

Coordinated Upregulation of a Series of Endogenous Antioxidants and Phase 2 Enzymes as a Novel Strategy for Protecting Renal Tubular Cells from Oxidative and Electrophilic Stress

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In view of the crucial involvement of oxidative and electrophilic stress in various kidney disorders, this study was undertaken to test the hypothesis that pharmacologically-mediated coordinated upregulation of endogenous renal antioxidants and phase 2 enzymes is an effective strategy for renal protection. Notably, studies on the pharmacological inducibility of a series of antioxidants and phase 2 enzymes in renal tubular cells are lacking. Here we reported that incubation of normal rat kidney (NRK-52E) proximal tubular cells with low micromolar concentrations (10–50 μ M) of the cruciferous nutraceutical, 1,2-dithiole-3-thione (D3T), led to a significant concentration-dependent induction of a wide spectrum of antioxidants and phase 2 enzymes, including catalase (CAT), reduced form of glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), and heme oxygenase (HO). We further observed that D3T treatment also increased the protein and mRNA expression for CAT, γ -glutamylcysteine ligase, GR, GST-A, GST-M, NQO1, and HO-1. Incubation of the renal tubular cells with H₂O₂, SIN-1-derived peroxynitrite, or 4-hydroxy-2-nonenal led to concentration-dependent decreases in cell viability. Pretreatment of the renal tubular cells with 10–50 μ M

D3T afforded remarkable protection against the nephrocytotoxicity elicited by the above oxidative and electrophilic species. The D3T-mediated cytoprotection showed a concentration-dependent relationship. Taken together, this study for the first time comprehensively characterized the inducibility by a unique nutraceutical of a wide spectrum of antioxidative and phase 2 defenses in renal tubular cells at the levels of enzyme activity as well as protein and mRNA expression, and demonstrated that such a coordinated upregulation of cellular defenses led to remarkable protection of renal tubular cell from oxidative and electrophilic stress. Because of the crucial role of oxidative and electrophilic stress in inflammatory injury, D3T-mediated coordinated induction of endogenous antioxidative and phase 2 defenses may also serve as an important anti-inflammatory mechanism in kidneys. *Exp Biol Med* 233:753–765, 2008

Key words: antioxidants; phase 2 enzymes; 1,2-dithiole-3-thione; renal tubular cells; oxidative stress; electrophilic stress

Introduction

Over twenty million American adults have kidney diseases. The number of people diagnosed with kidney diseases has doubled each decade for the last two decades. Each year, kidney diseases kill more than 14 people out of every 100,000, making them America's ninth leading cause of death (1). Although the mechanisms underlying kidney diseases are complex and diverse, substantial evidence supports a critical involvement of reactive oxygen species (ROS) and electrophilic species in the pathogenesis of various kidney disorders, including renal ischemia-reperfusion injury, kidney transplantation rejection, as well as drug-induced nephrotoxicity (2–7).

ROS include superoxide (O₂^{•-}), hydrogen peroxide

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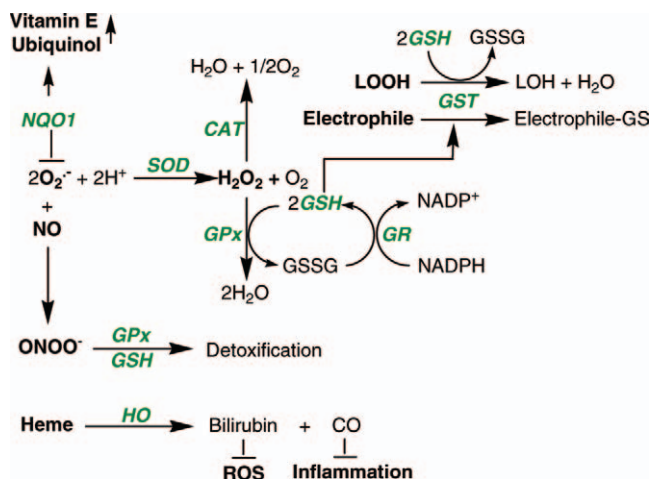


Figure 1. Schematic illustration of the coordinated actions of antioxidants and phase 2 enzymes in detoxification of oxidative and electrophilic species, as well as in protecting against inflammatory stress. LOOH, lipid hydroperoxide; LOH, lipid alcohol; electrophile-GS, GSH conjugate of electrophilic species. A color version of this figure is available in the online journal.

(H_2O_2), hydroxyl radical ($\cdot OH$), lipid peroxy radical ($LO_2^{\cdot -}$), lipid hydroperoxide (LOOH), and peroxynitrite ($ONOO^-$). $ONOO^-$ along with other nitrogen-containing reactive species is more commonly classified as reactive nitrogen species (RNS). ROS/RNS attack cellular constituents, including lipids and proteins, leading to the formation of secondary reactive species, such as electrophilic aldehydes (8–10). Due largely to the detrimental nature of ROS/RNS, mammalian cells have evolved a number of antioxidants and phase 2 enzymes to protect against oxidative cell damage (Fig. 1). As illustrated in Figure 1, superoxide dismutase (SOD) catalyzes the dismutation of superoxide to form H_2O_2 . The H_2O_2 is further decomposed to water by catalase (CAT) or glutathione peroxidase (GPx). During GPx-catalyzed decomposition of H_2O_2 , the reduced form of glutathione (GSH) is oxidized to GSSG, which can then be reduced to GSH by glutathione reductase (GR). On the other hand, glutathione S-transferase (GST) utilizing GSH as a cofactor is crucially involved in detoxification of organic hydroperoxides and other electrophiles, such as reactive aldehydes derived from lipid peroxidation (11). GSH (or GSH plus GSH peroxidase) is also a major cellular factor for detoxification of $ONOO^-$ (12). NAD(P)H: quinone oxidoreductase 1 (NQO1) catalyzes the two-electron reduction of electrophilic quinone compounds, thus limiting the formation of semiquinone radicals through one-electron reduction, and the subsequent generation of ROS (13). Moreover, NQO1 has been suggested to maintain the cellular levels of ubiquinol and vitamin E, two important antioxidants (13). Recently, NQO1 is also found to be able to scavenge superoxide (14, 15). HO metabolizes heme to form bilirubin and carbon monoxide (CO). Bilirubin is a potent ROS-scavenger, whereas CO has anti-inflammatory/antioxidative effects (16–18). In view of the complementary

effects of various antioxidants and phase 2 enzymes in detoxification of ROS and other reactive species (Fig. 1), the coordinated actions of a spectrum of cellular antioxidative and phase 2 defenses are essential for protecting against oxidative and electrophilic stress in mammalian tissues, including kidneys.

The increasing recognition of the causal role for oxidative and electrophilic stress in the pathophysiological processes of various kidney disorders has led to extensive investigation on the potential renal protective effects of exogenous antioxidative compounds. For example, antioxidant vitamins and flavonoids have been used for intervention of kidney disorders, including ischemia-reperfusion injury, transplantation rejection, and cyclosporine A-induced nephrotoxicity (19–23). However, using exogenous antioxidants for renal protection has yielded inconsistent results, which might be related to the pro-oxidative properties of these compounds (20, 21). In addition to the use of exogenous antioxidative compounds, overexpression of endogenous antioxidative proteins by genetic approaches has also been applied to the protection of oxidative renal injury in transgenic mice. For instance, it has been reported that overexpression of Cu, ZnSOD, HO-1, or GPx in mice attenuates renal ischemia-reperfusion injury (23–25). However, at the present time using the above genetic approaches for renal protection in humans is not practical. As aforementioned, efficient detoxification of ROS/RNS and other reactive intermediates relies on the coordinated actions of various endogenous antioxidants and phase 2 enzymes in mammalian cells (Fig. 1). In this context, an innovative strategy for effectively protecting against oxidative/electrophilic renal injury may be through pharmacologically-mediated coordinated upregulation of a spectrum of endogenous antioxidants and phase 2 enzymes in kidneys. Such a novel strategy relies on a profound understanding of both constitutive and inducible expression of a series of renal antioxidants and phase 2 enzymes. Accordingly, in this study we have used normal rat kidney (NRK-52E) proximal tubular cells as a model system to determine the constitutive and inducible expression of a wide spectrum of antioxidants and phase 2 enzymes, and the significance of upregulation of these cellular defenses in protecting renal tubular cells from oxidative and electrophilic stress.

Materials and Methods

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). Anti- γ -glutamylcysteine ligase (GCL) antibody was from Lab Vision (Fremont, CA). Anti-GR antibody was from Abcam (Cambridge, MA). Anti-GST-A, -M, and -P antibodies were from Alpha Diagnostic (San Antonio, TX). Anti-NQO1 and β -actin antibodies were from

Santa Cruz Biotech (Santa Cruz, CA). Anti-HO-1 antibody was from Stressgen (Ann Arbor, MI). All other chemicals and agents were from Sigma-Aldrich (St. Louis, MO).

Culture of NRK-52E Cells. The cloned normal rat renal NRK-52E cells, which maintain characteristics of normal renal proximal tubular cells (26), were obtained from American Type Culture Collection (Manassas, VA). The renal tubular cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in 75 or 150 cm² tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂. The cells were fed every 3–4 days and subcultured once they reached 80–90% confluence.

Preparation of Cell Extract for Measurement of Antioxidants and Phase 2 Enzymes. The renal tubular 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 assay dye (Hercules, CA) using bovine serum albumin as the standard. The supernatants were then used for measurement of the antioxidants and phase 2 enzymes except for HO.

Measurement of Cellular SOD Activity. The total cellular SOD activity was determined by the method of Spitz and Oberley (27) with slight modifications, as described before (28). This method is based on the inhibition of the superoxide-mediated reduction of nitroblue tetrazolium to formazan by SOD. In brief, the reaction mix contained in 50 mM potassium phosphate buffer, pH 7.8, 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/ml CAT, 70 µM nitroblue tetrazolium, 0.2 mM xanthine, 50 µM bathocuproinedisulfonic acid, and 0.13 mg/ml bovine serum albumin. The reaction mix (0.8 ml) was added to each cuvette, followed by addition of 100 µl of sample. The cuvettes were pre-warmed at 37°C for 3 min. The reaction was then initiated by adding 100 µl of xanthine oxidase (0.1–0.2 U/ml). The formation of formazan blue was measured at 560 nm, 37°C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma-Aldrich) standard curve, and expressed as units per milligram of cellular protein.

Measurement of Cellular CAT Activity. The cellular CAT activity was measured according to the method of Aebi (29). 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 H₂O₂. The decomposition of H₂O₂ was monitored at 240 nm, 25°C for 2 min. The cellular CAT activity was expressed as micromoles of H₂O₂ consumed per minute per milligram of cellular protein.

Measurement of Cellular GSH Content. The cellular GSH content was measured according to the *o*-phthalaldehyde-based fluorometric method, which is spe-

cific for the determination of GSH at pH 8.0 (28). Briefly, 10 µl of the sample was incubated with 12.5 µl of 25% metaphosphoric acid, and 37 µl of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0 at 4°C for 10 min. The samples were centrifuged at 13,000 g for 5 min at 4°C. The resulting supernatant (10 µl) was incubated with 0.1 ml of *o*-phthalaldehyde solution (0.1% in methanol) and 1.89 ml of the above phosphate buffer for 15 min at room temperature. Fluorescence intensity was then measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The cellular GSH content was calculated using a GSH (Sigma-Aldrich) standard curve, and expressed as nanomoles of GSH per milligram of cellular protein.

Measurement of Cellular GR Activity. The method based on the NADPH consumption coupled with the reduction of oxidized form of glutathione (GSSG) to GSH by GR, as described before (28) was followed to measure the cellular GR activity. Briefly, to an assay cuvette containing 0.46 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA, 20 µl of sample and 60 µl of 20 mM GSSG were added. The cuvettes were pre-warmed at 37°C for 3 min. The reaction was started by adding 60 µl of 1.5 mM NADPH (prepared in 0.1% NaHCO₃). The subsequent consumption of NADPH was monitored at 340 nm, 37°C for 5 min. The cellular GR activity was calculated using the extinction coefficient of 6.22 mM⁻¹cm⁻¹ and was expressed as nanomoles of NADPH consumed per minute per milligram of cellular protein.

Measurement of Cellular GPx Activity. The cellular GPx activity was measured based on the formation of GSSG from GPx-catalyzed oxidation of GSH by H₂O₂, coupled with NADPH consumption in the presence of exogenously added GR (30). In brief, to an assay cuvette containing 0.34 ml of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA and 2 mM sodium azide, 20 µl of sample, 60 µl of 10 mM GSH, 60 µl of glutathione reductase (2.4 U/ml) and 60 µl of 1.5 mM NADPH (prepared in 0.1% NaHCO₃) were added. The cuvette was incubated at 37°C for 3 min. After addition of 60 µl of 2 mM H₂O₂, the rate of NADPH consumption was monitored at 340 nm, 37°C for 5 min. The cellular GPx activity was calculated using the extinction coefficient of 6.22 mM⁻¹cm⁻¹, and expressed as nanomoles of NADPH consumed per minute per milligram of cellular protein.

Measurement of Cellular GST Activity. The cellular GST activity was measured according to the method of Habig *et al.* (31) 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 cellular GST activity was calculated using the extinction coefficient of 9.6 mM⁻¹cm⁻¹ and expressed as nanomoles of CDNB-GSH

conjugate formed per minute per milligram of cellular protein.

Measurement of Cellular NQO1 Activity. The cellular NQO1 activity was determined using dichloroindophenol (DCIP) as the two-electron acceptor, as described before (28). In brief, the reaction mix contained 50 mM Tris-HCl, pH 7.5, 0.08% Triton X-100, 0.25 mM NADPH, 80 μ M 2,6-dichloroindophenol (DCIP) in the presence or absence of 60 μ M dicumarol, a potent inhibitor of NQO1. To an assay cuvette, 0.695 ml of the reaction mix was added. The reaction was started by adding 5 μ l of sample, and the two-electron reduction of DCIP was monitored at 600 nm, 25°C for 3 min. 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 Cellular HO Activity. The cellular HO activity was measured according to the procedures described by Naughton et al. (32) with slight modifications. Briefly, the harvested cells were resuspended in 100 mM potassium phosphate buffer, pH 7.4 containing 2 mM MgCl₂ 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 μ l (200 μ g protein) of the supernatant was added to 200 μ l 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 MgCl₂. The reaction was conducted for 1 h at 37°C in the dark and terminated by addition of 300 μ l 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.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of mRNA Expression of Antioxidative and Phase 2 Genes. Total RNA from the renal tubular cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis and subsequent PCR reaction were performed using Superscript II One-Step system (Invitrogen), as described before (28). PCR products were separated by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide and analyzed under ultraviolet light using an Alpha Innotech Imaging system (San Leandro, CA). Various amounts of total RNA were used for each of the antioxidative and phase 2 genes to demonstrate a linear amplification of the specific mRNA. The quantitative capacity of RT-PCR in conjunction with standard curves for detecting mRNA levels has previously been characterized by our laboratory and others (33–35).

Immunoblot Analysis of Antioxidative and Phase 2 Enzymes. The procedures described before (36) were followed to detect protein expression by immunoblot analysis. Briefly, the renal tubular 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 SDS-PAGE on 10% gels, and transferred electrophoretically to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% non-fat dried milk in TTBS buffer at room temperature for 1.5 h. The membrane was then incubated with the individual primary antibody overnight at 4°C, followed by incubation with a horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) at room temperature for another 1.5 h. The membrane was visualized using an enhanced chemiluminescence system (Amersham Biosciences), and the blots were quantified by Gel-Pro Analyzer version 4.5 (Media Cybernetics, Silver Spring, MD).

Determination of Cell Injury Caused by Oxidative and Electrophilic Species. The renal tubular cell injury was determined by a slightly modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay, as described before (28). In brief, the cells were plated into 48-well tissue culture plates. After incubation of the cells with the oxidative and electrophilic species in DMEM 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.

Statistical Analysis. All data are expressed as means $\pm$ SE 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* < 0.05.

Results

SOD and CAT and Their Inducibility by D3T in the Renal Tubular Cells. The renal tubular cells expressed measurable activities for both SOD and CAT (Fig. 2A and B). Incubation of the cells with D3T (10, 25, and 50 μ M) for 24 h caused a significant concentration-dependent induction of the activity of CAT but not SOD. In consistence with the induction of the enzyme activity of CAT, the protein expression of CAT was also induced by D3T in a concentration-dependent fashion (Fig. 2C).

GSH and GCL and Their Induction by D3T in the Renal Tubular Cells. As shown in Figure 3A, the renal tubular cells expressed a basal level of GSH (~50 nmol/mg protein) similar to that in many other types of cells (28, 33, 36, 37). Incubation of the cells with D3T (10, 25, and 50 μ M) for 24 h led to a significant elevation of cellular GSH

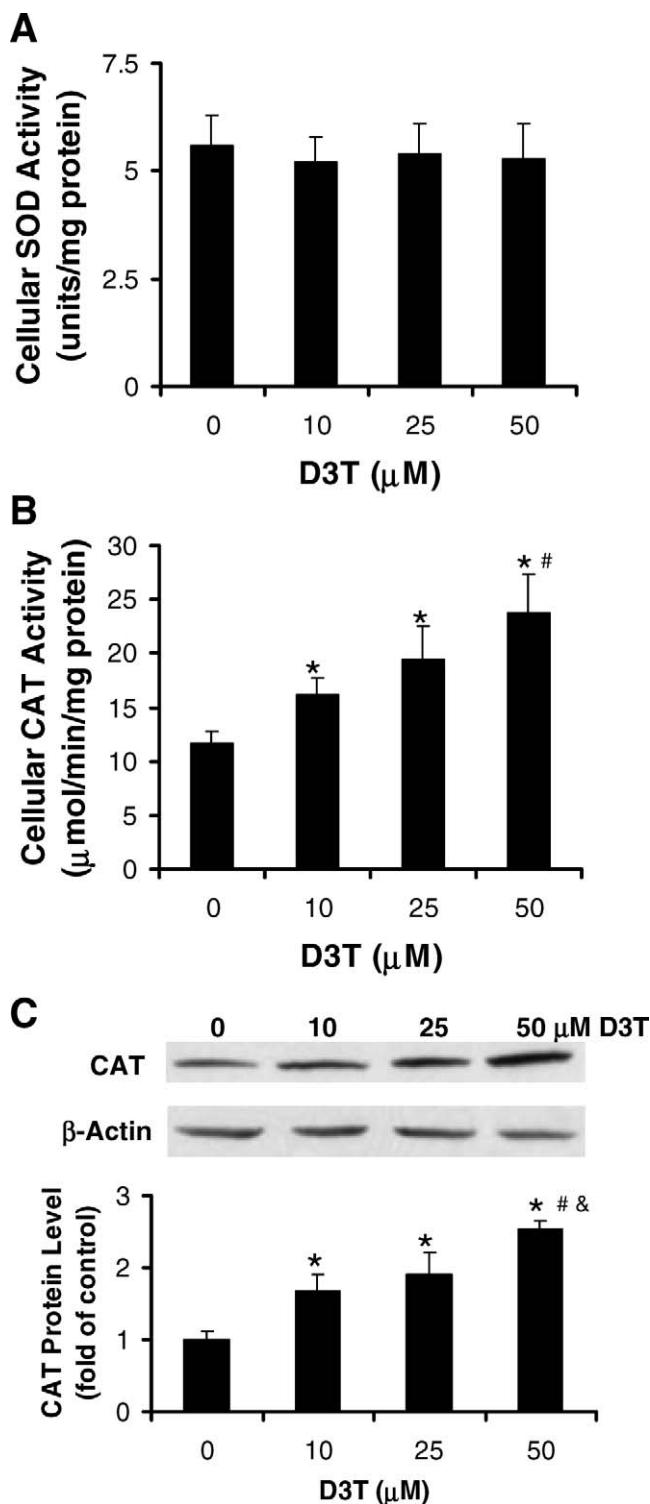


Figure 2. SOD and CAT and their inducibility by D3T in the renal tubular cells. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by determination of the enzyme activities of SOD (A) and CAT (B), as well as the protein level of CAT (C). Data represent means $\pm$ SE from three separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μ M D3T; & significantly different from 25 μ M D3T.

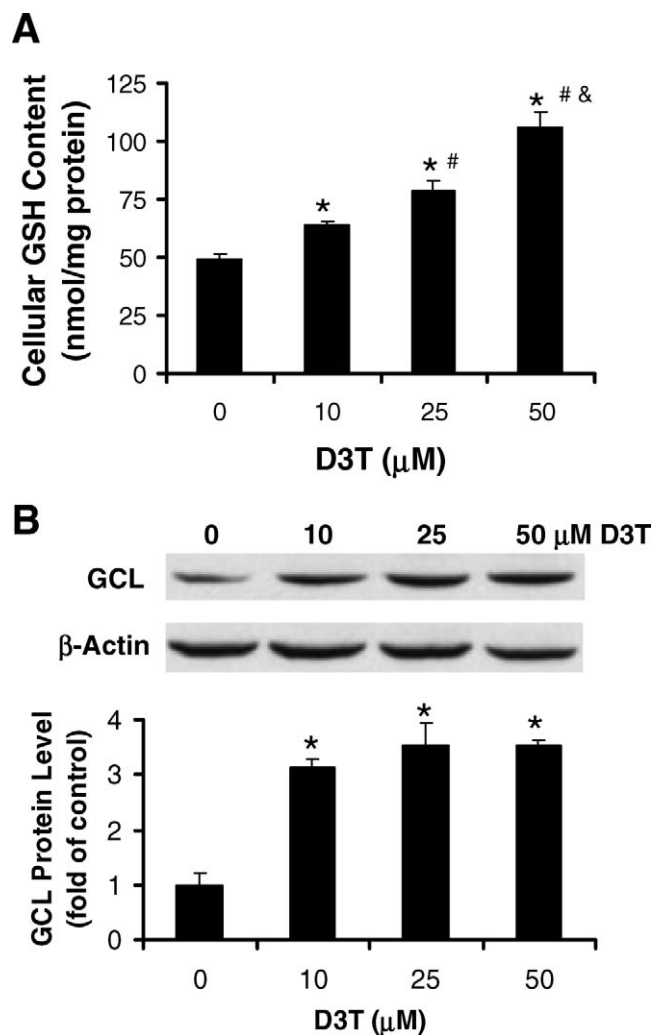


Figure 3. GSH and GCL and their inducibility by D3T in the renal tubular cells. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by determination of cellular GSH content (A) and the protein level of GCL (B). Data represent means $\pm$ SE from three separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μ M D3T; & significantly different from 25 μ M D3T.

content in a concentration-dependent manner. Incubation of the renal tubular cells with D3T also caused increased protein expression of GCL, the key enzyme in GSH synthesis (38). A remarkable 3.5-fold induction of GCL protein expression was observed with 25 and 50 μ M D3T. In contrast to the induction of GSH, induction of GCL protein expression did not show a clear D3T concentration-dependent relationship (Fig. 3B).

GPx and GR and Their Induction by D3T in the Renal Tubular Cells. Similar to GSH, the basal activities of GPx and GR in the renal tubular cells were also similar to those found in many other types of cells (28, 33, 36, 37). Incubation of the cells with D3T (10, 25, and 50 μ M) for 24 h led to a significant 20–25% induction of GPx activity (Fig. 4A). Notably, the same D3T treatment of the renal tubular cells caused marked concentration-dependent in-

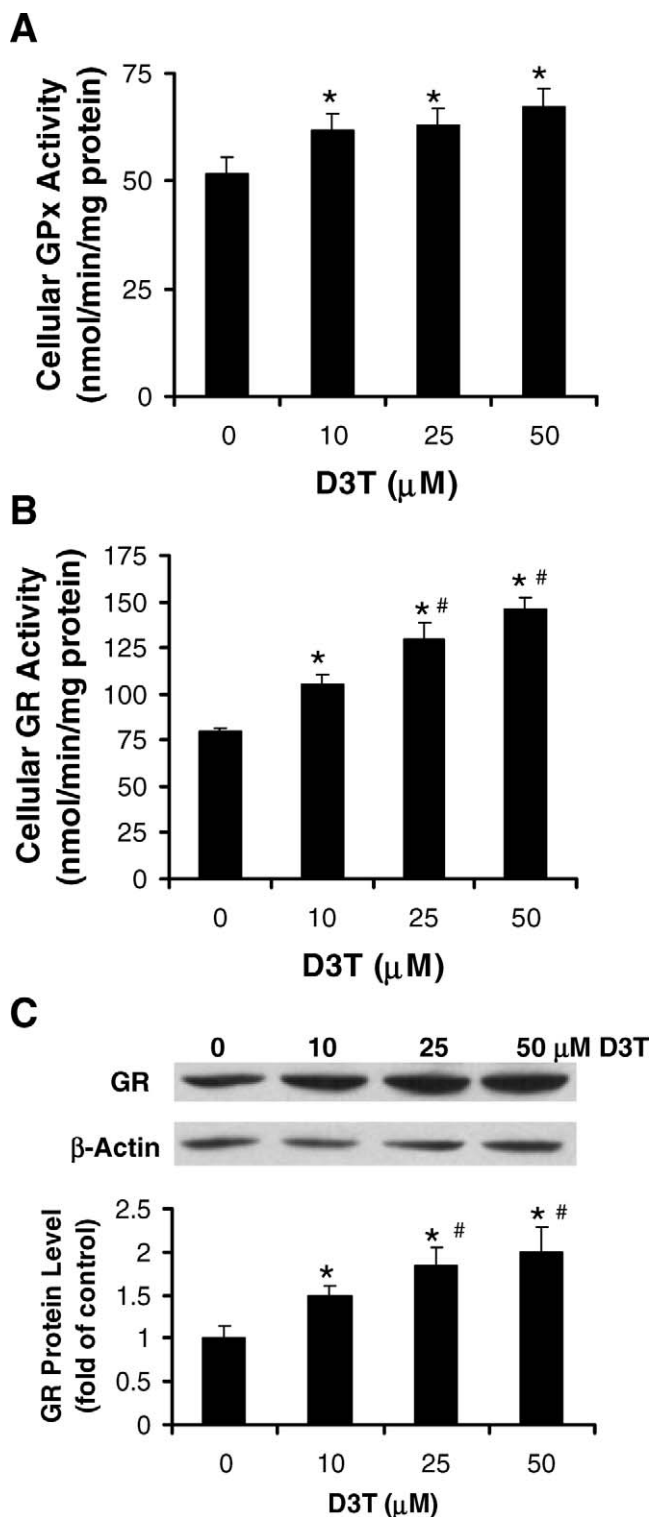


Figure 4. GPx and GR and their inducibility by D3T in the renal tubular cells. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by determination of the enzyme activities of GPx (A) and GR (B), as well as the protein level of GR (C). Data represent means $\pm$ SE from three separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μ M D3T.

creases in GR activity (Fig. 4B). In line with the increased GR activity, D3T treatment of the renal tubular cells also resulted in significant increases in GR protein expression in a concentration-dependent manner (Fig. 4C).

GST and Its Induction by D3T in the Renal Tubular Cells. The constitutive activity of GST in the renal tubular cells was relatively low as compared to that in most other types of cells (37). Incubation of the cells with D3T (10, 25, and 50 μ M) for 24 h caused a remarkable induction of cellular GST activity in a concentration-dependent manner (Fig. 5A). Since GST exists in various isozymes with GST-A, -M, and -P being the major forms in many types of cells (11), we next examined the expression of GST-A, -M, and -P, and their inducibility by D3T in the renal tubule cells. Although the basal protein level of GST-A was not detectable in the renal tubular cells, treatment with D3T (10, 25, and 50 μ M) led to significant increases in GST-A protein expression in a concentration-dependent manner (Fig. 5B). The renal tubular cells constitutively expressed a measurable level of GST-M protein, and the GST-M protein expression in these cells was also significantly induced by D3T in a concentration-dependent fashion (Fig. 5C). In data not shown, neither the basal expression nor the D3T-mediated induction of GST-P protein was detected by the immunoblot in the renal tubular cells under our experimental conditions.

NQO1 and Its Induction by D3T in the Renal Tubular Cells. The basal activity of NQO1 in the renal tubular cells was $\sim$ 500 nmol/min/mg protein, which was close to that in human vascular cells, known to constitutively express extremely high activity of NQO1 (15). Notably, incubation of the renal tubular cells with D3T (10, 25, and 50 μ M) for 24 h resulted in a significant concentration-dependent induction of NQO1 activity; a 2.3-, 3.2-, and 4.8-fold induction of NQO1 activity was observed with 10, 25, and 50 μ M D3T, respectively (Fig. 6A). In agreement with the induction of enzyme activity, D3T treatment also caused increased expression of NQO1 protein in a concentration-dependent manner (Fig. 6B).

HO and Its Induction by D3T in the Renal Tubular Cells. As shown in Fig. 7A, the renal tubular cells expressed a basal HO activity of $\sim$ 400 pmol/h/mg protein. Treatment of the cells with D3T (10, 25, and 50 μ M) for 24 h caused a 2- to 3-fold induction of HO activity in a concentration-dependent manner. Immunoblot analysis showed that D3T treatment of the renal tubular cells also led to increased expression of HO-1 protein; a remarkable $>$ 5-fold induction of HO-1 protein was seen with 50 μ M D3T (Fig. 7B).

Induction of mRNA Expression for Antioxidative and Phase 2 Genes by D3T in the Renal Tubular Cells. Since D3T treatment of the renal tubular cells caused significant increases in enzyme activity and protein expression for a series of antioxidative and phase 2 enzymes, we next examined the induction of mRNA expression for the above enzyme genes. As shown in Figure

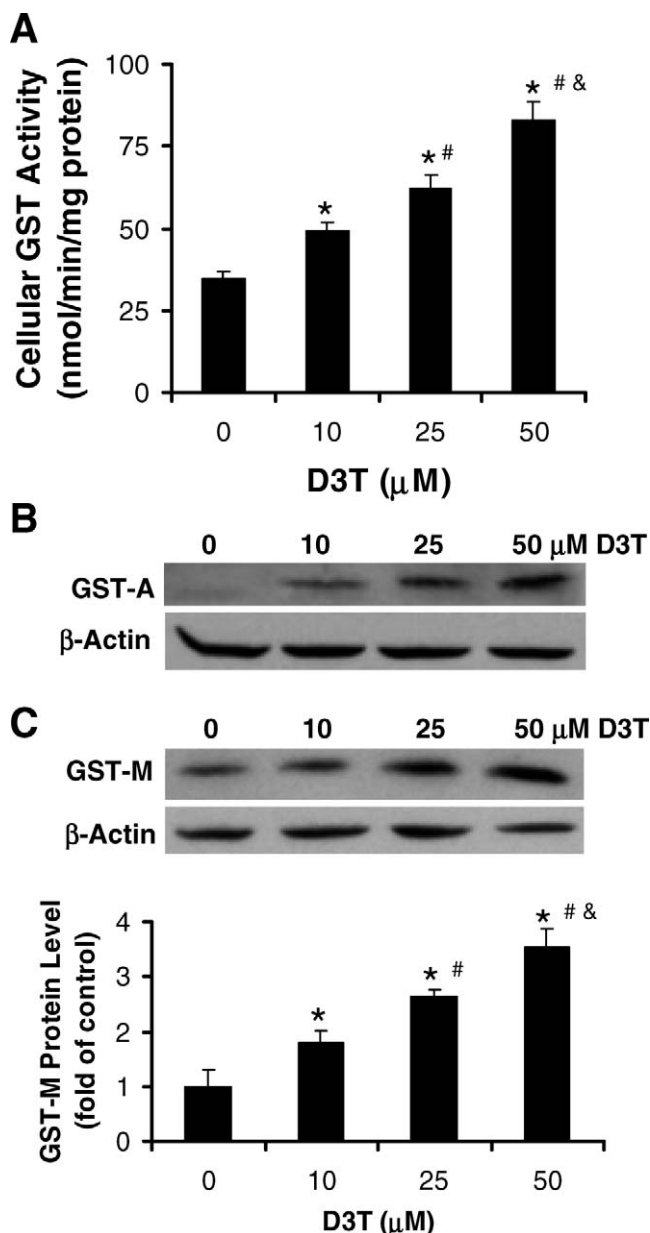


Figure 5. GST and its inducibility by D3T in the renal tubular cells. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by determination of activity of GST (A) as well as the protein levels of GST-A (B) and GST-M (C). Data represent means $\pm$ SE from three separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μ M D3T; & significantly different from 25 μ M D3T.

8, incubation of the renal tubular cells with 50 μ M D3T led to a significant time-dependent induction of the mRNA expression for CAT, GCL-catalytic subunit (GCLC), GR, GST-A1, GST-M1, NQO1 and HO-1. In contrast, the mRNA expression for the house-keeping gene, β -actin, remained unchanged after D3T treatment. Notably, the induction of the mRNA expression for all of the above antioxidative/phase 2 genes remained elevated for at least 48 h after D3T treatment (Fig. 8).

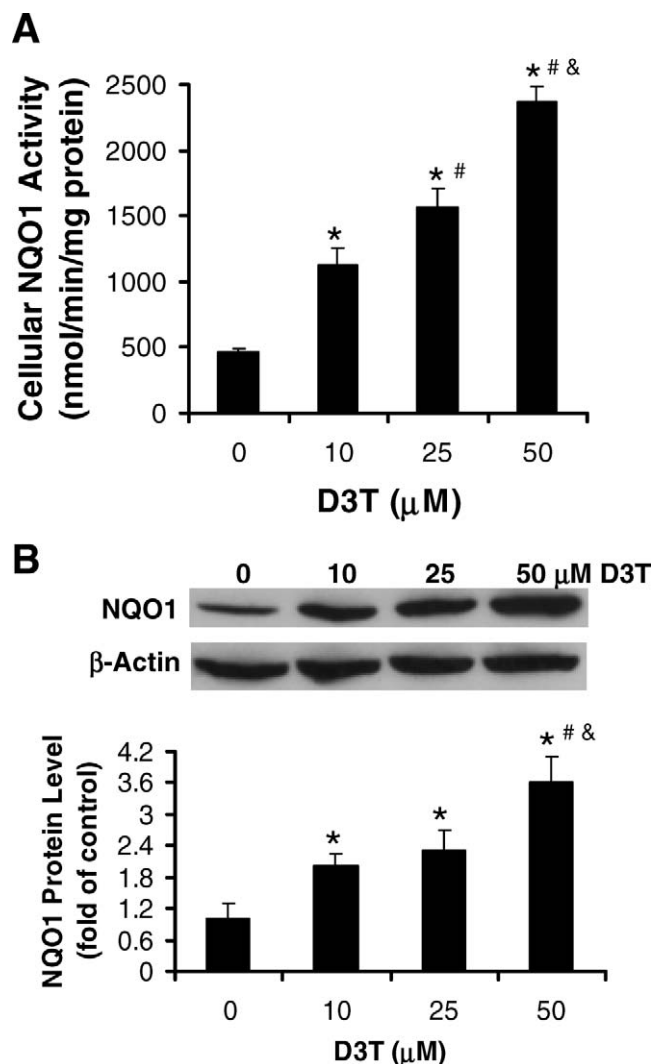


Figure 6. NQO1 and its inducibility by D3T in the renal tubular cells. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by determination of the activity of NQO1 (A) and its protein level (B). Data represent means $\pm$ SE from three separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μ M D3T; & significantly different from 25 μ M D3T.

H₂O₂-Mediated Cytotoxicity and the Cytoprotection by D3T Pretreatment in the Renal Tubular Cells. H₂O₂ is one of the most frequently encountered ROS under various pathophysiological conditions, including renal ischemia-reperfusion (2). As shown in Fig. 9, exposure of the renal tubular cells to lower micromolar concentrations of H₂O₂ caused significant concentration-dependent decreases in cell viability, as assessed by MTT reduction. To determine the cytoprotective effects of D3T-mediated upregulation of antioxidants and phase 2 enzymes on H₂O₂-induced cytotoxicity, the renal tubular cells were pretreated with D3T (10, 25, and 50 μ M) for 24 h, followed by exposure to H₂O₂. As shown, pretreatment of the cells with D3T afforded remarkable cytoprotection against H₂O₂-mediated cytotoxicity. The cytoprotective effect of D3T

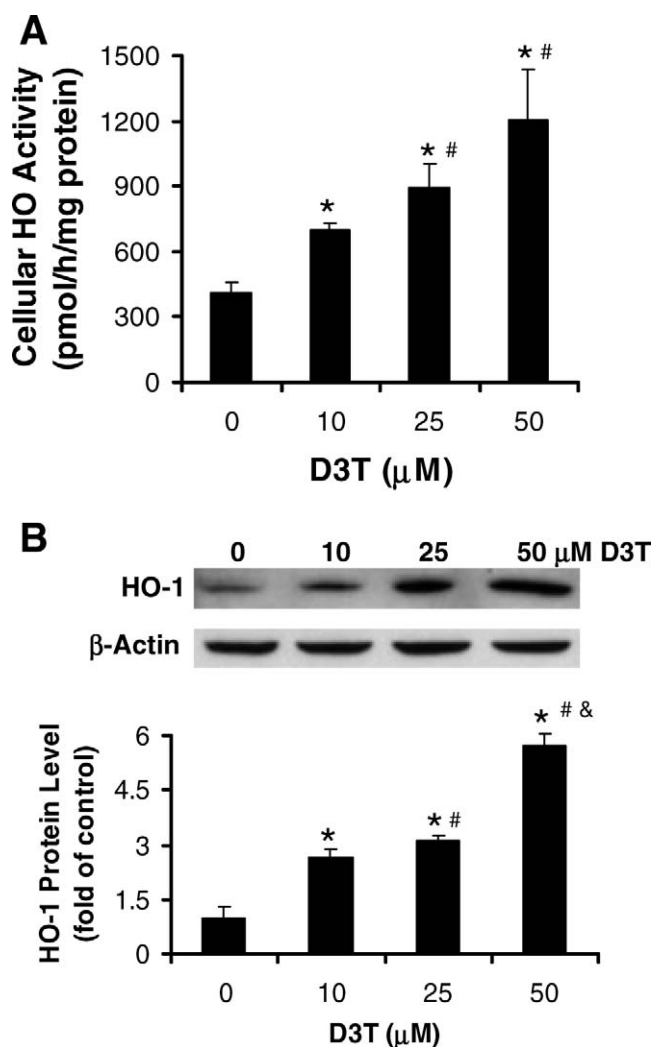


Figure 7. HO and its inducibility by D3T in the renal tubular cells. Cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by determination of the activity of HO (A) and the protein level of HO-1 (B). Data represent means $\pm$ SE from three separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μ M D3T; & significantly different from 25 μ M D3T.

showed a concentration-dependent relationship. Notably, a complete cytoprotection was observed with 50 μ M D3T for cytotoxicity induced by H_2O_2 at 25–100 μ M (Fig. 9).

3-Morpholininosydnonimine (SIN-1)-Mediated Cytotoxicity and the Cytoprotection by D3T Pretreatment in the Renal Tubular Cells. Exposure of the renal tubular cells to SIN-1, a $ONOO^-$ generator (33), caused significant decreases in cell viability in a concentration-dependent fashion (Fig. 10). Pretreatment of the cells with D3T (10, 25, and 50 μ M) for 24 h resulted in dramatic cytoprotection against SIN-1-induced cytotoxicity in a D3T concentration-dependent relationship. Again, pretreatment of the cells with 50 μ M D3T afforded a complete cytoprotection against the cytotoxicity elicited by SIN-1 at all of the toxic concentrations examined (Fig. 10).

4-Hydroxy-2-Nonenal (HNE)-Mediated Cytotoxicity and the Cytoprotection by D3T Pretreatment in the Renal Tubular Cells. In addition to D3T-mediated cytoprotection against ROS/RNS-induced nephrocytotoxicity, D3T pretreatment of the renal tubular cells also led to dramatic cytoprotection against HNE-elicited cytotoxicity. HNE is a potent electrophilic aldehyde involved in renal disorders (39–41). As shown in Figure 11, exposure of the renal tubular cells to lower micromolar concentrations of HNE for 24 h provided significant concentration-dependent decreases in cell viability. Pretreatment of the renal tubular cells with D3T (10, 25, and 50 μ M) for 24 h afforded remarkable protection against the cell injury elicited by all of the toxic concentrations of HNE examined (Fig. 11). The D3T-afforded cytoprotection also exhibited a concentration-dependent relationship; a notable complete cytoprotection was observed with 50 μ M D3T (Fig. 11).

Discussion

Although it is well recognized that antioxidants and phase 2 enzymes play important roles in detoxification of oxidants and electrophiles in various types of tissues and cells, careful studies on the constitutive expression and pharmacological inducibility of these cellular defenses in renal tubular cells are lacking. Understanding of the basal as well as the coordinated inducible expression of a series of renal cellular antioxidants and phase 2 enzymes is of importance for developing effective strategies for intervention of the oxidative and electrophilic stress-mediated cell degeneration that occurs in various renal disorders, including ischemia-reperfusion injury, transplantation rejection, and drug-induced nephrotoxicity. The results of this study for the first time comprehensively characterized the constitutive as well as D3T-mediated coordinated upregulation of a wide spectrum of endogenous antioxidants and phase 2 enzymes in normal rat kidney (NRK-52E) tubular cells, a commonly used *in vitro* model for studying physiology and pathophysiology of renal proximal tubular cells (26, 42–44). The renal tubular cells expressed measurable levels of SOD and CAT, two important antioxidative enzymes involved in detoxification of superoxide and H_2O_2 (37, 45). The induction of CAT but not SOD activity by D3T in the renal tubular cells (Fig. 2) suggested that these two enzymes might be regulated *via* different signaling pathways. In this context, we previously observed that in bone marrow cells and macrophages CAT but not SOD was induced by D3T *via* an Nrf2-dependent mechanism (28, 46). The increased protein and mRNA expression for CAT in D3T-treated renal tubular cells (Figs. 2 and 8) suggested that the induction of CAT activity by D3T most likely resulted from the increased transcription and translation.

GSH is one of the most abundant non-protein antioxidants in mammalian cells, which occurs intracellularly in millimolar concentrations (38). Consistent with what seen in many other types of cells, GSH in the renal tubular

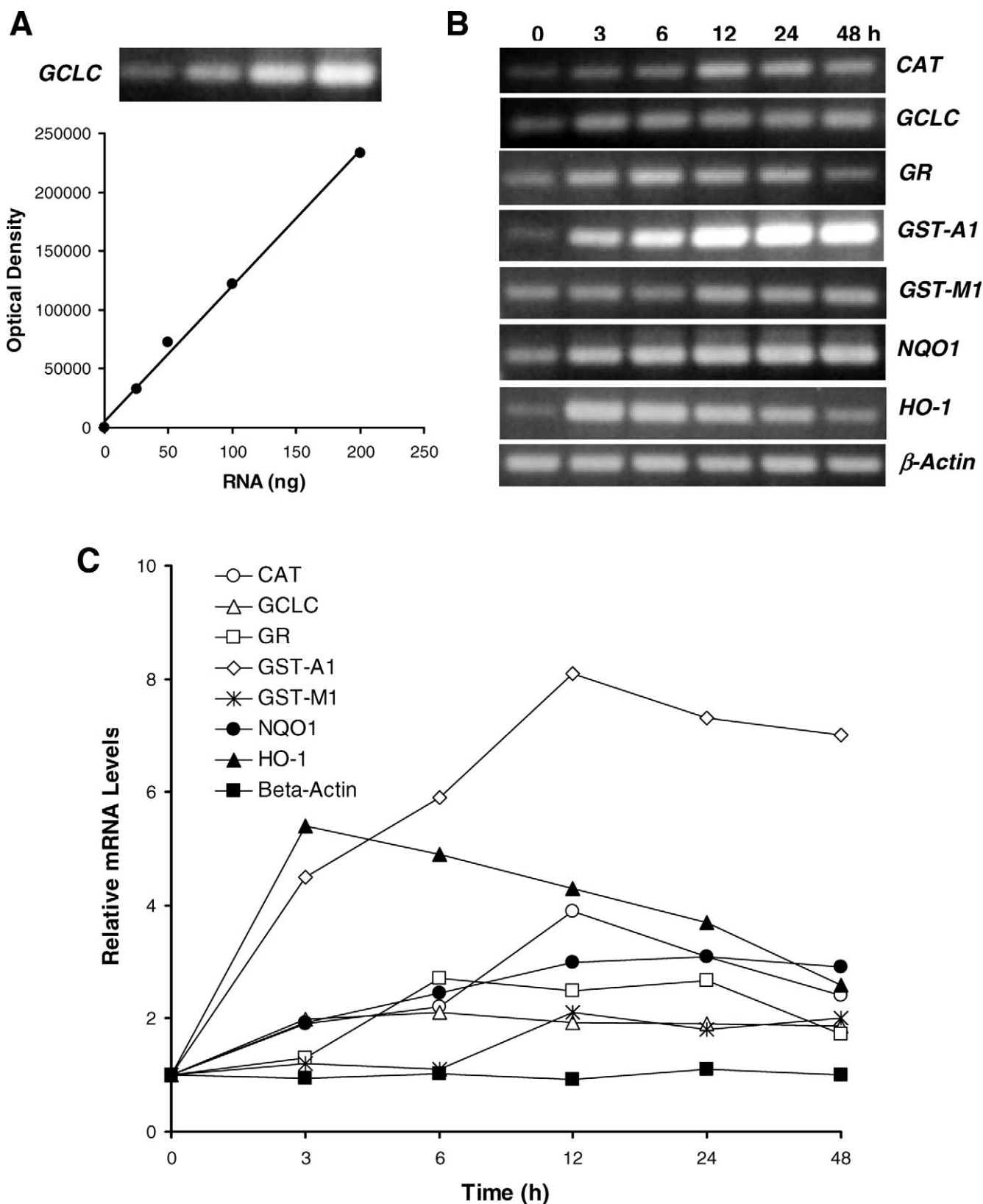


Figure 8. Effects of D3T treatment on mRNA expression of various antioxidative and phase 2 genes in the renal tubular cells. The cells were incubated with 50 μ M D3T for various time points, followed by detection of cellular mRNA levels for the indicated genes. Gel DNA band picture and graph in panel A show the linear amplification of the GCLC 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.

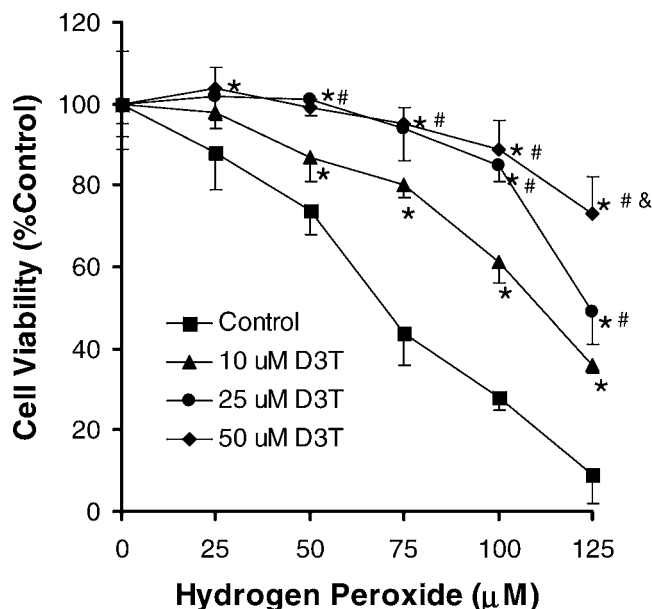


Figure 9. H_2O_2 -induced cytotoxicity in the renal tubular cells, and the cytoprotective effects of D3T pretreatment. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of H_2O_2 for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means $\pm$ SE from four separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μM D3T; & significantly different from 25 μM D3T.

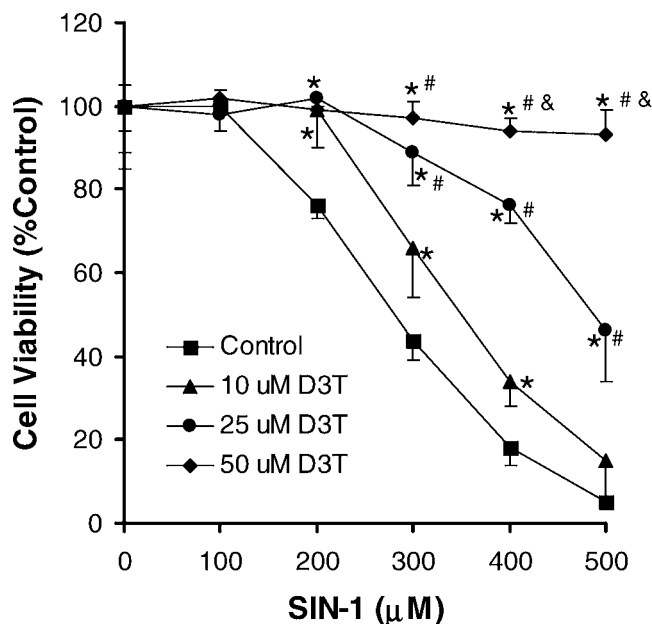


Figure 10. SIN-1-induced cytotoxicity in the renal tubular cells, and the cytoprotective effects of D3T pretreatment. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of SIN-1 for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means $\pm$ SE from four separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μM D3T; & significantly different from 25 μM D3T.

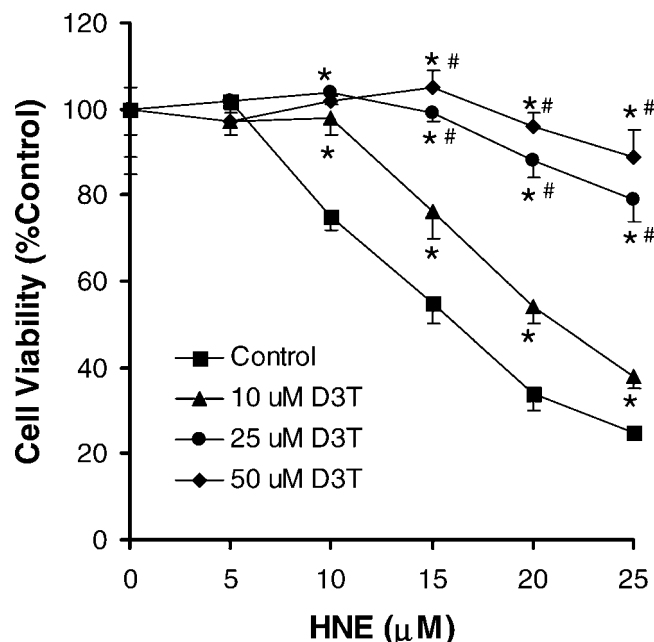


Figure 11. HNE-induced cytotoxicity in the renal tubular cells, and the cytoprotective effects of D3T pretreatment. The cells were incubated with or without the indicated concentrations of D3T for 24 h, followed by incubation with various concentrations of HNE for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means $\pm$ SE from four separate experiments. * significantly different from control (without D3T treatment); # significantly different from 10 μM D3T.

cells was highly inducible by D3T (Fig. 3). GSH is synthesized from glutamate, cysteine and glycine, with GCL being the key enzyme (38). The increased protein expression of GCL caused by D3T treatment (Fig. 3B) appeared to be responsible for the induction of cellular GSH in the renal tubular cells. GCL consists of two subunits, namely, catalytic and modulatory subunits. The mRNA expression for the catalytic subunit of GCL (GCLC) was also inducible by D3T in the renal tubular cells (Fig. 8), suggesting that D3T-mediated GSH induction might result from the initial increased transcription of GCLC. D3T treatment of the renal tubular cells caused increased induction of GPx and GR activities (Fig. 4). Although GR is found to be highly inducible by D3T in various types of cells, the inducibility of GPx by D3T varies greatly with cell types (28, 46–48). Indeed, as compared to GR, GPx was less inducible by D3T in the renal tubular cells. Similar to the induction of CAT, the increased protein expression of GR appeared to be responsible for the induction of GR activity by D3T in the renal tubular cells. In this regard, the extent of the D3T-mediated induction of GR protein expression was similar to that of the induction of GR enzyme activity (Fig. 4). The increased mRNA expression of GR by D3T (Fig. 8) was also in line with the notion that induction of GR activity by D3T occurred most likely *via* enhanced transcription of the GR gene in the renal tubular cells.

GST and NQO1 are the two most extensively studied phase 2 enzymes in mammalian cells (49, 50). The basal and

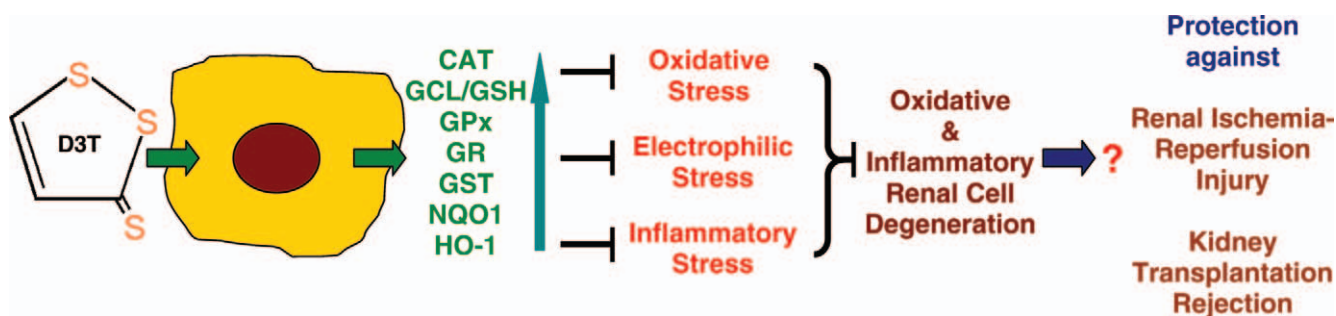


Figure 12. A schematic illustration of the role of D3T-mediated coordinated induction of endogenous antioxidative and phase 2 defenses in protecting against oxidative and inflammatory renal cell degeneration, as well as the potential implications in *in vivo* renal protection against ischemia-reperfusion injury and transplantation rejection. A color version of this figure is available in the online journal.

inducible expression of these two enzymes in the renal tubular cells has not been reported in literature. As compared to most other types of cells, the renal tubular cells constitutively expressed a relatively low GST activity, but an extremely high NQO1 activity (Figs. 5 and 6). The high inducibility of the total GST activity by D3T in the renal tubular cells was also in line with what observed in other types of cells (28, 46, 47). It is known that GST exists as a family of various members, with GST-A, -M and -P being the three major isozymes in many types of cells (11). Consistent with the induction of total cellular GST activity by D3T, D3T treatment also led to significant increases in the protein levels of GST-A and -M, as well as the mRNA levels of GST-A1 and -M1 in the renal tubular cells (Figs. 5 and 8). Notably, treatment of the renal tubular cells with D3T did not result in any changes in the protein expression of GST-P (data not shown). Previous studies also suggested that GST-P was not readily inducible by chemoprotectants, including D3T in various types of cells (28, 47). These observations suggested that different isozymes of GST might be regulated by distinct mechanisms. Although the basal expression of NQO1 in the renal tubular cells was extremely high, treatment with D3T resulted in a further remarkable ~2.2- to 4.8-fold induction of NQO1 activity, which was accompanied by dramatic increases in the NQO1 protein and mRNA expression (Figs. 6 and 8). The high basal and inducible expression of NQO1 in the renal tubular cells is of importance in view of the crucial roles of NQO1 in detoxification of ROS as well as in regulation of immunity and stabilization of the tumor suppressor p53 protein (13–15, 51, 52).

This study also for the first time demonstrated that HO activity as well as the protein and mRNA expression of HO-1 were highly inducible by D3T in the renal tubular cells (Figs. 7 and 8). HO; particularly, HO-1 has been demonstrated to play a critical role in protecting against oxidative and inflammatory stress in kidneys (16, 17). It has been reported that high expression of HO-1 in donor kidneys is associated with a decreased kidney transplantation rejection (53). Transgenic overexpression or induction of HO-1 has also been shown to attenuate renal ischemia-reperfusion injury as well as renal transplantation

rejection (24, 54, 55). Moreover, expression of HO-1 also appears to protect animals from cyclosporine A-induced nephrotoxicity (56). Thus, potent upregulation of HO-1 by D3T in renal tubular cells may have important implications with regard to protection against renal ischemia-reperfusion injury, transplantation rejection, as well as drug-induced nephrotoxicity.

Pretreatment of the renal tubular cells with D3T resulted in marked protection against H_2O_2 - or peroxynitrite-mediated cytotoxicity (Figs. 9 and 10). H_2O_2 and ONOO^- are two most commonly encountered ROS/RNS, which have been extensively implicated in the pathogenesis of various kidney disorders, including ischemia-reperfusion injury, transplantation rejection, and drug-induced nephrotoxicity (2–7). CAT and GPx/GSH are key cellular defenses involved in the detoxification of H_2O_2 (45). In addition to being involved in detoxifying H_2O_2 , the GPx/GSH system is also found to be a major pathway for detoxification of ONOO^- in mammalian cells (12, 33). Thus, the simultaneous induction of the above cellular factors by D3T may largely contribute to the increased resistance of the D3T-pretreated renal tubular cells to the above ROS/RNS-mediated cytotoxicity. Furthermore, the induction of GR by D3T may lead to increased regeneration of GSH from GSSG produced during GPx-catalyzed decomposition of H_2O_2 in the renal tubular cells. As depicted in Figure 1, GSH is also a cofactor for GST, an abundant cellular enzyme in mammalian tissues. GST is generally viewed as a phase 2 enzyme, primarily involved in the detoxification of electrophilic compounds *via* catalyzing the formation of GSH-electrophile conjugates (11). Several recent studies have also demonstrated that GST plays a critical role in protecting cells from oxidant-mediated injury through catalyzing the decomposition of lipid hydroperoxides generated from oxidative damage of cellular lipid molecules (Fig. 1; Refs. 57, 58). Accordingly, the marked induction of GST by D3T in the renal tubular cells may also contribute partially to the increased resistance of the D3T-pretreated cells to the oxidant-elicited cytotoxicity. As mentioned above, NQO1 not only detoxifies electrophilic quinones but also plays a critical role in controlling oxidative stress *via* maintaining high levels of cellular vitamin E and ubiquinol,

two important antioxidants (Fig. 1; Ref. 13). This is particularly relevant for the involvement of NQO1 in protecting against oxidative nephrocytotoxicity in view of the extremely high constitutive and D3T-inducible expression of NQO1 in the renal tubular cells (Fig. 6).

The increased resistance of the D3T-pretreated renal tubular cells to oxidative stress may also result partially from the potent induction of HO by D3T (Fig. 7). In this context, as illustrated in Figure 1, HO catalyzes the decomposition of heme to produce the potent antioxidant, bilirubin and the anti-inflammatory/antioxidative molecule, CO. In this regard, overexpression of HO-1 has been demonstrated extensively to protect cells from ROS-induced injury (17).

D3T pretreatment also led to marked protection against HNE-induced nephrocytotoxicity (Fig. 11). HNE is a potent electrophilic, α,β -unsaturated aldehyde, which is formed during lipid peroxidation in biological systems (10). HNE has been implicated in the pathogenesis of various renal disorders, including ischemia-reperfusion injury (39–41). Although a number of cellular factors have been proposed to participate in metabolism of HNE in biological systems, detoxification of HNE heavily relies on cellular GSH system in mammalian cells (11). In this context, due to its high electrophilic property, HNE readily reacts with GSH to form a less reactive GSH-conjugate, leading to its detoxification (11). The presence of GST has also been found to promote the conjugation reaction between HNE and GSH (11, 36). Thus, upregulation of both GSH and GST by D3T would largely account for the augmented resistance of the renal tubular cells to HNE-induced cytotoxicity.

In conclusion, as summarized in Figure 12, this study demonstrates that a number of endogenous antioxidants and phase 2 enzymes in cultured renal tubular cells can be simultaneously induced by low micromolar concentrations of D3T and that this pharmacologically-mediated coordinated upregulation of renal cellular defenses is accompanied by a remarkably increased resistance to oxidative and electrophilic stress. As aforementioned, oxidative/electrophilic stress and inflammation are intimately related processes contributing to various renal disorders, including ischemia-reperfusion injury and transplantation rejection (2–5, 59–61). In this context, the results of this study not only demonstrate the feasibility for protecting against *in vitro* oxidative/electrophilic as well as inflammatory renal cell degeneration *via* simultaneously upregulating a spectrum of endogenous antioxidative and phase 2 defenses by pharmacological inducers but also provide a potential strategy which could be used for intervention of the oxidative and inflammatory processes underlying renal diseases, especially, ischemia-reperfusion injury and transplantation rejection (Fig. 12).

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