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Chemical Induction of Cellular Antioxidants Affords Marked Protection against Oxidative Injury in Vascular Smooth Muscle Cells

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Extensive evidence suggests that reactive oxygen species are critically involved in the pathogenesis of cardiovascular diseases, such as atherosclerosis and myocardial ischemia–reperfusion injury. Consistent with this concept, administration of exogenous antioxidants has been shown to be protective against oxidative cardiovascular injury. However, whether induction of endogenous antioxidants by chemical inducers in vasculature also affords protection against oxidative vascular cell injury has not been extensively investigated. In this study, using rat aortic smooth muscle A10 cells as an *in vitro* system, we have studied the induction of cellular antioxidants by the unique chemoprotector, [^{3}H]-1,2-dithiole-3-thione (D3T) and the protective effects of the D3T-induced cellular antioxidants against oxidative cell injury. Incubation of A10 cells with micromolar concentrations of D3T for 24 h resulted in a significant induction of a battery of cellular antioxidants in a concentration-dependent manner. These included reduced glutathione (GSH), GSH peroxidase, GSSG reductase, GSH *S*-transferase, superoxide dismutase, and catalase. To further examine the protective effects of the induced endogenous antioxidants against oxidative cell injury, A10 cells were pretreated with D3T and then exposed to either xanthine oxidase (XO)/xanthine, 4-hydroxynonenal, or cadmium. We observed that D3T pretreatment of A10 cells led to significant protection against the cytotoxicity induced by XO/xanthine, 4-hydroxynonenal or cadmium, as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium reduction assay. Taken together, this study demonstrates for the first time that a number of endogenous antioxidants in vascular smooth muscle cells can be induced by exposure to D3T, and that this chemical induction of cellular antioxidants is accompanied by markedly increased resistance to oxidative vascular cell injury.

Key Words: **antioxidants; reactive oxygen species; [^{3}H]-1,2-dithiole-3-thione; vascular smooth muscle; xanthione oxidase; 4-hydroxynonenal; cadmium.**

Abbreviations used: ROS, reactive oxygen species; GSH, reduced glutathione; GST, GSH *S*-transferase; SOD, superoxide dismutase; GSSG, oxidized glutathione; D3T, [^{3}H]-1,2-dithiole-3-thione; XO, xanthine oxidase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CDNB, 1-chloro-2,4-dinitrobenzene; BSA, bovine serum albumin; FBS, fetal bovine serum; PBS, phosphate buffered saline.

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Cardiovascular diseases remain a leading cause of morbidity and mortality of the human population (1, 2). Extensive studies over the last several years have suggested that oxidant and/or electrophilic stress may be critically involved in the pathogenesis of human cardiovascular diseases, particularly arterial atherosclerosis (1–3). In this regard, reactive oxygen species (ROS) produced by various vascular and blood cells have been implicated in the pathological processes leading to production of atherosclerotic plaques (3). On the other hand, electrophilic stress induced by reactive aldehydes and environmental toxicants, such as cadmium has also been suggested to play a role in the development of human atherosclerotic lesions (4–6). Four major types of cells have been shown to be involved in the development of atherosclerotic plaques. They are vascular endothelial cells, monocytes/macrophages, lymphocytes, and vascular smooth muscle cells (7, 8). Among the above cell types, smooth muscle cells have been demonstrated to play a critical role in the production and stability of atherosclerotic plaques (9, 10). Indeed, smooth muscle cells are a major type of cells present in atherosclerotic plaques (7–10). Both ROS and electrophiles, including reactive aldehydes and cadmium, can cause dysregulated growth and death of vascular smooth muscle cells,

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presumably contributing to the pathogenesis of atherosclerotic lesions, as well as other vascular disorders (3, 4, 8, 11). The involvement of an oxidative process in atherosclerosis was further supported by recent observations that glutathione-related antioxidant defenses were markedly reduced in human atherosclerotic plaques (12).

By understanding the involvement of ROS/electrophilic stress in cardiovascular cell pathology, it has become possible to devise rational strategies to prevent or modify this oxidative cellular damage. In this regard, administration of a variety of exogenous antioxidants, including antioxidant vitamins, has been shown to be protective against oxidative cardiovascular injury in animal models and/or human trials (13, 14). However, a major drawback associated with using exogenous antioxidants is the limited cell permeability and short half-life of these molecules *in vivo*, which may contribute to the inconsistency in protection against oxidative cardiovascular injury (13). Another strategy to protect against oxidative cardiovascular injury, including atherosclerosis, may be to increase the endogenous cellular antioxidants via the use of chemical inducers, such as 1,2-dithiole-3-thiones in vasculature. 1,2-Dithiole-3-thiones are five-membered cyclic sulfur-containing compounds, some of which are constituents of cruciferous vegetables (15). Several substituted 1,2-dithiole-3-thiones exhibit chemotherapeutic, radioprotective, and chemoprotective properties with little side effects (16–18). Extensive studies over the last several years have demonstrated that 1,2-dithiole-3-thiones, particularly 3H-1,2-dithiole-3-thione (D3T), are potent inducers of enzymes important to electrophile detoxification, notably, glutathione *S*-transferase (GST), NADPH:quinone reductase, epoxide hydratase, and UDP-glucuronosyltransferase in liver cells (19, 20). In addition, D3T has also recently been reported to induce several cellular antioxidative enzymes, including γ-glutamylcysteine synthetase, Mn-superoxide dismutase (SOD) and catalase, in rodent hepatic tissue (21, 22). However, the inducibility of endogenous antioxidants in vascular cells by D3T, and the protective effects of the chemically induced cellular antioxidants on oxidative vascular cell injury have not been previously investigated. In this study, using rat aortic smooth muscle A10 cells as an *in vitro* system, we have investigated the induction by D3T of cellular antioxidants, including reduced glutathione (GSH), GSH peroxidase, GSSG reductase, GST, SOD and catalase, and their protective effects on oxidant/electrophile-induced cellular injury. Our results demonstrate for the first time that a battery of cellular antioxidants can be induced by D3T in cultured A10 cells, which is accompanied by dramatically increased resistance to cellular injury caused by ROS, 4-hydroxynonenal and cadmium.

MATERIALS AND METHODS

Materials. D3T was a kind gift from Dr. James Crowell at National Institute of Cancer. Xathine oxidase (XO), xanthine, *tert*-butylhydroperoxide, oxidized glutathione (GSSG), GSSG reductase, 1-chloro-2,4-dinitrobenzene (CDNB), GSH, *o*-phthalaldehyde, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cadmium chloride, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxynonenal was from Cayman Chem. (Ann Arbor, MI). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline (PBS) were from Gibco Life Technologies (Grand Island, NY). Tissue culture flasks and 24-well tissue culture plates were from Corning Inc. (Corning, NY).

Cell culture. Rat aortic A10 cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in 150 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were fed every 2–3 days, and subcultured once they reached 90–100% confluence.

Assay of cellular GSH. The cellular GSH content was measured according to the method of Hissin and Hilf, which is specific for the determination of GSH at pH 8.0 (23). The procedures described previously by Zhu *et al.* (24) were followed. In brief, cells were pelleted by centrifugation and washed once in PBS. To the cell pellet, 50 μl of 25% HPO_3 and 188 μl of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0 were added, and then the cells were homogenized on ice. After homogenization, the samples were centrifuged at 13,000*g* for 5 min at 4°C. The supernatant was diluted 10 times with above phosphate buffer; 0.1 ml of diluted sample was incubated with 0.1 ml of *o*-phthalaldehyde solution (0.1% in methanol) and 1.8 ml of phosphate buffer for 15 min at room temperature. Fluorescence was then read with 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 standard curve and expressed as nanomoles of GSH per milligram of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method of Bradford (25) with BSA as the standard.

Assay of cellular GSH peroxidase. GSH peroxidase activity was measured by the method described by Flohe and Gunzler (26). Briefly, the cells were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% Triton X-100 on ice. The homogenate was kept on ice for subsequent measurement of GSH peroxidase activity within 1 h. To an assay cuvette containing 0.5 ml of 50 mM potassium phosphate (pH 7.0) and 1 mM EDTA, 100 μl of sample, 100 μl of 10 mM GSH, 100 μl of GSSG reductase (2.4 U/ml) and 100 μl of 1.5 mM NADPH were added. The cuvette was incubated at 37°C for 5 min. After addition of 100 μl of 12 mM *tert*-butylhydroperoxide, 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 assay 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. GSH peroxidase activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nmoles of NADPH consumed per min per milligram of cellular protein.

Assay of cellular GSSG reductase. GSSG reductase activity was measured by the method of Wheeler *et al.* (27). Briefly, the cells were homogenized as described in the above GSH peroxidase assay. To an assay cuvette containing 0.7 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA, 100 μl of sample and 100 μl of 20 mM GSSG were added. The reaction was started by adding 100 μl of

1.5 mM NADPH. The subsequent consumption of NADPH was monitored at 340 nm, 37°C for 5 min. The GSSG reductase activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per m per milligram of cellular protein.

Assay of cellular GST. Cellular GST activity was measured according to the method of Habig *et al.* (28) using CDNB as a substrate. Briefly, the cells were homogenized in 10 mM sodium phosphate buffer, pH 6.5 on ice. The homogenate was then centrifuged at 13,000*g* for 5 min at 4°C. The resulting supernatant was collected and kept on ice for subsequent measurement of GST activity within 1 h. The reaction mixture (to be freshly prepared) contained 1 mM GSH, 1 mM CDNB and 3 mg/ml of BSA in 0.1 M sodium phosphate buffer, pH 6.5. 0.95 ml of the above reaction mixture was added to each cuvette. The reaction was started by adding 50 μl of sample, and the reduction of CDNB was monitored at 340 nm, 25°C for 5 min. The GST activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}, and expressed as nmoles of CDNB reduced per min per milligram of cellular protein.

Assay of cellular SOD. Total cellular SOD activity was determined by the method of Spitz and Oberley (29) with slight modifications. Briefly, Cells were homogenized in 50 mM potassium phosphate buffer (pH 7.8) on ice. The reaction mixture (to be 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, 0.05 mM bathocuproinedisulfonic acid, and 0.13 mg/ml BSA. 0.8 ml of the reaction mixture was added to each cuvette, followed by addition of 100 μl of sample. The reaction was started by adding 100 μl of xanthine oxidase (0.05 U/ml). The formation of formazan blue was monitored at 560 nm, 25°C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve, and expressed as units per milligram of cellular protein.

Assay of cellular catalase. The method of Aebi was used to measure the catalase activity (30). In brief, the cells were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 on ice. To a quartz cuvette, 0.65 ml of 50 mM potassium phosphate buffer 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 micromoles of H_2O_2 consumed per m per milligram of cellular protein.

MTT reduction assay. Cell viability was determined by a slightly modified MTT cytotoxicity assay as originally described by Mosmann (31). In brief, A10 cells (4×10^4 cells/well in 0.5 ml culture medium) were plated into 24-well tissue culture plates. After incubation of the cells with XO/xanthine, 4-hydroxynonenal or cadmium 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 for another 2 h at 37°C. Media were removed and wells were rinsed twice with PBS. To each well 60 μl of DMSO, 240 μl of isopropanol and 300 μl of deionized water were added at room temperature to solubilize the formazan crystals. The dissolved formazan was then transferred into semi-microcuvettes, and the absorbance measured at 570 nm.

Statistical analyses. All data are expressed as means ± SEM. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) or Student's *t* test. Statistical significance was considered at $P < 0.05$.

RESULTS

Induction of cellular GSH and GSH-related antioxidant enzymes by D3T. Although D3T has been reported to induce several cellular antioxidant enzymes in hepatic tissue (21, 22), the inducibility of antioxidants in vasculature, such as smooth muscle cells, by D3T has not been previously investigated. As shown in Fig. 1, incubation of aortic smooth muscle A10 cells with 25–100 μM D3T for 24 h resulted in a significant induction of cellular GSH, GSH peroxidase, GSSG reductase and GST in a concentration-dependent manner. A significant induction of cellular GSH and GST was observed with 25 μM D3T. A 2- to 3-fold increase in cellular GSH, GSH peroxidase and GST was noted with 100 μM D3T (Fig. 1). In contrast to the induction of GSH, GSH peroxidase and GST, incubation of A10 cells with 50 and 100 μM D3T only resulted in a 30% and 37% increase (both were statistically significant) in cellular GSSG reductase, respectively (Fig. 1).

Induction of cellular SOD and catalase by D3T. Besides GSH and GSH-associated antioxidative enzymes, SOD and catalase are also key cellular antioxidant defenses. As shown in Fig. 2, incubation of A10 cells with 100 μM D3T led to a 1.7-fold increase in total cellular SOD. A significant induction of cellular catalase was observed with 25 μM D3T. A 2-fold induction of cellular catalase was achieved following incubation of A10 cells with 100 μM D3T (Fig. 2).

Protective effects of D3T pretreatment on XO/xanthine-, 4-hydroxynonenal- or cadmium-induced cell injury. GSH, GSH peroxidase, GSSG reductase, SOD and catalase are principal cellular antioxidants, which act coordinately to protect cells against ROS-mediated injury (32, 33). We examined if induction of the above cellular antioxidants by D3T in A10 cells also afforded protection against ROS-mediated toxicity. To this end, A10 cells were pretreated with 100 μM D3T for 24 h and then exposed to XO/xanthine, a ROS-generating system, for another 24 h. As shown in Fig. 3, incubation of A10 cells with various concentrations of XO in the presence of 0.5 mM xanthine for 24 h resulted in a significant decrease of cell viability in a concentration-dependent manner. Pretreatment of A10 cells with D3T led to significant protection against XO/xanthine-induced decrease of cell viability (Fig. 3).

Since both GSH and GST have been shown to be major cellular defenses against reactive aldehyde-induced cell injury (34, 35), we determined if D3T-pretreated cells also acquired increased resistance to toxicity induced by 4-hydroxynonenal. As shown in Fig. 4, incubation of A10 cells with 4-hydroxynonenal at 5 and 10 μM for 24 h did not cause any significant decrease of cell viability. However, 4-hydroxynonenal at 15–25 μM induced a marked decrease of cell viability in a concentration-dependent fashion. Pretreatment of A10 cells with D3T resulted in great protection against 4-hydroxynonenal-induced decrease of cell viability (Fig. 4).

In view of the critical involvement of cellular glutathione in the detoxification of cadmium, a cardiovascular toxin (5, 36), in mammalian cells, we determined if induction of cellular GSH by D3T also offered protec-

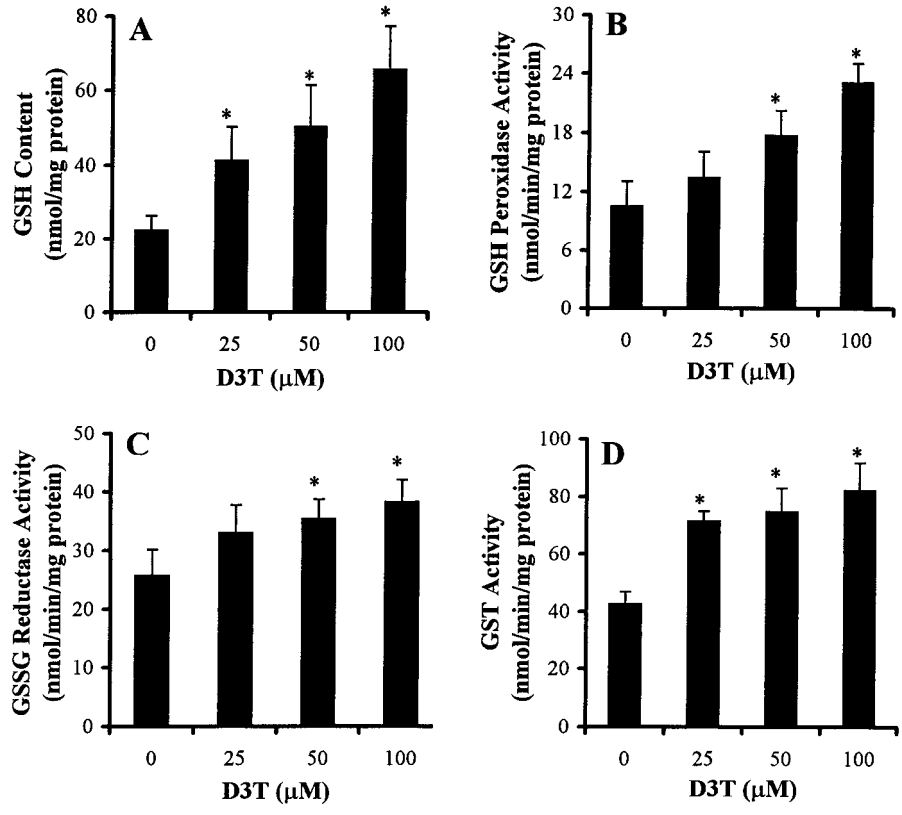

FIG. 1. Induction of cellular GSH (A), GSH peroxidase (B), GSSG reductase(C) and GST (D) by D3T in A10 cells. A10 cells were incubated with the indicated concentrations of D3T in DMEM supplemented with 10% FBS at 37°C for 24 h. Cellular GSH content, and GSH peroxidase, GSSG reductase and GST activity were measured as described under Materials and Methods. Values represent means ± SEM from at least 3 independent experiments. *, significantly different from 0 μM D3T.

tive effects on cadmium-induced A10 cell injury. As shown in Fig. 5, incubation of A10 cells with cadmium (2.5–15 μM) for 24 h caused a concentration-dependent decrease in cell viability. Similar to the protective effects on ROS- and 4-hydroxynonenal-induced cell toxicity (Figs. 3 and 4), D3T pretreatment of A10 cells also resulted in significant protection against cadmium-induced decrease of cell viability (Fig. 5).

DISCUSSION

ROS, including superoxide and H_2O_2, can be generated by a number of cellular sources (32, 33). Because ROS are reactive to cellular macromolecules, including lipids, proteins and nucleic acids, mammalian cells have evolved a variety of antioxidative enzymes to detoxify the ROS produced during normal cellular metabolism and/or by various pathophysiological processes (32, 33). Among these cellular antioxidants, SOD, catalase, GSH, GSH peroxidase, and GSSG reductase have received extensive studies (32, 33). SOD converts superoxide into H_2O_2 and molecular oxygen. Since H_2O_2 is still toxic to the cells, catalase and GSH peroxidase further catalyze the decomposition of H_2O_2 to water. In the reaction catalyzed by GSH peroxidase, GSH is oxidized to GSSG, which can be reduced back to GSH via the action of GSSG reductase (32, 33).

The protective effects of the above cellular antioxidants in oxidative cardiovascular injury have been investigated over the last decade. For example, overexpression of SOD, catalase, and GSH peroxidase in transgenic models was reported to afford protection against myocardial ischemia-reperfusion injury and/or atherosclerotic lesions (37–40). However, use of the above transgenic approach to protect against oxidative cardiovascular injury in humans is not yet feasible. As such, extensive studies have focused on identification of exogenous antioxidants, including antioxidative vitamins, that can be administrated to protect against oxidative cardiovascular injury in both experimental animals and humans (13, 14). As aforementioned, another way to protect against oxidative cardiovascular injury is to chemically induce the endogenous antioxidative enzymes in cardiovascular cells. However, studies on the chemical induction of endogenous antioxidants in vascular cells are currently lacking. The results in this study demonstrate for the first time that a number of cellular antioxidants, including GSH,

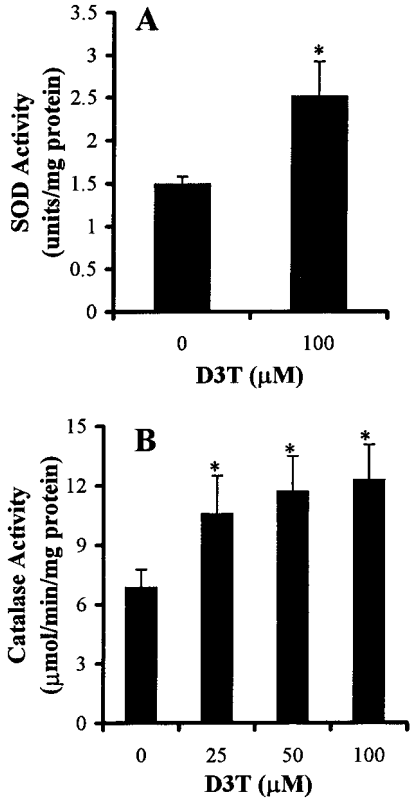

FIG. 2. Induction of cellular SOD and catalase by D3T in A10 cells. A10 cells were incubated with the indicated concentrations of D3T in DMEM supplemented with 10% FBS at 37°C for 24 h. Cellular SOD and catalase activity were measured as described under Materials and Methods. Values represent means ± SEM from at least 3 independent experiments *, significantly different from 0 μM D3T.

GSH peroxidase, GSSG reductase, GST, SOD and catalase, can be induced by D3T at micromolar concentrations in rat aortic A10 cells, a commonly used model of vascular smooth muscle cells (41, 42). The induction of cellular antioxidants by D3T in A10 cells was accompanied by markedly increased resistance to oxidative injury caused by XO/xanthine (Fig. 3), a system that produces both superoxide and H_2O_2, and that has been implicated in various cardiovascular pathological processes (2). In addition, this study also for the first time revealed two additional D3T-inducible cellular antioxidant enzymes: GSH peroxidase and GSSG reductase (Fig. 1). Induction of these two enzymes by D3T has not been previously reported in any types of cells/tissues. As mentioned above, GSH peroxidase and catalase are two critical cellular enzymes involved in the decomposition of H_2O_2, and have been demonstrated to protect against oxidative cardiovascular cell injury in animals and/or cell cultures (37–40). The increased resistance of the D3T-pretreated A10 cells to XO/xanthine-mediated injury may thus be largely attributable to the simultaneous induction of these two H_2O_2-detoxification enzymes plus SOD and GSH by D3T (Figs. 1 and 2). Furthermore, the induction of GSSG reductase by D3T may lead to increased regeneration of GSH from GSSG produced during GSH peroxidase-catalyzed decomposition of H_2O_2 in A10 cells. GSH is also a cofactor for GST, an abundant cellular enzyme in mammalian tissues (42).

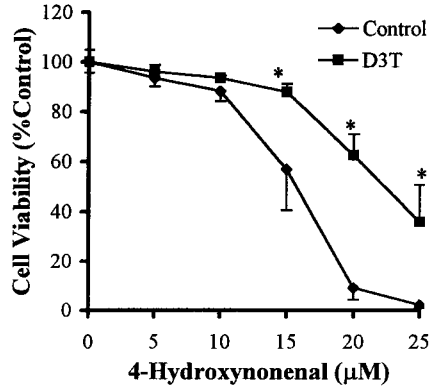

FIG. 3. Effects of D3T pretreatment on XO/xanthine-induced cytotoxicity in A10 cells. A10 cells (4×10^4 cells/well in 0.5 ml culture medium) were incubated with or without 100 μM D3T in 24-well culture plates in DMEM supplemented with 10% FBS at 37°C for 24 h. The media were then removed and cells washed once with DMEM supplemented with 0.5% FBS (0.5% FBS medium). The cells were continuously cultured in the 0.5% FBS media containing the indicated concentrations of XO in the presence of 0.5 mM xanthine for another 24 h. Following the incubation with XO/xanthine, cell viability was determined using MTT reduction assay as described under Materials and Methods. Values represent means ± SEM from at least 3 independent experiments. *, significantly different from the respective control group.

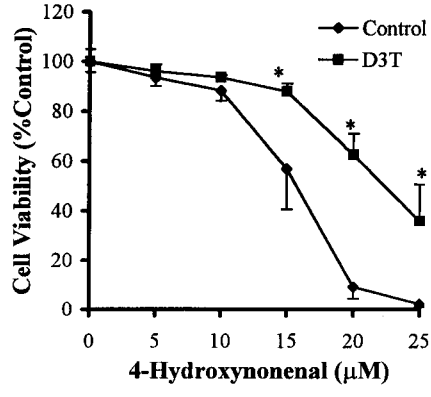

FIG. 4. Effects of D3T pretreatment on 4-hydroxynonenal-induced cytotoxicity in A10 cells. A10 cells (4×10^4 cells/well in 0.5 ml culture medium) were incubated with or without 100 μM D3T in 24-well culture plates in DMEM supplemented with 10% FBS at 37°C for 24 h. The media were then removed and cells washed once with DMEM supplemented with 0.5% FBS (0.5% FBS medium). The cells were continuously cultured in the 0.5% FBS media containing the indicated concentrations of 4-hydroxynonenal for another 24 h. Following the incubation with 4-hydroxynonenal, cell viability was determined using MTT reduction assay as described under Materials and Methods. Values represent means ± SEM from at least 3 independent experiments. *, significantly different from the respective control group.

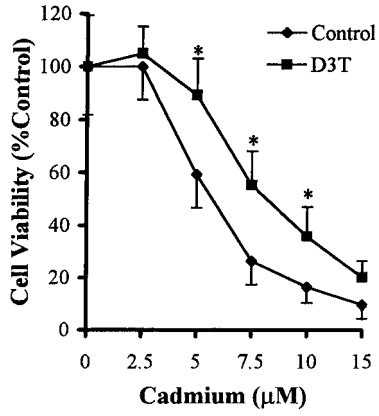

FIG. 5. Effects of D3T pretreatment on cadmium-induced cytotoxicity in A10 cells. A10 cells (4×10^4 cells/well in 0.5 ml culture medium) were incubated with or without 100 μM D3T in 24-well culture plates in DMEM supplemented with 10% FBS at 37°C for 24 h. The media were then removed and cells washed once with DMEM supplemented with 0.5% FBS (0.5% FBS medium). The cells were continuously cultured in the 0.5% FBS media containing the indicated concentrations of cadmium chloride ($CdCl_2$) for another 24 h. Following the incubation with $CdCl_2$, cell viability was determined using MTT reduction assay as described under Materials and Methods. Values represent means ± SEM from at least 3 independent experiments. *, significantly different from the respective control group.

GST is generally viewed as a phase II enzyme, primarily involved in the detoxification of electrophilic xenobiotics via catalyzing the formation of GSH-electrophile conjugation (42). Recent studies have also demonstrated that GST plays an important role in protecting cells against ROS-mediated injury through catalyzing the decomposition of lipid hydroperoxides derived from ROS-induced cellular lipid peroxidation (43, 44). Accordingly, the induction of GST by D3T in A10 cells may also contribute to the increased resistance of the D3T-pretreated cells to XO/xanthine-caused toxicity.

Pretreatment of A10 cells with D3T also resulted in increased resistance to 4-hydroxynonenal-induced cytotoxicity (Fig. 4). 4-Hydroxynonenal can be produced by oxidation of low-density lipoprotein (4). 4-Hydroxynonenal is known to stimulate vascular smooth muscle cell growth at lower concentrations and elicit smooth muscle cell apoptosis at higher levels, and has been implicated in the pathogenesis of atherosclerosis (4). Both GSH and GST are principal defenses against 4-hydroxynonenal-induced cell injury (34, 35). In this regard, Hubatsch *et al.* (45) recently reported that an alpha class of human GST exhibited high catalytic efficiency in the conjugation of 4-hydroxynonenal. Thus, it appears that induction of GSH and GST by D3T may largely account for the increased resistance of D3T-pretreated cells to 4-hydroxynonenal-induced cytotoxicity (Fig. 4).

Epidemiological studies have suggested that exposure to cadmium is associated with an increased incidence of atherosclerosis in humans (5). In animal models, cadmium has been reported to induce atherosclerotic lesions (46). Several recent studies have shown that vascular smooth muscle cells preferentially accumulate cadmium and may thus represent a major cellular target of cadmium-induced vascular injury (11, 47, 48). More recently, Abu-Hayyes *et al.* (49). Further reported cadmium accumulation in the media smooth muscle in aortas of smokers, which might contribute to the degenerative aortic disease in smokers. Regarding the cellular metabolism of cadmium, early studies have demonstrated that GSH is a major cellular defense against cadmium toxicity (36). In this context, cadmium toxicity in various cell cultures has been shown to be modulated by the alteration of intracellular GSH levels (50). As such, the increased resistance of D3T-pretreated A10 cells to cadmium toxicity observed in this study (Fig. 5) most likely resulted from the elevated levels of intracellular GSH.

In summary, this study demonstrates that a number of endogenous antioxidants in cultured aortic smooth muscle cells can be induced by exposure to D3T, and that this chemical-mediated induction of cellular antioxidants is accompanied by markedly increased resistance to oxidative cell injury induced by ROS as well as 4-hydroxynonenal and cadmium. As mentioned above, efficient detoxification of ROS requires the coordinative actions of various cellular antioxidants. Therefore, simultaneous induction of a battery of key cellular antioxidant enzymes by D3T in vascular cells may represent an effective approach to protecting against oxidative vascular pathogenesis.

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