Cruciferous Dithiolethione-Mediated Coordinated Induction of Total Cellular and Mitochondrial Antioxidants and Phase 2 Enzymes in Human Primary Cardiomyocytes: Cytoprotection Against Oxidative/Electrophilic Stress and Doxorubicin Toxicity

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3*H*-1,2-dithiole-3-thione (D3T), a cruciferous organosulfur compound, induces cytoprotective enzymes in animal cardiovascular cells. However, it remains unknown if D3T also upregulates antioxidants and phase 2 enzymes in human cardiomyocytes, and protects against cell injury induced by oxidative/electrophilic species as well as doxorubicin. In this study, we found that D3T (10–50 μM) potently induced a series of antioxidants and phase 2 enzymes in primary cultured human cardiomyocytes, including superoxide dismutase (SOD), glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx) glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), aldose reductase (AR), and heme oxygenase (HO). D3T treatment also caused elevation of SOD, GSH, GR, GPx and GST in the isolated mitochondria. We also observed a time-dependent induction by D3T of mRNA expression for Cu,ZnSOD, MnSOD, γ-glutamylcysteine ligase, GR, GSTA1, GSTM1, NQO1, AR, and HO-1. Pretreatment with D3T conferred concentration-dependent protection against cell injury induced by xanthine oxidase (XO)/xanthine, H_2O_2, 3-morpholinosydnonimine, 4-hydroxy-2-nonenal, and doxorubicin. Pretreatment with D3T also reduced the formation of intracellular reactive oxygen species by XO/xanthine, H_2O_2, and doxorubicin. In conclusion, this study demonstrated that D3T potently upregulated many antioxidants and phase 2 enzymes in human cardiomyocytes, which was accompanied by increased resistance to oxidative/electrophilic stress and doxorubicin toxicity. Exp Biol Med 234:418–429, 2009

Key words: 3*H*-1,2-dithiole-3-thione; human cardiomyocytes; antioxidants; reactive oxygen species; 4-hydroxy-2-nonenal; doxorubicin

Introduction

Cardiovascular disease remains the leading cause of death in the developed countries. It is estimated that more than 80 million American adults have cardiovascular disease, which accounts for ~36% of all deaths in the United States (1). Substantial evidence supports that oxidative/electrophilic stress and inflammation are intimately involved in the pathogenesis of various forms of cardiovascular disease, including hypertension, atherosclerotic coronary heart disease, heart failure, as well as

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doxorubicin-induced cardiomyopathy (2, 3). The evidence for a critical role of oxidative/electrophilic stress in cardiovascular disease includes that (i) increased formation of oxidative and electrophilic species occurs during the disease process (1); (ii) consumption of vegetables and fruits rich in antioxidants is associated with decreased risk of cardiovascular disease (4, 5); and (iii) transgenic overexpression of antioxidant enzymes in animals confers protection in various experimental models of cardiovascular disease (6). However, for the most part, clinical trials have failed to demonstrate a beneficial effect of antioxidant supplements on cardiovascular disease morbidity and mortality (7). In this regard, another strategy for intervention of cardiovascular disease may be via coordinated induction of endogenous antioxidants and phase 2 enzymes in cardiovascular tissue by chemoprotective agents, such as dithiolethiones.

Dithiolethiones are organosulfur compounds present in cruciferous vegetables. These compounds, particularly 3*H*-1,2-dithiole-3-thione (D3T) potently induce cellular detoxification enzymes and protect against chemical carcinogenesis (8). Recently, we have demonstrated that D3T also upregulates antioxidants and phase 2 enzymes in animal cardiovascular cells, and protects these cells from oxidative and electrophilic injury (9, 10). However, it remains unknown if D3T induces the above cellular defenses in human cardiomyocytes. It is noteworthy that the inducibility of cellular antioxidants and phase 2 enzymes varies greatly among different animal species. As such, this study was undertaken to determine the inducibility by D3T of a series of important antioxidants and phase 2 enzymes in primary cultured human cardiomyocytes, and the accompanying cytoprotective effects against oxidative/electrophilic stress as well as doxorubicin toxicity.

Materials and Methods

Chemicals and Reagents. 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). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Preparation of Cell Extract. Primary human cardiomyocytes were purchased from ScienCell Research Laboratories (San Diego, CA) and cultured according to the manufacturer's protocol in a recommended cardiomyocyte medium (ScienCell) at 37°C in a humidified atmosphere of 5% CO_2. To prepare cell extract for assays, cells were collected and resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA and 0.1% Triton X-100. The cell suspensions were sonicated, followed by centrifugation at 13,000 *g* for 10 min at 4°C. The resulting supernatants were collected and the protein concentrations were quantified with Bio-Rad protein dye-binding assay (Hercules, CA) using bovine serum albumin (BSA) as the standard. The supernatants were then used for measurement of the antioxidants and phase 2 enzymes.

Isolation of Mitochondria. Mitochondria were isolated from freshly harvested cardiomyocytes according to the method described previously (11). Briefly, cells ($\sim 5 \times 10^6$ cells per sample) were washed once with phosphate-buffered saline (PBS). The cell pellet was resuspended in 5 ml sucrose buffer (0.25 M sucrose, 10 mM Hepes, 1 mM EGTA and 0.5% BSA, pH 7.4), homogenized in a Dounce tissue grinder on ice. The homogenate was centrifuged at 1,500 *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 10,000 *g* for 10 min at 4°C. The resulting mitochondrial pellet was washed twice with sucrose buffer and then resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA and 0.1% Triton X-100, followed by sonication to lyse the mitochondria. The protein concentrations of mitochondrial lysates were measured as above.

Measurement of Superoxide Dismutase (SOD) Activity. Total cellular and mitochondrial SOD activity was determined by the method of Spitz and Oberley (12). This method is based on the inhibition of the superoxide-mediated reduction of nitroblue tetrazolium to formazan by SOD. The sample SOD activity was calculated using a concurrently run SOD (Sigma-Aldrich) standard curve, and expressed as units per milligram of sample protein.

Measurement of Catalase Activity. Total cellular and mitochondrial catalase activity was measured according to the method of Aebi (13). In brief, to a quartz cuvette, 0.4 ml of 50 mM potassium phosphate buffer (pH 7.0) and 20 μl of sample were added. The reaction was initiated by adding 0.18 ml of 30 mM H_2O_2. The decomposition of H_2O_2 was monitored at 240 nm, 25°C for 2 min. The sample catalase activity was expressed as micromoles of H_2O_2 consumed per minute per milligram of sample protein.

Measurement of Reduced Glutathione (GSH) Content. Total cellular and mitochondrial GSH content was measured according to the *o*-phthalaldehyde-based fluorometric method, which is specific for the determination of GSH at pH 8.0 (14). The sample GSH content was calculated using a GSH (Sigma-Aldrich) standard curve, and expressed as nanomoles of GSH per milligram of sample protein.

Measurement of Glutathione Reductase (GR) Activity. The method as described before (15) was followed to measure total cellular and mitochondrial GR activity. This assay is based on NADPH consumption coupled with reduction of the oxidized form of glutathione (GSSG) to GSH by GR. The sample GR activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per minute per milligram of sample protein.

Measurement of Glutathione Peroxidase (GPx) Activity. Total cellular and mitochondrial GPx activity was measured based on the formation of GSSG from GPx-

catalyzed oxidation of GSH by H_2O_2, coupled with NADPH consumption in the presence of exogenously added GR (16). The sample GPx activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per minute per milligram of sample protein.

Measurement of Glutathione S-Transferase (GST) Activity. Total cellular and mitochondrial GST activity was measured according to the method of Habig *et al*. (17) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Briefly, the reaction mix contained 1 mM GSH, 1 mM CDNB and 3 mg/ml of bovine serum albumin in 0.1 M sodium phosphate buffer, pH 6.5. The above reaction mix (0.59 ml) was added to each cuvette, followed by addition of 10 μl of sample to start the reaction. The rate of formation of CDNB-GSH conjugate was monitored at 340 nm, 25°C for 5 min. The sample GST activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}, and expressed as nanomoles of CDNB-GSH conjugate formed per minute per milligram of sample protein.

Measurement of NAD(P)H:Quinone Oxidoreductase 1 (NQO1) Activity. Cellular NQO1 activity was determined using dichloroindophenol (DCIP) as the two-electron acceptor, as described before (15). The dicumarol-inhibitable cellular NQO1 activity was calculated using the extinction coefficient of 21.0 mM^{-1} cm^{-1}, and expressed as nanomoles of DCIP reduced per minute per milligram of cellular protein.

Measurement of Aldose Reductase (AR) Activity. Cellular AR activity was measured according to the method described before (18). In brief, the reaction mix (prepared freshly) contained 50 mM potassium phosphate buffer, pH 6.0, 0.4 M Li_2SO_4, 10 mM D,L-glyceraldehyde and 100 μM NADPH. To an assay cuvette 0.95 ml of the reaction mix was added. The reaction was started by adding 50 μl of sample, and the subsequent consumption of NADPH was monitored at 340 nm, 25°C for 5 min. The cellular AR activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per minute per milligram of cellular protein.

Measurement of Heme Oxygenase (HO) Activity. Cellular HO activity was measured according to the procedures described by Naughton *et al*. (19) with slight modifications. Briefly, the harvested cells were resuspended in 100 mM potassium phosphate buffer, pH 7.4 containing 2 mM $MgCl_2$, and subjected to three cycles of freeze-thawing and finally sonicated on ice before centrifugation at 18,000 *g* for 10 min at 4°C. As much as 100 μl (200 μg protein) of the supernatant was added to 200 μl of reaction mix containing 0.5 mM NADPH, 2 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 0.2 mM hemin, and 1 mg/ml rat liver cytosol (as a source of biliverdin reductase) in 100 mM potassium phosphate buffer, pH 7.4, containing 2 mM $MgCl_2$. The reaction was conducted for 1 h at 37°C in the dark and terminated by addition of 300 μl chloroform. The extracted bilirubin was measured by the difference in absorbance between 464 and 530 nm (extinction coefficient = 40 mM^{-1} cm^{-1}). The cellular HO activity was expressed as picomoles of bilirubin formed per hour per milligram of cellular protein.

Detection of mRNA Levels by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA from freshly harvested cells 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 the 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 PCR primers were designed based on sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank). PCR products were separated by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide and 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 and phase 2 enzyme mRNAs using various amounts of total RNA was included in each assay to estimate changes in mRNA levels.

Detection of Cytotoxicity. Cytotoxicity was determined by a slightly modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) reduction assay, as described before (15). In brief, the cells were plated into 48-well tissue culture plates. After incubation of the cells with the oxidative and electrophilic species in culture medium supplemented with 0.5% FBS at 37°C for 24 h, media were discarded, followed by addition to each well of 0.5 ml of fresh medium containing MTT (0.2 mg/ml). The plates were incubated at 37°C for another 2 h. Then, media were completely removed followed by addition to each well of 0.25 ml of mix of dimethyl sulfoxide, isopropanol and deionized water (1:4:5) to solubilize the formazan crystals. The amount of the dissolved formazan was then detected at 570 nm.

Detection of Intracellular Reactive Oxygen Species (ROS). 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) was used to detect intracellular ROS levels according to the procedures described before (15). Briefly, cells of equal number grown on 6-well tissue culture plates were rinsed once with serum-free culture medium and then incubated with 10 μM DCF-DA in serum-free culture medium at 37°C for 30 min. After this incubation, the cells were washed once with serum-free culture medium followed by incubation with XO/xanthine or H_2O_2 in culture medium supplemented with 0.5% FBS at 37°C for another 30 min. For detection of doxorubicin-induced ROS formation, cells were exposed to doxorubicin for 12 h, followed by incubation with 10 μM DCF-DA for 30 min, as described above. Then, the cells were washed twice with PBS and

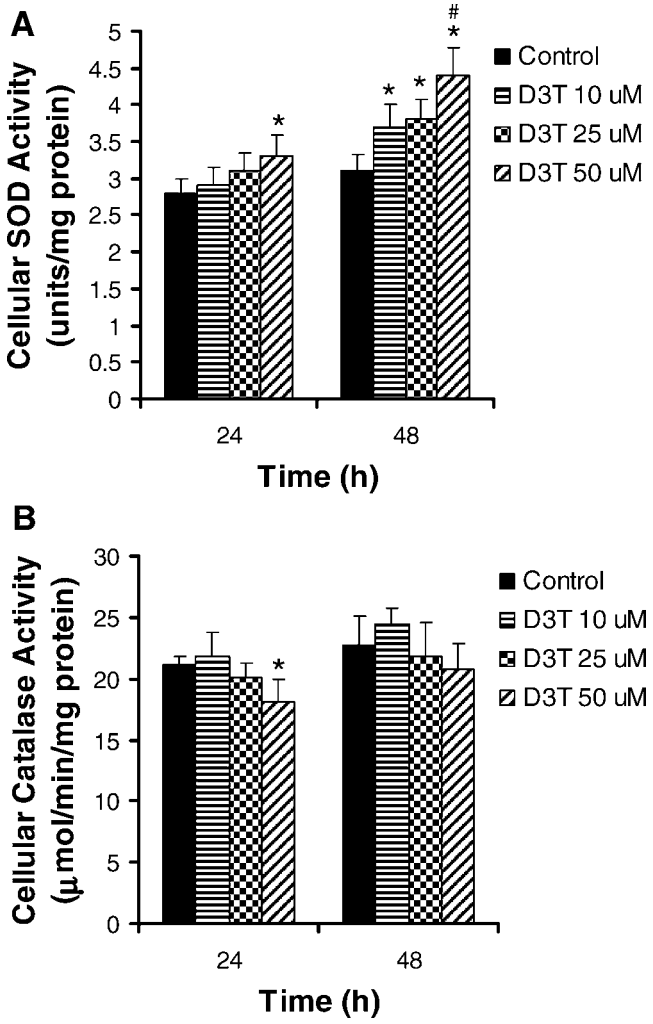

Figure 1. Effects of D3T on cellular activities of SOD (A) and catalase (B) in human primary cardiomyocytes. The cells were incubated with the indicated concentrations of D3T for 24 and 48 h, followed by measurement of enzyme activities. Values represent means ± SEM, $n = 3$. * Significantly different from control; # significantly different from 10 μM D3T.

lysed in 3 ml ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 0.2% SDS. The cell lysates were collected and centrifuged at 2000 *g* for 5 min. The fluorescence intensity of the supernatants was measured at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

Statistical Analysis. All data are expressed as means ± SEM from at least three separate experiments unless otherwise indicated. Differences between mean values of multiple groups were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test. Differences between two groups were analyzed by Student's *t* test. Statistical significance was considered at $P \leq 0.05$.

Results

Inducibility of Antioxidants and Phase 2 Enzymes by D3T. The basal levels of antioxidants and phase 2 enzymes in human primary cardiomyocytes were not reported in literature. As shown in Figure 1, human cardiomyocytes constitutively expressed measurable activities for both SOD and catalase. Treatment of the cells with D3T at 10, 25, and 50 μM for 24 and 48 h led to significant induction of cellular SOD in a concentration- and time-dependent manner. However, the same D3T treatment did not induce cellular catalase; a significant decrease of catalase activity was observed in the cells treated with 50 μM D3T for 24 h (Fig. 1B). Human cardiomyocytes contained a basal level of GSH similar to that found in many other types of cells (Fig. 2A). Incubation with D3T (10–50 μM) for 24–48 h caused significant 40–130% increases in cellular GSH content in a concentration-, but not time-dependent manner. Human cardiomyocytes also expressed basal activities of GR and GPx similar to those found in other cell types (Fig. 2B and 2C). D3T treatment resulted in concentration- and time-dependent induction of GR. A more than 2-fold induction of GR was observed in the cells after treatment with 50 μM D3T for 48 h. In contrast, D3T treatment for 48 h caused small, but statistically significant, induction of GPx (Fig. 2C).

Human cardiomyocytes expressed relatively high activities for both GST and NQO1, as compared to most other cell types. As shown in Figure 3, D3T treatment resulted in significant induction of both GST and NQO1 in a concentration- and time-dependent manner. Notably, a 4.3-fold induction of NQO1 was observed in the cells treated with 50 μM D3T for 48 h.

As shown in Figure 4, incubation of human cardiomyocytes with D3T at 10, 25, and 50 μM also led to marked induction of AR and HO in a concentration-dependent manner. The induction of AR and HO by D3T also showed a time dependency.

Inducibility of Mitochondrial Antioxidants and Phase 2 Enzymes by D3T. Because SOD, catalase, GSH, GR, GPx, and GST are present in mitochondria of mammalian cells, we examined the levels/activities of these antioxidants and phase 2 enzymes in this cellular compartment after treatment of human cardiomyocytes with D3T. As shown in Figure 5, treatment of the cells with 50 μM D3T for 48 h caused significant induction of mitochondrial SOD, GSH, GR, GPx, and GST.

Inducibility of the mRNAs for Antioxidant and Phase 2 Enzyme Genes by D3T. Since the levels/activities of various antioxidant and phase 2 enzymes were induced by D3T, we determined if D3T treatment of human cardiomyocytes increased levels of their respective mRNAs. As shown in Figure 6, D3T treatment caused significant time-dependent increases in the levels of the mRNA for Cu,ZnSOD, MnSOD, the catalytic subunit (GCLC) and modifier subunit (GCLM) of γ-glutamylcysteine ligase, the key enzyme in GSH synthesis, GR, GSTA1, GSTM1, NQO1, AR, and HO-1. D3T-mediated induction of the mRNA expression for all of the above antioxidant and phase 2 enzyme genes remained elevated for up to 48 h after addition of D3T to the cell culture. Notably, MnSOD

mRNA showed a biphasic induction by D3T. A marked induction of GSTM1 mRNA was observed only at 48 h of D3T treatment.

Protective Effects of D3T Pretreatment on Cytotoxicity and ROS Generation by Xanthine Oxidase (XO)/Xanthine, H_2O_2, 3-Morpholinosydnonimine (SIN-1), 4-Hydroxy-2-Nonenal (HNE) and Doxorubicin. To determine if D3T treatment, which upregulated antioxidant and phase 2 defenses in human cardiomyocytes, could confer cytoprotection against oxidative stress, the cardiomyocytes were pretreated with D3T for 24 h, followed by exposure for another 24 h to H_2O_2, and XO/xanthine, a system that generates both superoxide and H_2O_2. As shown in Figures 7 and 8, pretreatment with D3T afforded marked concentration-dependent protection against the above oxidant-mediated cytotoxicity. D3T pretreatment also caused decreased formation of intracellular ROS by XO/xanthine and H_2O_2.

SIN-1 is a commonly used peroxynitrite generator for studying the biological effects of peroxynitrite (9, 10). As shown in Figure 9A, SIN-1-mediated cytotoxicity was remarkably protected by D3T pretreatment (10–50 μM for 24 h) in a concentration-dependent manner. Similarly, the above D3T pretreatment also resulted in dramatic concentration-dependent protection against HNE-elicited cytotoxicity in human cardiomyocytes (Fig. 9B).

Doxorubicin is a widely used anticancer drug, which induces cardiotoxicity via generation of free radicals and oxidative stress (20, 21). Studies on doxorubicin toxicity in primary cultured human cardiomyocytes are currently lacking in literature. As shown in Figure 10, incubation of human cardiomyocytes with pharmacologically relevant concentrations (0.1 and 0.25 μM) for 48 h decreased cell viability by 45–65%. Higher concentrations of doxorubicin caused further decreases in cell viability. Doxorubicin treatment for 12 h resulted in increased cellular ROS formation. Pretreatment with D3T for 24 h conferred remarkable concentration-dependent protection against doxorubicin cytotoxicity. Doxorubicin-induced ROS formation was also reduced by D3T pretreatment.

Discussion

Mammalian cells express a number of cytoprotective enzymes, each catalyzing a distinct reaction. The coordinated actions of a variety of different antioxidants and phase 2 enzymes ensure efficient detoxification of oxidative and electrophilic species as well as other reactive metabolites that contribute to the pathogenesis of cardiovascular disease (2, 3, 22). Since oxidative/electrophilic stress and inflammation are intimately related processes underlying various degenerative diseases, especially cardiovascular disorders, chemoprotectants that cause coordinated upregulation of the above cellular defenses would be of great value for intervention of cardiovascular degeneration. However, the basal expression as well as chemical inducibility of

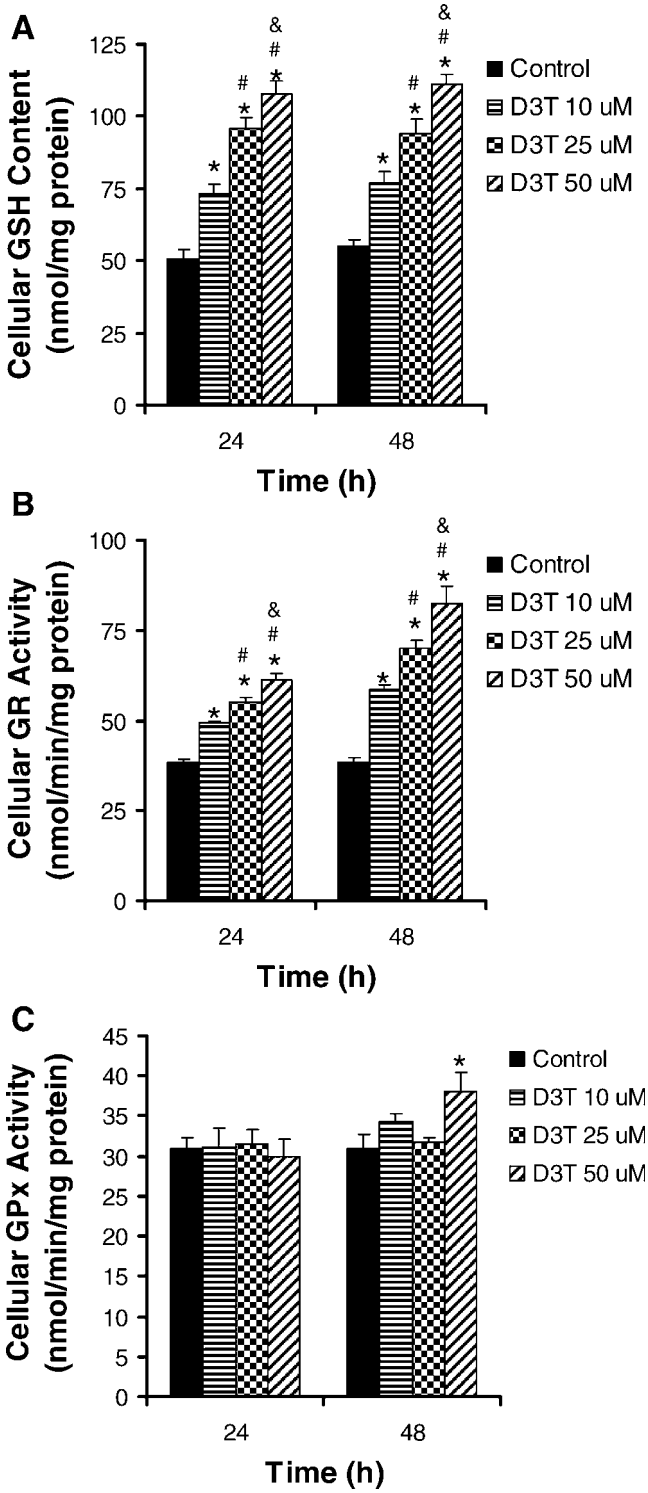

Figure 2. Effects of D3T on cellular GSH content (A) and the activities of GR (B) and GPx (C) in human primary cardiomyocytes. The cells were incubated with the indicated concentrations of D3T for 24 and 48 h, followed by measurement of the total cellular GSH content, and GR and GPx activities. Values represent means ± SEM, $n = 3$. * Significantly different from control; # significantly different from 10 μM D3T; & significantly different from 25 μM D3T.

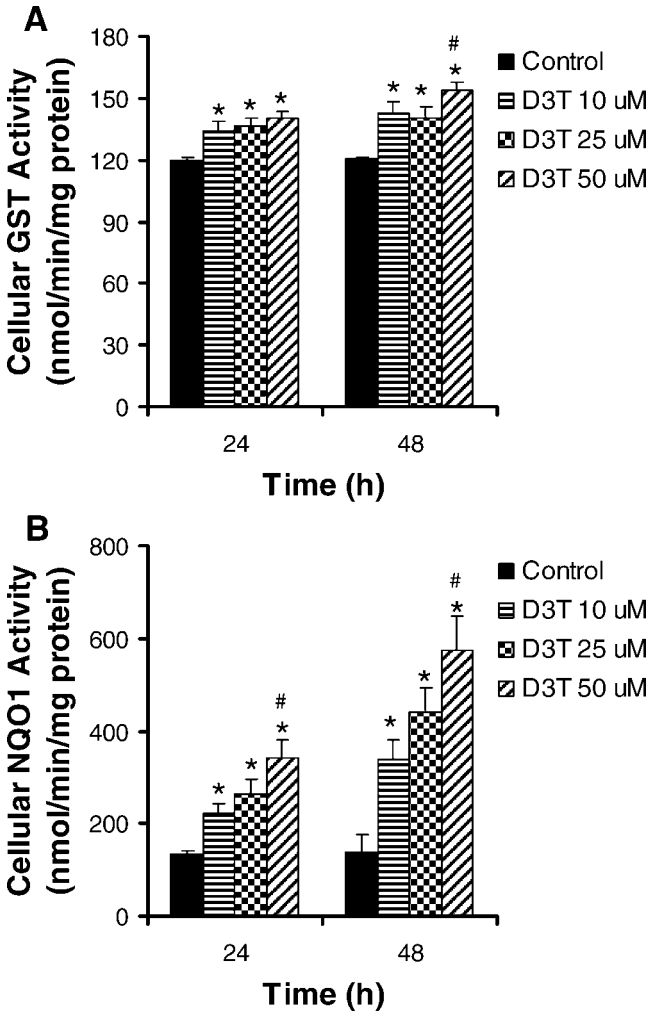

Figure 3. Effects of D3T on cellular activities of GST (A) and NQO1 (B) in human primary cardiomyocytes. The cells were incubated with the indicated concentrations of D3T for 24 and 48 h, followed by measurement of the enzyme activities. Values represent means ± SEM, $n = 3$. * Significantly different from control; # significantly different from 10 μM D3T.

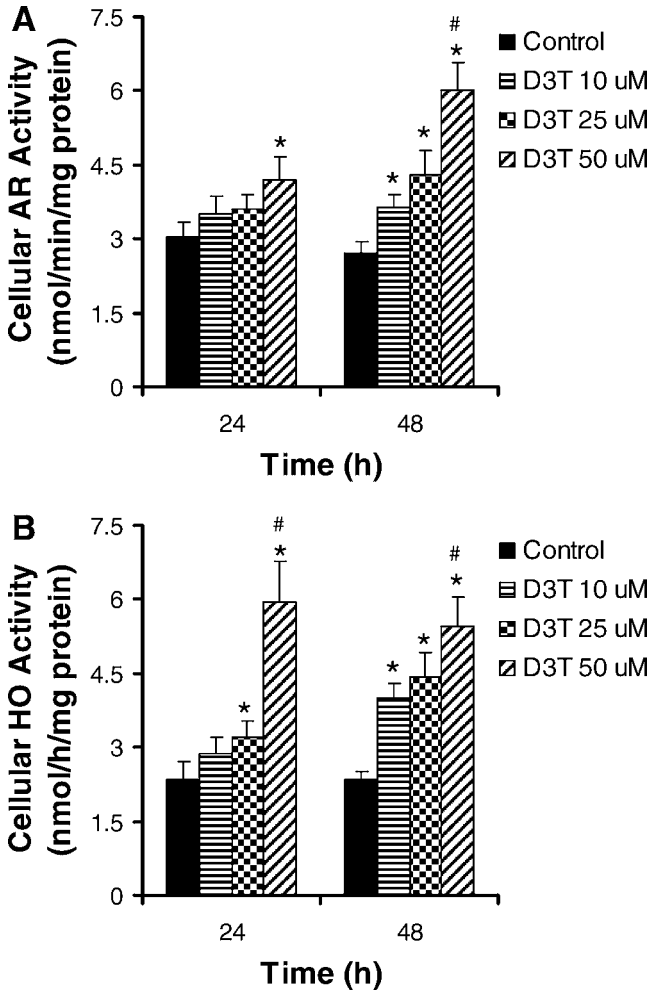

Figure 4. Effects of D3T on cellular activities of AR (A) and HO (B) in human primary cardiomyocytes. The cells were incubated with the indicated concentrations of D3T for 24 and 48 h, followed by measurement of the enzyme activities. Values represent means ± SEM, $n = 3$. * Significantly different from control; # significantly different from 10 or 25 μM D3T.

antioxidants and phase 2 enzymes in normal human cardiomyocytes had not been previously investigated.

The results of this study demonstrated for the first time that a series of important antioxidants and phase 2 enzymes in primary cultured human cardiomyocytes were significantly induced by D3T at low micromolar concentrations. A notable finding of this study was that D3T also caused significant induction of a number of mitochondrial antioxidants and phase 2 enzymes, including SOD, GSH, GR, GPx, and GST. Induction of the above cytoprotective defenses in mitochondria would render the mitochondria resistance to oxidative and electrophilic stress. Indeed, due to the high ROS levels combined with insufficient repairing mechanisms for oxidative damage, mitochondria are particularly vulnerable to oxidative and electrophilic stress. Oxidative and electrophilic damage of mitochondria are involved in the pathophysiological processes of various forms of cardiovascular disorders (23, 24). In this regard, potent induction of antioxidants and phase 2 defenses in mitochondria of cardiomyocytes by D3T may provide any important pathway for protecting against oxidative myocardiac cell injury.

The coordinated upregulation of a spectrum of important antioxidants and phase 2 enzymes by D3T in human cardiomyocytes was accompanied by increased resistance of these cells to cytotoxicity induced by XO/xanthine, H_2O_2, SIN-1-derived peroxynitrite, and the lipid peroxidation end-product HNE. XO generates both superoxide and H_2O_2, which along with peroxynitrite have been extensively implicated in myocardial injury (25). While a number of cellular factors are involved in metabolism of superoxide and H_2O_2, SOD, catalase and GPx/GSH are the predominant systems to detoxify these ROS. In addition to SOD, NQO1 when expressed at high levels also scavenges superoxide (26). In this context, human cardiomyocytes

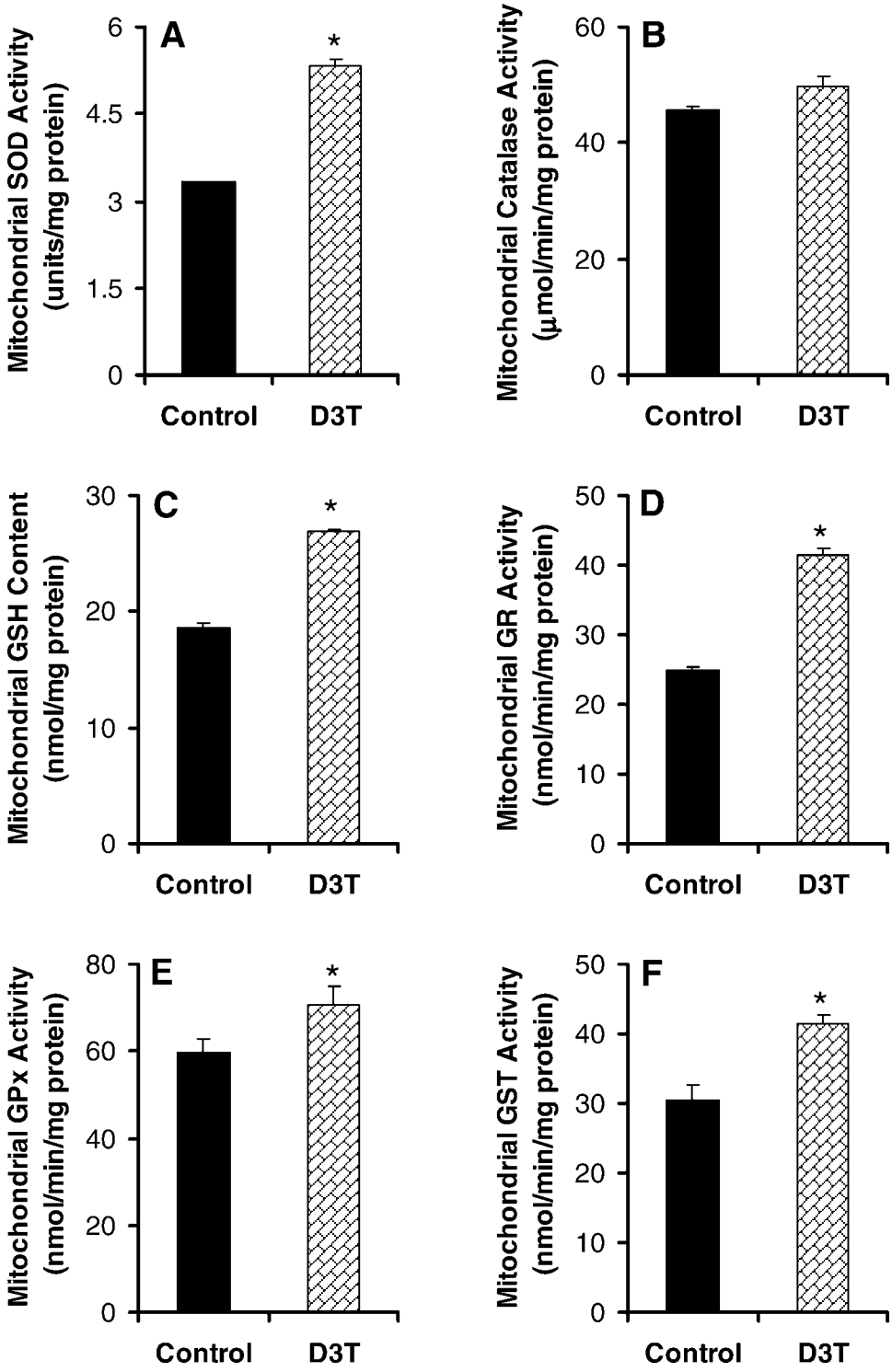

Figure 5. Effects of D3T on mitochondrial levels/activity of SOD (A), catalase (B), GSH (C), GR (D), GPx (E) and GST (F) in human primary cardiomyocytes. The cells were incubated with 50 μM D3T for 48 h, and then the mitochondria were isolated for measurement of the above antioxidants and phase 2 enzymes. Values represent means ± SEM, $n = 4$. * Significantly different from control.

expressed a high basal activity of NQO1, which was dramatically upregulated by D3T. Thus, the coordinated upregulation of SOD, NQO1, catalase, and GPx/GSH by D3T created a complementary pathway for detoxifying both superoxide and H_2O_2. In addition to detoxification of H_2O_2, the GPx/GSH system is found to metabolize peroxynitrite in mammalian cells (9, 27). As such, the potent induction of both total cellular and mitochondrial GSH as well as GPx may largely contribute to the protective effects of D3T on SIN-1-mediated cardiocytotoxicity. In addition to the above

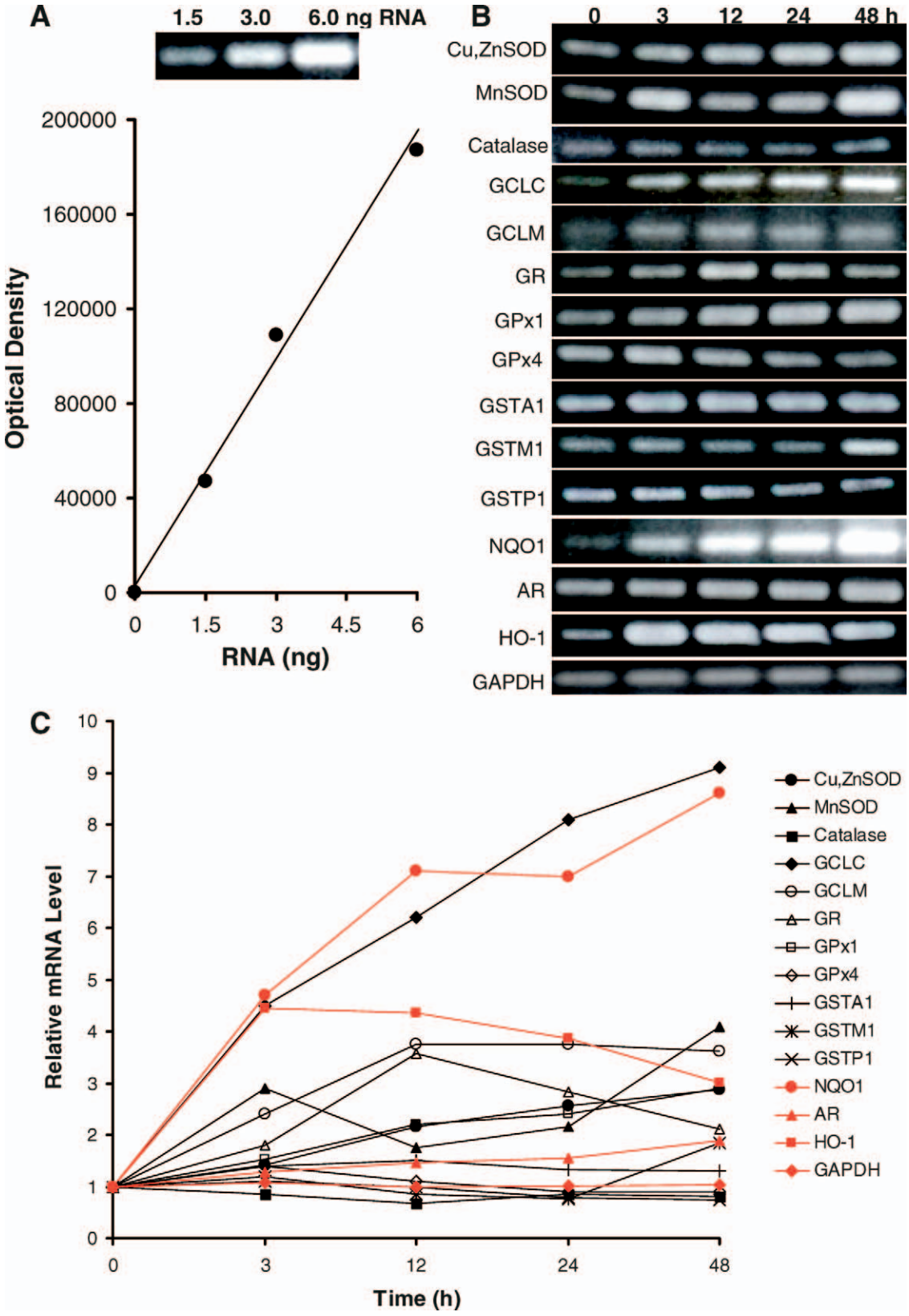

Figure 6. Effects of D3T on mRNA expression of various antioxidative and phase 2 genes in human primary cardiomyocytes. The cells were incubated with 50 μM D3T for various time points, followed by detection of cellular mRNA levels for the indicated genes. Gel DNA band picture and graph in panel A show the linear amplification of the Cu,ZnSOD mRNA. Gel DNA band pictures in panel B are representative of two separate experiments. Values in panel C represent averages of the relative density of the gel DNA bands from two separate experiments. A color version of the figure is available in the online journal.

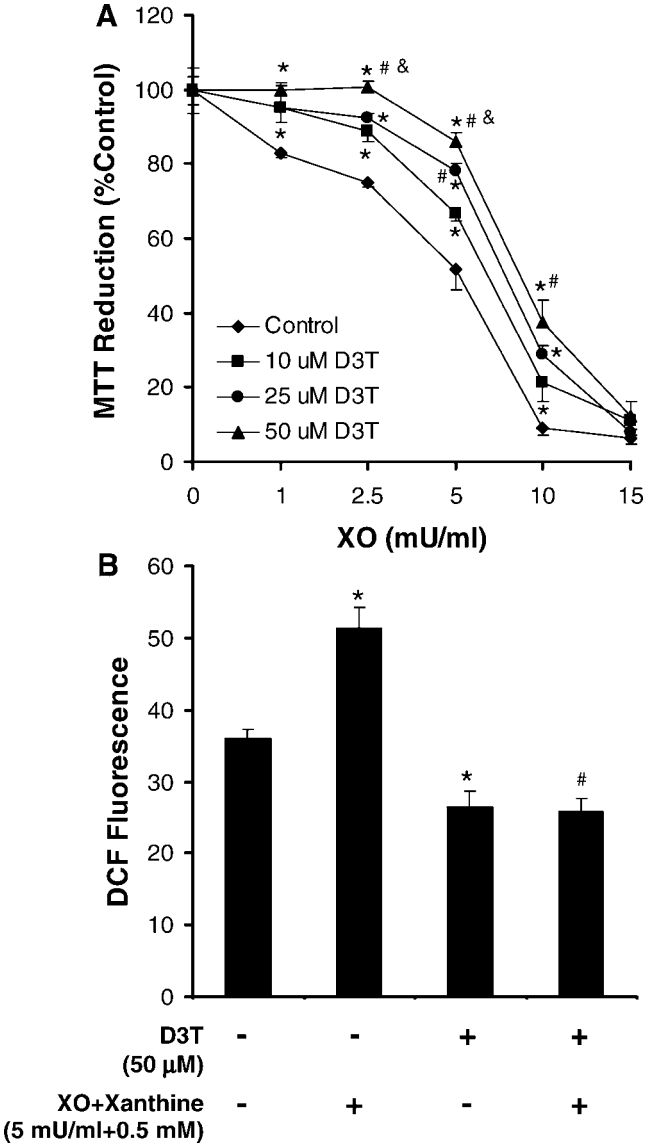

Figure 7. Effects of D3T pretreatment on XO/xanthine-mediated cytotoxicity (A) and intracellular ROS accumulation (B) in human primary cardiomyocytes. In panel A, the cells were pretreated with the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of XO in the presence of 0.5 mM xanthine for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. In panel B, the cells were pretreated with 50 μM D3T for 24 h, followed by incubation with 10 μM DCF-DA for 30 min. The intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of the DCF-loaded cells with XO (5 mU/ml) and xanthine (0.5 mM) for another 30 min. Values represent means ± SEM, $n = 3–5$. In panel A, * significantly different from control; # significantly different from 10 μM D3T; & significantly different from 25 μM D3T. In panel B, * significantly different from control (without D3T and XO/xanthine); # significantly different from "XO/xanthine".

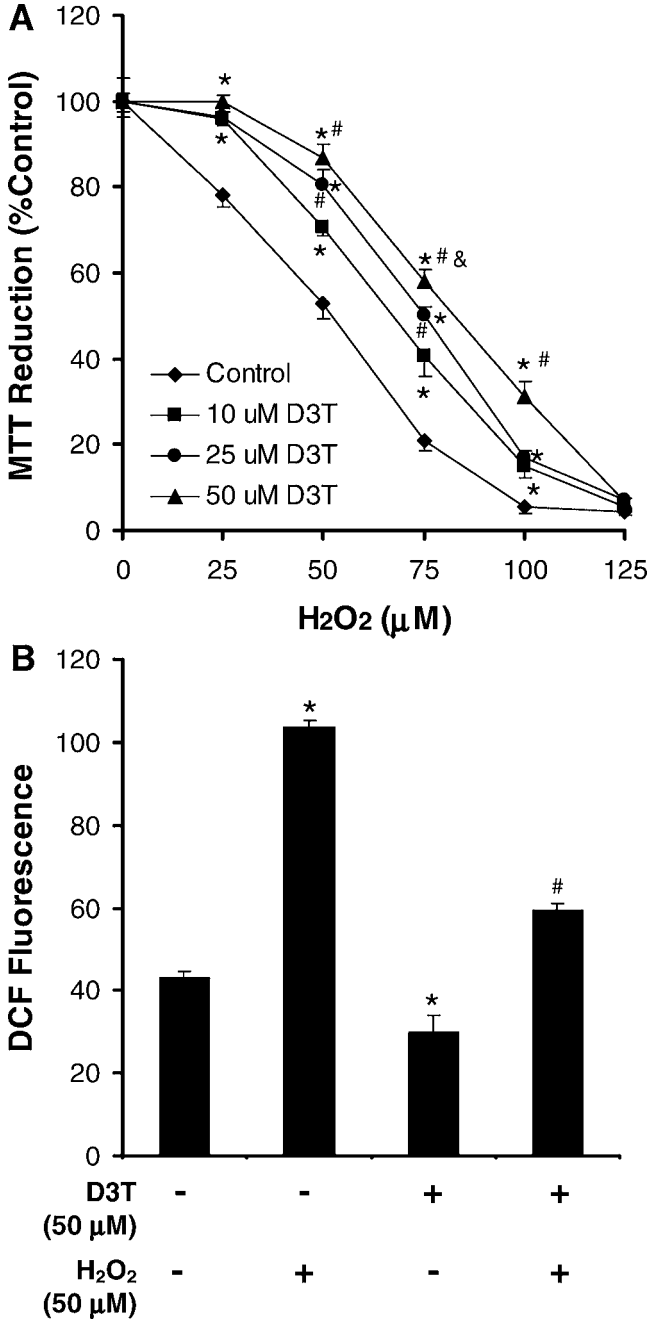

Figure 8. Effects of D3T pretreatment on H_2O_2-mediated cytotoxicity (A) and intracellular ROS accumulation (B) in human primary cardiomyocytes. In panel A, the cells were pretreated with the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of H_2O_2 for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. In panel B, the cells were pretreated with 50 μM D3T for 24 h, followed by incubation with 10 μM DCF-DA for 30 min. The intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of the DCF-loaded cells with 50 μM H_2O_2 for another 30 min. Values represent means ± SEM, $n = 3–5$. In panel A, * significantly different from control; # significantly different from 10 μM D3T; & significantly different from 25 μM D3T. In panel B, * significantly different from control (without D3T and H_2O_2); # significantly different from "H_2O_2".

antioxidants, GST may also protect cells from ROS-induced injury. In this regard, GST detoxifies lipid peroxidation-derived hydroperoxides and reactive aldehydes, especially HNE (18, 28). The induction of mitochondrial GSH was particularly relevant for D3T-mediated cytoprotection against HNE considering that electrophilic attack of

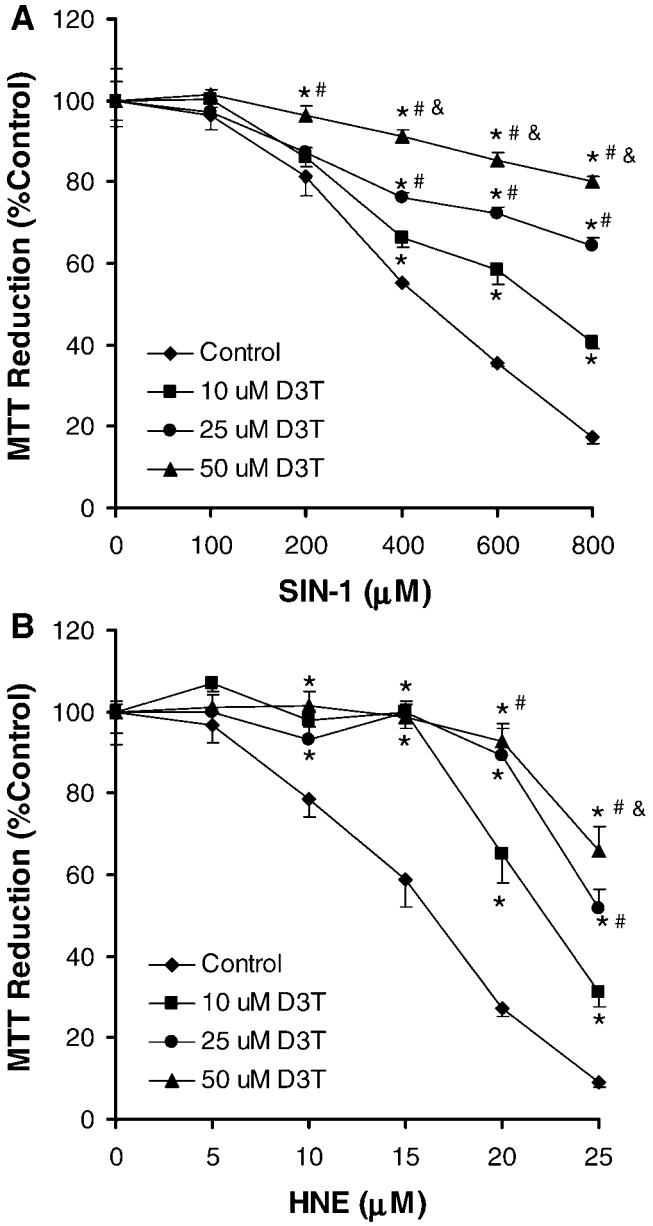

Figure 9. Effects of D3T pretreatment on cytotoxicity induced by SIN-1 (A) and HNE (B) in human primary cardiomyocytes. The cells were pretreated with the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of SIN-1 or HNE for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent means ± SEM, $n = 4$. * Significantly different from control; # significantly different from 10 μM D3T; & significantly different from 25 μM D3T.

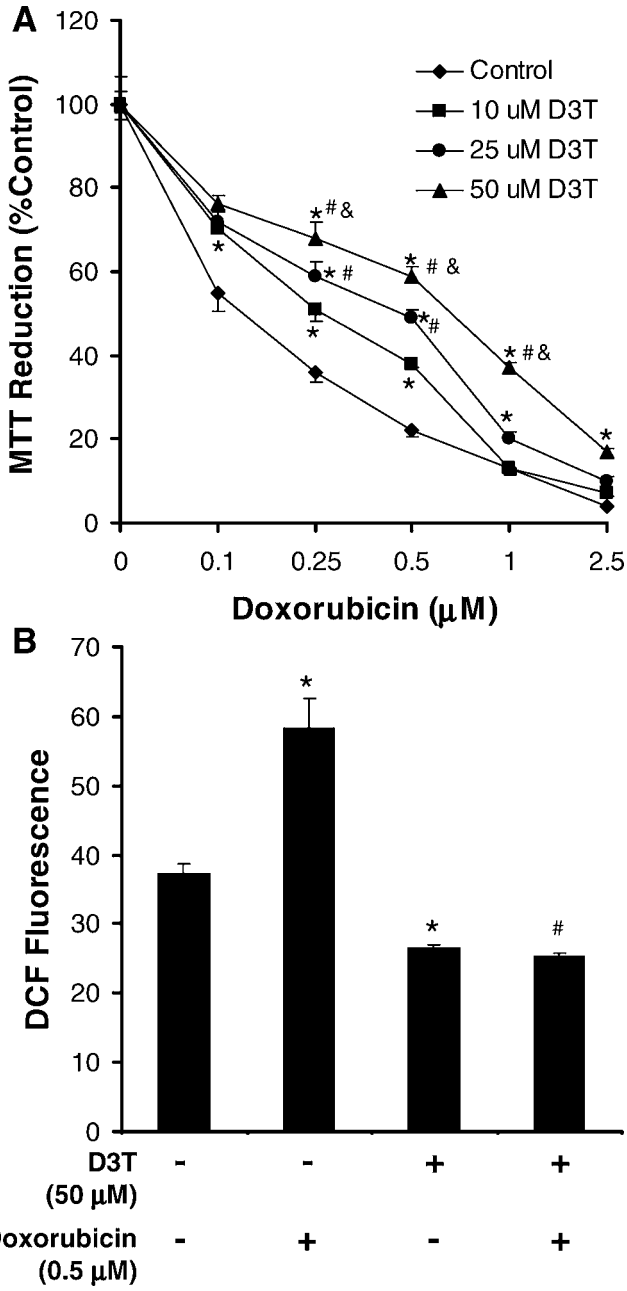

Figure 10. Effects of D3T pretreatment on doxorubicin-mediated cytotoxicity (A) and intracellular ROS accumulation (B) in human primary cardiomyocytes. In panel A, the cells were pretreated with the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of doxorubicin for another 48 h. After this incubation, cell viability was determined using MTT reduction assay. In panel B, the cells were pretreated with 50 μM D3T for 24 h, followed by exposure to 0.5 μM doxorubicin for 12 h. Then, the cells were incubated with 10 μM DCF-DA for 30 min followed by measuring the DCF-derived fluorescence. Values represent means ± SEM, $n = 3$. In panel A, * significantly different from control; # significantly different from 10 μM D3T; & significantly different from 25 μM D3T. In panel B, * significantly different from control (without D3T and doxorubicin); # significantly different from "doxorubicin".

mitochondrial enzymes is a critical mechanism for HNE-mediated cardiocytotoxicity (29). The increased resistance of D3T-pretreated human cardiomyocytes to HNE toxicity may also result from the induction of AR. Previous studies demonstrated that AR was a secondary defense mechanism for HNE in rat cardiomyocytes (18).

Cardiotoxicity is a major adverse effect that limits the clinical use of doxorubicin in cancer patients (30). This study for the first time showed that D3T pretreatment conferred marked protection of human cardiomyocytes

against doxorubicin-induced cytotoxicity. Oxidative stress is believed to be a major mechanism underlying doxorubicin cardiotoxicity (20, 21). In this context, ROS, peroxynitrite, as well as HNE are implicated in doxorubicin-induced cardiac cell injury. Indeed, overexpression of SOD and use of scavengers of ROS and peroxynitrite were found to protect against doxorubicin cardiotoxicity in vivo and/or in vitro (31–33). Thus, the coordinated induction of the many antioxidant enzymes most likely accounted for the increased resistance of the D3T-pretreated cardiomyocytes to doxorubicin toxicity.

In summary, this study demonstrates that a number of cellular and mitochondrial antioxidants and phase 2 enzymes in human cardiomyocytes are induced by cruciferous D3T at low micromolar concentrations. This chemoprotectant-mediated coordinated upregulation of cellular defenses is accompanied by remarkably increased resistance of the cardiomyocytes to oxidative and electrophilic stress, as well as doxorubicin toxicity. These findings may provide a mechanism for the decreased risk of cardiovascular events associated with consumption of cruciferous vegetables rich in organosulfur compounds (4, 5), and set a stage for development of a cruciferous dithiolethione-based strategy for protecting against doxorubicin cardiotoxicity in cancer patients. It should be noted that both the induction of endogenous myocardial antioxidants and phase 2 enzymes by cruciferous dithiolethiones and the direct scavenging of ROS by other dietary antioxidants appear to be effective approaches to the intervention of cardiovascular diseases. In this context, extensive animal studies have conclusively demonstrated the ability of a wide variety of direct antioxidant compounds from diet, including vitamins C and E to protect against cardiovascular disorders (34, 35). Thus, studies on both of the above aspects of novel dietary components will contribute to the development of more effective strategies for protecting against human cardiovascular diseases in general.

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