2 enzymes in the D3T-pretreated cells (Figs. 1, 3, and 5).

Inhibitory Effects of D3T Pretreatment on Cell Injury and ROS Formation Induced by Simulated Ischemia-Reperfusion. The causal involvement of oxidative stress in myocardial ischemia-reperfusion injury has been demonstrated in various experimental models, including simulated ischemia-reperfusion in cultured cells (6, 8, 25). Thus, we examined whether upregulation of cellular antioxidants and phase 2 enzymes by D3T in H9c2 cells

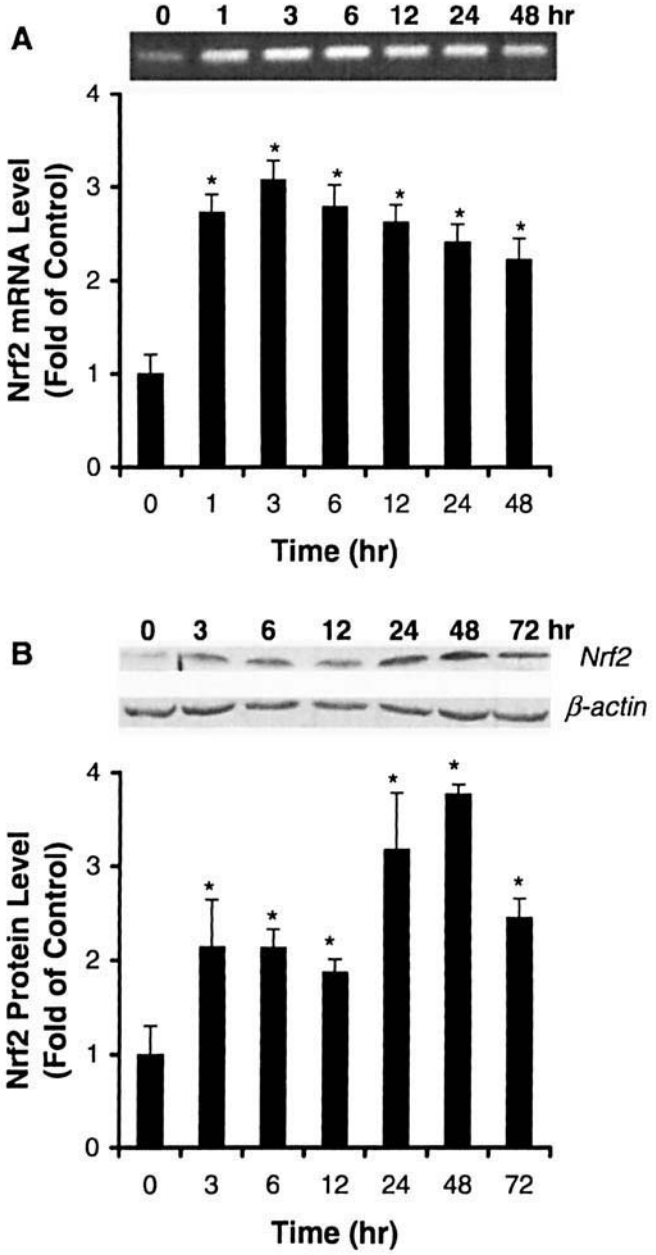

Figure 7. Time-dependent induction of Nrf2 mRNA (A) and protein (B) expression by D3T in H9c2 cells. In panels A and B, the top panel shows a representative gel picture of the Nrf2 mRNA (A) or protein (B) expression at the indicated times after treatment of H9c2 cells with 100 μM D3T; the bottom panel shows quantitative analysis of the Nrf2 mRNA (A) or protein (B) expression. Values represent means ± SEM from 3 independent experiments. * Significantly different from 0 hrs.

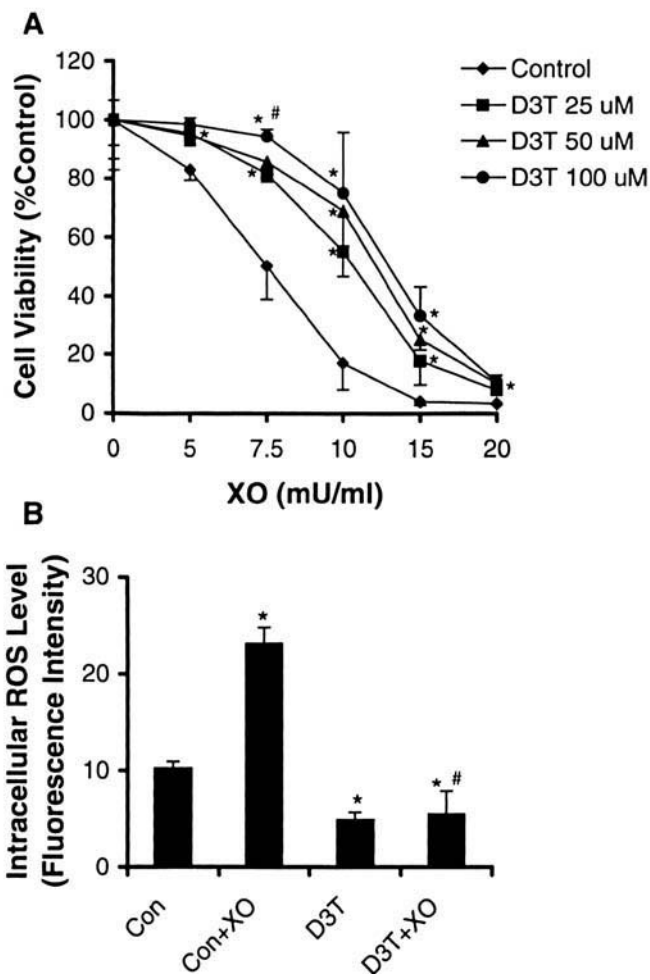

Figure 8. Inhibitory effects of D3T pretreatment on XO/xanthine-mediated cytotoxicity (A) and intracellular ROS accumulation (B) in H9c2 cells. (A) Cells were incubated with or without the indicated concentrations of D3T for 24 hrs, followed by incubation with various concentrations of XO in the presence of 0.5 mM xanthine for another 24 hrs. After this incubation, cell viability was determined using MTT reduction assay. *Significantly different from control. #Significantly different from 25 and 50 μM D3T. (B) Cells were incubated with or without 100 μM D3T for 24 hrs, followed by incubation with 10 μM DCF-DA for 30 mins. The intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of the cells with XO (10 mU/ml) and xanthine (0.5 mM) for another 30 mins. * Significantly different from control. #Significantly different from control + XO. In each panel values represent means ± SEM from 4 independent experiments.

could afford protection against simulated ischemia-reperfusion injury. As shown in Figure 11A, D3T pretreatment of H9c2 cells resulted in a significant protection against cell injury induced by simulated ischemia-reperfusion in a D3T concentration–dependent manner. A nearly complete protection was observed in H9c2 cells pretreated with 100 μM D3T. A significantly increased formation of ROS as detected by DCF assay was observed in ischemic cells following 1 hr of reperfusion. This ROS generation was remarkably reduced in D3T-pretreated cells in a D3T concentration–dependent fashion (Fig. 11B).

Discussion

Although it has been demonstrated that 1,2-dithiole-3-thiones, particularly D3T, are capable of inducing endogenous antioxidants and phase 2 enzymes in various cells and tissues (12), the concentration- and time-dependent inducibility of the above cellular defenses in cardiac cells by D3T has not been carefully studied. The results of the present study demonstrated that incubation of rat cardiac H9c2 cells with low micromolar concentrations of D3T resulted in a

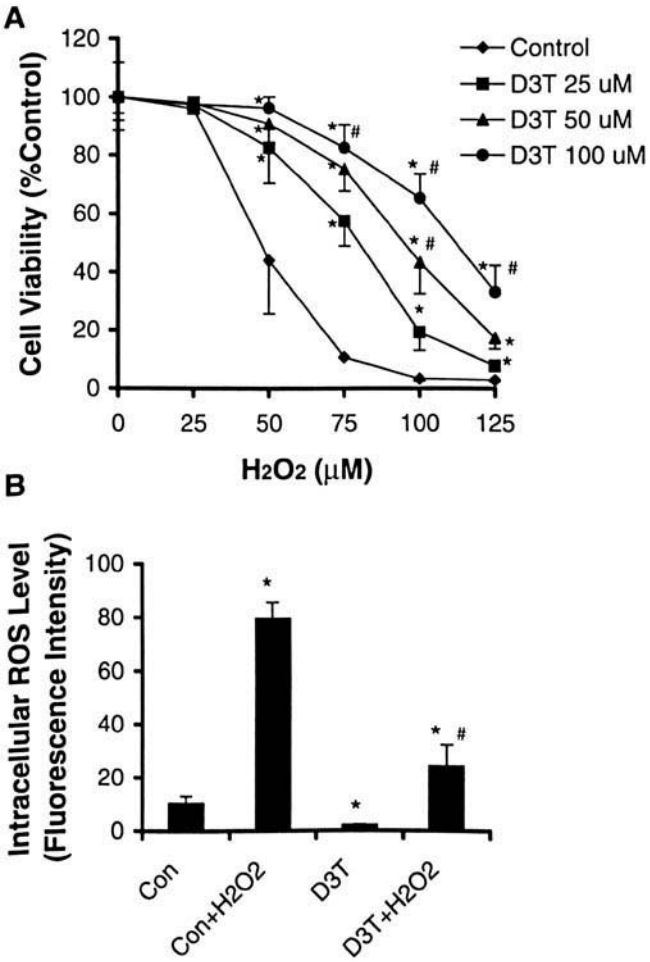

Figure 9. Inhibitory effects of D3T pretreatment on H_2O_2-mediated cytotoxicity (A) and intracellular ROS accumulation (B) in H9c2 cells. (A) Cells were incubated with or without the indicated concentrations of D3T for 24 hrs, followed by incubation with various concentrations of H_2O_2 for another 24 hrs. After this incubation, cell viability was determined using MTT reduction assay. *Significantly different from control. #Significantly different from 25 μM D3T. (B) Cells were incubated with or without 100 μ*M* D3T for 24 hrs, followed by incubation with 10 μ*M* DCF-DA for 30 mins. The intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of the cells with 75 μ*M* H_2O_2 for another 30 mins. * Significantly different from control. #Significantly different from control + H_2O_2. In each panel values represent means ± SEM from 3 to 4 independent experiments.

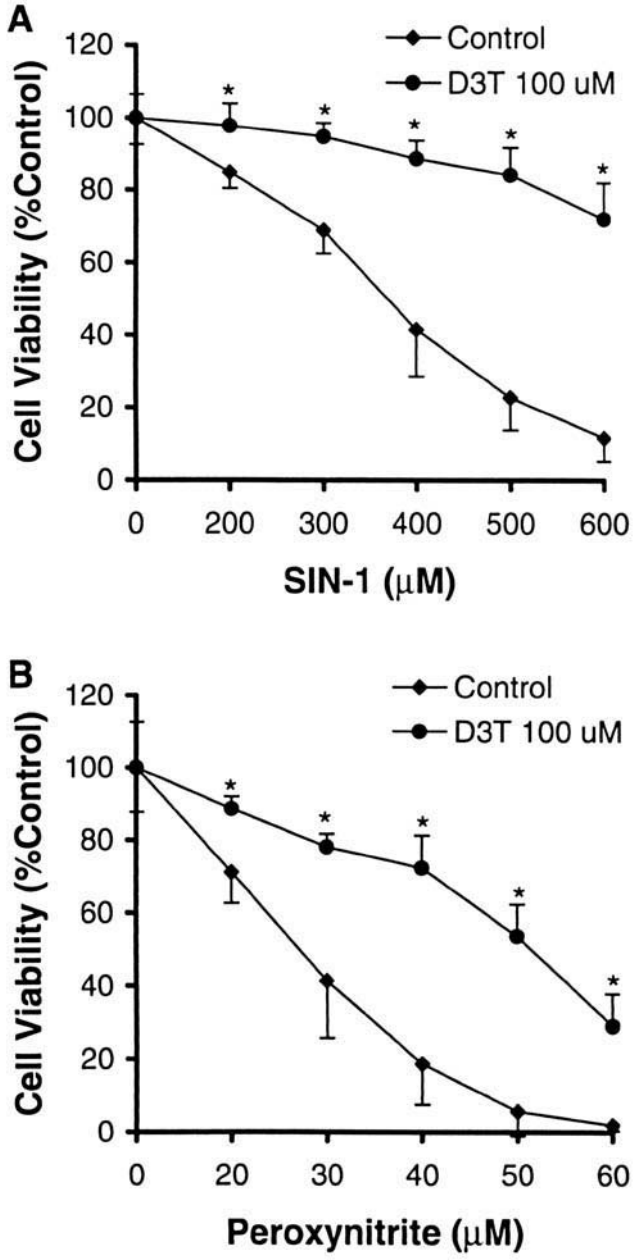

Figure 10. Protective effects of D3T pretreatment on cytotoxicity induced by SIN-1 (A) or authentic peroxynitrite (B) in H9c2 cells. Cells were incubated with or without 100 μ*M* D3T for 24 hrs, followed by incubation with various concentrations of SIN-1 or authentic peroxynitite for another 24 hrs. After this incubation cell viability was determined using MTT reduction assay. Values represent means ± SEM from 4 independent experiments. * Significantly different from control.

significant induction of a series of cellular antioxidants and phase 2 enzymes, including catalase, GSH, GR, GPx, GST, and NQO1 in a concentration- and/or time-dependent fashion (Figs. 1, 3, and 5). The concentrations of D3T (25–100 μ*M*) described in the present study are similar to those used in previously published studies (20, 24, 35). Treatment of H9c2 cells with D3T also led to significantly increased levels of mRNA for catalase, γGCL catalytic subunit, GR, GSTA1, M1, P1, and NQO1 (Figs. 2, 4, and 6). It remains unknown why SOD was not inducible by D3T in H9c2 cells (Fig. 1A). In contrast to D3T, another chemoprotective agent, resveratrol was found to potently induce SOD in H9c2 cells (36), suggesting that different signaling pathway(s) may be employed by different chemical inducers to upregulate SOD in cardiac cells.

The induction of the antioxidants and phase 2 enzymes and their gene expression by D3T in H9c2 cells was long lasting (Figs. 1–6). To investigate the possible mechanism(s) underlying this long-lasting induction, we determined whether D3T treatment of H9c2 cells led to increased expression of Nrf2. Nrf2 is an indispensable regulator of cytoprotective gene expression in mammalian cells (34).

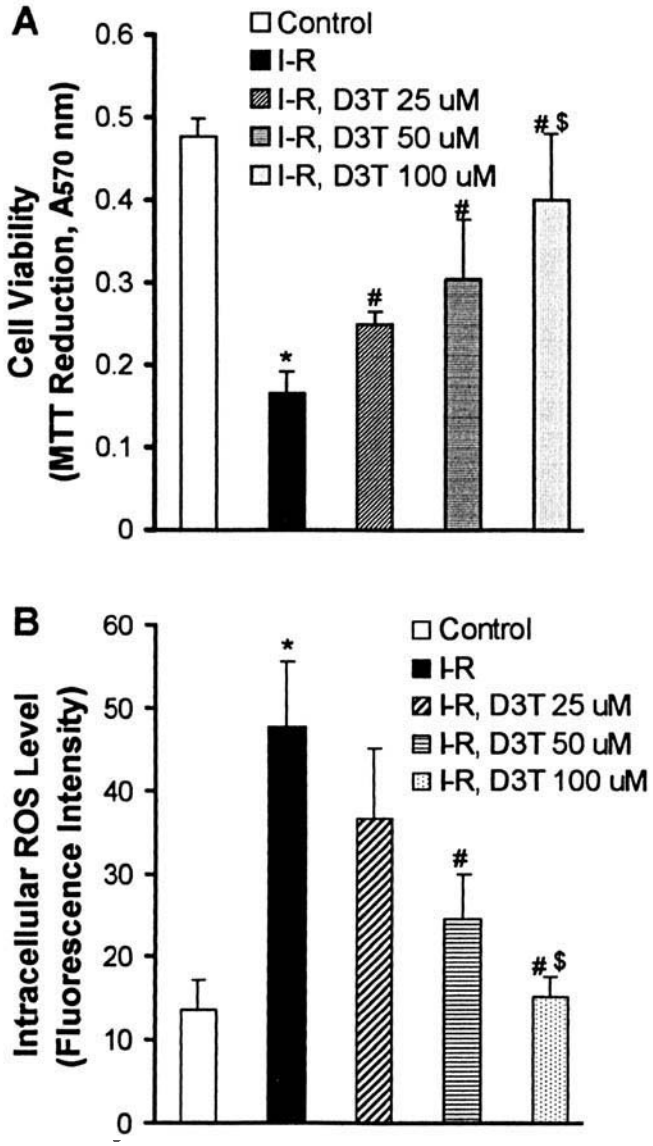

Figure 11. Protective effects of D3T pretreatment on cytotoxicity (A) and ROS formation (B) induced by simulated ischemia-reperfusion in H9c2 cells. (A) Cells were incubated with or without the indicated concentrations of D3T for 24 hrs and then subjected to 10 hrs simulated ischemia and 16 hrs reperfusion. After the simulated ischemia-reperfusion (I-R), cell viability was determined using MTT reduction assay. (B) Cells were incubated with or without the indicated concentrations of D3T for 24 hrs and then subjected to 10 hrs simulated ischemia, and 1 hr reperfusion in the presence of 10 μM DCF. Values represent means ± SEM from 3 (A) or 4 (B) independent experiments. * Significantly different from control. #Significantly different from I-R. $Significantly different from I-R, 25 μM D3T.

Recently, Nrf2 also was found to be inducible by cancer chemoprotective agents in murine keratinocytes (37). This may serve as a positive feedback mechanism for prolonged induction of cytoprotective genes by chemoprotective agents (37). Thus, the remarkable induction of both mRNA and protein expression of Nrf2 by D3T in H9c2 cells (Fig. 7) most likely accounted for the long-lasting elevation of the antioxidants and phase 2 enzymes and their mRNA expression in the D3T-treated cardiac cells (Figs. 1–6). Although D3T was shown to upregulate a series of antioxidants and phase 2 enzymes in H9c2 cells (Figs. 1–6), the effects of D3T on activity and expression of ROS-generating enzymes, including xanthine oxidase and NAD(P)H oxidase, remain to be investigated. In this context we recently observed that D3T treatment of H9c2 cells did not affect the formation of $O_2^{\cdot-}$ by mitochondria (data not shown), a major source of cellular ROS.

Pretreatment of H9c2 cells with D3T resulted in a marked protection against XO/xanthine-, H_2O_2-, or peroxynitrite-mediated cytotoxicity (Figs. 8–10). XO can generate both $O_2^{\cdot-}$ and H_2O_2, which, along with peroxynitrite, have been extensively implicated in the pathogenesis of various cardiovascular diseases, including ischemia-reperfusion injury (3, 8, 38). On the other hand, catalase, GPx/GSH, and NQO1 are cellular defenses involved in the detoxification of H_2O_2 and $O_2^{\cdot-}$ (29, 33). The significance of NQO1 as a $O_2^{\cdot-}$ scavenger (33) in protection against $O_2^{\cdot-}$-mediated cardiac cell injury was strengthened by the observation that cardiac H9c2 cells expressed extremely high basal levels of NQO1, which were further elevated 5-fold following D3T treatment (Fig. 5B). Thus, NQO1 might function as an effective $O_2^{\cdot-}$ scavenger in cardiac H9c2 cells. In addition to being involved in detoxifying H_2O_2, the GPx/GSH system also has been found to be a major pathway for detoxification of peroxynitrite in mammalian cells (24, 39). Thus, the simultaneous induction of the above cellular factors by D3T may largely contribute to the increased resistance of the D3T-pretreated H9c2 cells to the above oxidant-mediated cytotoxicity. Furthermore, the induction of GR by D3T may lead to increased regeneration of GSH from the oxidized form of glutathione (GSSG) produced during GPx-catalyzed decomposition of H_2O_2 in cardiac cells. GSH also is a cofactor for GST, an abundant cellular enzyme in mammalian tissues. GST generally is viewed as a phase 2 enzyme, primarily involved in the detoxification of electrophilic compounds by catalyzing the formation of GSH–electrophile conjugates (28). Several recent studies also have demonstrated that GST plays a critical role in protecting cells against oxidant-mediated injury by catalyzing the decomposition of lipid hydroperoxides generated from oxidative damage of cellular lipid molecules (30, 31). Accordingly, the marked induction of GST by D3T in H9c2 cells may also contribute partially to the increased resistance of the D3T-pretreated cells to the oxidant-elicited cytotoxicity.

Upregulation of H9c2 cellular antioxidant and phase 2 enzymes by D3T also led to cytoprotection against simulated ischemia-reperfusion injury (Fig. 11). As mentioned previously, oxidative stress plays a critical role in ischemia-reperfusion injury (6, 8, 25). The augmented formation of a number of oxidant species, including $O_2^{\cdot-}$, H_2O_2, and peroxynitrite has been observed during myocardial ischemia-reperfusion (6, 8). It is believed that the interaction of the above reactive species with myocardial cellular constituents leads to injury or death of cardiomyo-

cytes (6, 8). The protective effects of D3T on H9c2 cell injury as well as ROS formation elicited by simulated ischemia-reperfusion suggested that chemoprotectant-mediated upregulation of endogenous antioxidants and phase 2 enzymes in cardiac cells might be a valid approach for protecting against *in vivo* myocardial ischemia-reperfusion injury. In this context, studies currently are underway in our laboratories to investigate the chemoprotective effects of D3T on regional myocardial ischemia-reperfusion injury in animals.

In conclusion, this study demonstrates that a number of endogenous antioxidants and phase 2 enzymes in cultured cardiac H9c2 cells can be induced by low micromolar concentrations of D3T, and that this chemoprotectant-mediated upregulation of cellular defenses is accompanied by a remarkably increased resistance to oxidative cardiac cell injury. Thus, this study demonstrates the feasibility for protecting against oxidative cardiac cell injury by upregulating endogenous antioxidative and phase 2 defenses by chemical inducers.

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