

Antioxidants and Phase 2 Enzymes in Macrophages: Regulation by Nrf2 Signaling and Protection Against Oxidative and Electrophilic Stress

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Macrophages play important roles in immunity and other physiological processes. They are also target cells of various toxic agents, including oxidants and electrophiles. However, little is known regarding the molecular regulation and chemical inducibility of a spectrum of endogenous antioxidants and phase 2 enzymes in normal macrophages. Understanding the molecular pathway(s) controlling the coordinated expression of various macrophage antioxidants and phase 2 defenses is of importance for developing strategies to protect against macrophage injury induced by oxidants and electrophiles. Accordingly, this study was undertaken to determine the role of the nuclear factor E2-related factor 2 (Nrf2) in regulating both constitutive and chemoprotectant-inducible expression of various antioxidants and phase 2 enzymes in mouse macrophages. The constitutive expression of a series of antioxidants and phase 2 enzymes was significantly lower in macrophages derived from Nrf2-null (Nrf2^{-/-}) mice than those from wild-type (Nrf2^{+/+}) littermates. Incubation of wild-type macrophages with 3H-1,2-dithiole-3-thione (D3T) led to significant induction of various antioxidants and phase 2 enzymes, including catalase,

glutathione, glutathione peroxidase (GPx), glutathione reductase, glutathione S-transferase, and NAD(P)H:quinone oxidoreductase 1. The inducibility of the above cellular defenses except for GPx by D3T was completely abolished in Nrf2^{-/-} macrophages. As compared with wild-type cells, Nrf2^{-/-} macrophages were much more susceptible to cell injury induced by reactive oxygen/nitrogen species, as well as two known macrophage toxins, acrolein and cadmium. Up-regulation of the antioxidants and phase 2 enzymes by D3T in wild-type macrophages resulted in increased resistance to the above oxidant- and electrophile-induced cell injury, whereas D3T treatment of Nrf2^{-/-} macrophages provided only marginal or no cytoprotection. This study demonstrates that Nrf2 is an indispensable factor in controlling both constitutive and inducible expression of a wide spectrum of antioxidants and phase 2 enzymes in macrophages as well as the susceptibility of these cells to oxidative and electrophilic stress. *Exp Biol Med* 233:463–474, 2008

Key words: Nrf2; macrophages; phase 2 enzymes; cytotoxicity; oxidants; electrophiles

This work was supported in part by R01 HL71190 from National Institutes of Health (Y.L.), and a grant from Harvey Peters Research Center Foundation (Y.L.).

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Received November 12, 2007.
Accepted December 7, 2007.

DOI: 10.3181/0711-RM-304
1535-3702/08/2334-0463\$15.00
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Introduction

Macrophages are widely distributed cells found in many mammalian tissues and organs. Macrophages are well known for their critical roles in both innate and adaptive immunity. Macrophages also play an important part in other physiological processes. For example, bone marrow stromal macrophages via releasing growth factors/cytokines support hematopoiesis (1). In bone, macrophages (osteoclasts) are central players in the regulation of bone remodeling and homeostasis (2).

Since macrophages possess important physiological

roles, macrophage dysfunction has been implicated in many pathophysiological processes, including inflammatory disorders, neurodegeneration, tumorigenesis, and atherosclerosis (3–5). Under these conditions, macrophages are usually found to be abnormally activated, releasing various inflammatory cytokines and reactive intermediates, which subsequently cause tissue injury (3–5). On the other hand, compromised macrophage function may increase the host's susceptibility to microorganism infection, and possibly tumorigenesis as well (6). In this context, macrophages have been demonstrated to be critical targets of environmental toxic agents, and as such macrophage injury has been implicated in environmental chemical-induced disorders. For instance, airborne particulates have been shown to inhibit the phagocytic function of alveolar macrophages, and furthermore, the oxidants derived from air pollutants, including airborne particulates are able to directly cause cytotoxicity to macrophages, thereby compromising pulmonary immunity (7, 8). It has also been shown that macrophages are susceptible to cell injury elicited by acrolein and cadmium, two electrophilic toxins in tobacco smoke and air pollution (9–12). In addition to alveolar macrophages, bone marrow macrophages are known to be a critical target cell population for benzene-derived quinoid compounds, and death/dysfunction of these cells has been implicated in benzene-induced hematotoxicity (13, 14). Similarly, death of lipid-laden macrophages due to oxidative/electrophilic stress in atherosclerotic lesions is believed to be a critical event, leading to plaque instability and the subsequent thrombosis (15, 16).

The identification of macrophages as critical targets for oxidative and electrophilic stress makes it important to determine the detoxification mechanisms and their regulation in these cells. In this regard, an increasing number of antioxidants and phase 2 enzymes have been implicated in detoxification of numerous oxidative and electrophilic species, including reactive oxygen/nitrogen species (ROS/RNS), aldehydes and heavy metals (17). Recently, the nuclear factor E2-related factor 2 (Nrf2) is found to be a critical transcription factor that binds to the antioxidant response element (ARE) in the promoter region of a number of genes encoding for antioxidative and phase 2 enzymes in several types of cells and tissues (18, 19). However, careful studies on the expression of a series of antioxidants and phase 2 enzymes as well as their molecular regulation by Nrf2 signaling in macrophages are currently lacking. Accordingly, in this study we determined the regulatory role of Nrf2 in constitutive expression as well as inducibility by the chemoprotectant, 3*H*-1,2-dithiole-3-thione (D3T) of various antioxidants and phase 2 enzymes, including superoxide dismutase (SOD), catalase, reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase 1 (NQO1) in primary cultured macrophages derived from Nrf2-null (Nrf2^{-/-}) and wild-type littermates. This study for the first time comprehensively

characterized a wide spectrum of important antioxidants and phase 2 enzymes in macrophages, and demonstrated a crucial role for Nrf2 in regulating both the constitutive and D3T-inducible expression of the antioxidative and phase 2 defenses, as well as in determining macrophage susceptibility to oxidative and electrophilic stress.

Materials and Methods

Chemicals and Materials. D3T with a purity of 99.8% was generously provided by Dr. Mary Tanga at SRI International (Menlo Park, CA) and Dr. Linda Brady at National Institute of Mental Health (Bethesda, MD). RPMI-1640 medium, penicillin, streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate buffered saline (PBS) were from Gibco-Invitrogen (Carlsbad, CA). Anti- γ -glutamylcysteine ligase (γ 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). All other chemicals and agents were from Sigma-Aldrich (St. Louis, MO).

Animals and Genotyping. Breeding pairs of Nrf2^{+/-} (ICR/Sv129) mice were obtained from a colony at Tsukuba University and maintained in the animal facility at Virginia Tech. Nrf2^{+/+} and Nrf2^{-/-} mice were generated following the breeding procedures described previously (20). Purina laboratory animal chow (Richmond, IN) and water were available ad libitum. Genotypes (Nrf2^{+/+}, Nrf2^{-/-}, and Nrf2^{+/-}) of the animals were determined by polymerase chain reaction (PCR) amplification of genomic DNA from tails as described before (20). All of the animal procedures were approved by the Institutional Animal Care and Use Committee.

Primary Macrophage Culture. Each of the female Nrf2^{-/-} and wild-type mice (25–30 g) mice was intraperitoneally injected 1 ml of 4% thioglycollate medium. Four days later, total peritoneal cells were harvested by washing the peritoneal cavity with serum-free RPMI-1640 medium. Following centrifugation, the cells were resuspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, and incubated in tissue culture flasks at 37°C for 1 h. Then, the non-adherent cells were removed by vigorous washing of the cultures three times with serum-free RPMI-1640 medium. The resultant macrophages (over 98% purity) were continuously cultured at 37°C in a humidified atmosphere of 5% CO₂. The macrophages were used for experiments within three passages following the initial isolation.

Preparation of Cell Extract. Macrophages 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.

Assay for Cellular SOD Activity. Total cellular SOD activity was determined by the method of Spitz and Oberley (21) with slight modifications, as described before (20). This method is based on the inhibition of the superoxide-mediated reduction of nitroblue tetrazolium to formazan by SOD. The sample total SOD activity was calculated using a concurrently run SOD (Sigma-Aldrich) standard curve, and expressed as units per mg of cellular protein.

Assay for Cellular Catalase Activity. Catalase activity was measured according to the method of Aebi (22). In brief, to a quartz cuvette, 0.4 ml of 50 mM potassium phosphate buffer (pH 7.0) and 20 μ l 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 catalase activity was expressed as μ mol of H_2O_2 consumed per min per mg of cellular protein.

Assay for Cellular GSH Content. The cellular GSH content was measured according to the *o*-phthalaldehyde-based fluorometric method, which is specific for the determination of GSH at pH 8.0 (23). Briefly, 10 μ l of the sample was incubated with 12.5 μ l of 25% metaphosphoric acid, and 37 μ l 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 μ l) 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. Cellular GSH content was calculated using a GSH (Sigma-Aldrich) standard curve, and expressed as nmol of GSH per mg of cellular protein.

Assay for 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 (20) was followed to measure cellular GR activity. Cellular GR activity was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

Assay for Cellular GPx Activity. Cellular 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 (20). GPx activity was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

Assay for Cellular GST Activity. Cellular GST activity was measured according to the method of Habig *et al.* (24) using 1-chloro-2,4-dinitrobenzene (CDNB) as a

substrate, as described before (20). GST activity was calculated using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$, and expressed as nmol of CDNB-GSH conjugate formed per min per mg of cellular protein.

Assay for Cellular NQO1 Activity. Cellular NQO1 activity was determined using dichloroindophenol (DCIP) as the two-electron acceptor, as described before (25). The dicumarol-inhibitable NQO1 activity was calculated using the extinction coefficient of 21.0 $mM^{-1}cm^{-1}$, and expressed as nmol of DCIP reduced per min per mg of cellular protein.

Reverse Transcriptase (RT)-PCR Analysis of mRNA Expression. Total RNA from macrophages 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 (20). The sequences for the PCR primers are shown in Table 1. 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 (26, 27).

Immunoblot Analysis of Antioxidative and Phase 2 Enzymes. The procedures described before (20) were followed to detect protein expression by immunoblot analysis. Briefly, macrophages 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).

Assay for Cell Injury. Macrophage injury was determined by a slightly modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) reduction assay, as described before (20). In brief, macrophages were plated into 48-well tissue culture plates. After incubation of the cells with the toxic agents in RPMI-1640 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

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

Genes	Primers	
Catalase	Sense	GACATGGTCTGGGACTTCTG
	Antisense	GTAGGGACAGTTCACAGGTA
GCLC	Sense	GGAGGCTACTTCTGTACTA
	Antisense	CGATGGTCAGGTCGATGTCATT
GR	Sense	TGCCTGCTCTGGGCCATT
	Antisense	CTCCTCTGAAGAGGTAGGAT
GST-A1	Sense	CCGTGCTTCACTACTTCAAT
	Antisense	GCATCCATGGGAGGCTTTCT
GST-M1	Sense	CTAGGAAGGGGAGTGCCTAA
	Antisense	CAGGCACTTGGGCTCAAACA
NQO1	Sense	CCATTCTGAAAGGCTGGTTTG
	Antisense	CTAGCTTTGATCTGGTTGTC
β -Actin	Sense	GACAACGGCTCCGGCATGT
	Antisense	GCAACATAGCACAGCTTCT

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 $\pm$ 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* < 0.05.

Results

Constitutive Levels/Activities of Antioxidants and Phase 2 Enzymes in Wild-Type and Nrf2^{-/-} Macrophages. The constitutive levels/activities of various antioxidants and phase 2 enzymes in macrophages from wild-type and Nrf2^{-/-} mice are shown in Table 2. As shown, except for SOD, the basal levels/activities of catalase, GSH, GPx, GR, GST and NQO1 in Nrf2^{-/-} macrophages were significantly lower than those in wild-type macrophages. Notably, wild-type macrophages constitutively expressed high activities of GPx, GR, and GST, whereas the activities of SOD and NQO1 in these cells were

relatively lower than those found in most other types of cells (17).

Inducibility of Cellular SOD and Catalase by D3T in Wild-Type and Nrf2^{-/-} Macrophages. Incubation with D3T (10–50 μ M) failed to cause any significant induction of SOD in either wild-type or Nrf2^{-/-} macrophages (Fig. 1A). In contrast to SOD, catalase in wild-type macrophages was significantly induced by D3T, with maximal induction seen after treatment with 10 and 25 μ M D3T. The inducibility of catalase by D3T was completely abolished in Nrf2^{-/-} macrophages (Fig. 1B). In line with the increased activity of catalase after D3T treatment, the protein expression of catalase was also significantly increased in wild-type macrophages after incubation with D3T. Again, the catalase protein expression was not affected by D3T treatment in Nrf2^{-/-} macrophages. Also consistent with the lower basal activity of catalase in Nrf2^{-/-} macrophages (Table 2), the constitutive protein expression of catalase was also significantly reduced in Nrf2^{-/-} macrophages (Fig. 1C).

Inducibility of Cellular GSH and GSH-Linked Enzymes by D3T in Wild-Type and Nrf2^{-/-} Macrophages. As shown in Figure 2A, incubation of wild-type macrophages with D3T (10–50 μ M) led to significant concentration-dependent induction of cellular GSH. This D3T-mediated induction of GSH was completely diminished in Nrf2^{-/-} macrophages. Similarly, the protein expression of γ GCL, the key enzyme in GSH synthesis, was also highly inducible by D3T in wild-type, but not Nrf2^{-/-} macrophages (Fig. 2B). Also notably, the basal protein level of γ GCL in Nrf2^{-/-} macrophages was significantly reduced, which was consistent with the reduced basal level of cellular GSH in Nrf2^{-/-} macrophages as compared with the wild-type cells.

Although the basal activity of GPx in Nrf2^{-/-} macrophages was lower than that in wild-type macrophages, treatment of both the wild-type and Nrf2^{-/-} macrophages with D3T (25 and 50 μ M) caused a similar extent of induction of GPx activity (Fig. 3A). In contrast, while both the activity and protein expression of GR were highly inducible by D3T (10–50 μ M) in wild-type macrophages in a concentration-dependent manner, the same D3T treatment

Table 2. Constitutive Levels/Activities of Antioxidants and Phase 2 Enzymes in Primary Macrophages Derived from Nrf2^{-/-} and Wild-Type (Nrf2^{+/+}) Mice. Data Represent Means $\pm$ SEM from Three Separate Experiments.

Antioxidants/phase 2 enzymes	Nrf2 ^{+/+}	Nrf2 ^{-/-}	<i>P</i> values
SOD (units/mg protein)	2.0 $\pm$ 0.3	1.9 $\pm$ 0.2	
Catalase (μ mol/min/mg protein)	31.9 $\pm$ 1.9	13.9 $\pm$ 1.3*	< 0.001
GSH (nmol/mg protein)	17.9 $\pm$ 3.1	11.5 $\pm$ 1.8*	< 0.05
GPx (nmol/min/mg protein)	160.1 $\pm$ 12.2	114.3 $\pm$ 7.6*	< 0.01
GR (nmol/min/mg protein)	101.7 $\pm$ 8.6	52.5 $\pm$ 3.2*	< 0.001
GST (nmol/min/mg protein)	169.9 $\pm$ 11.6	40.1 $\pm$ 5.5*	< 0.001
NQO1 (nmol/min/mg protein)	7.0 $\pm$ 0.8	2.1 $\pm$ 0.6*	< 0.002

*, significantly different from Nrf2^{+/+} cells.

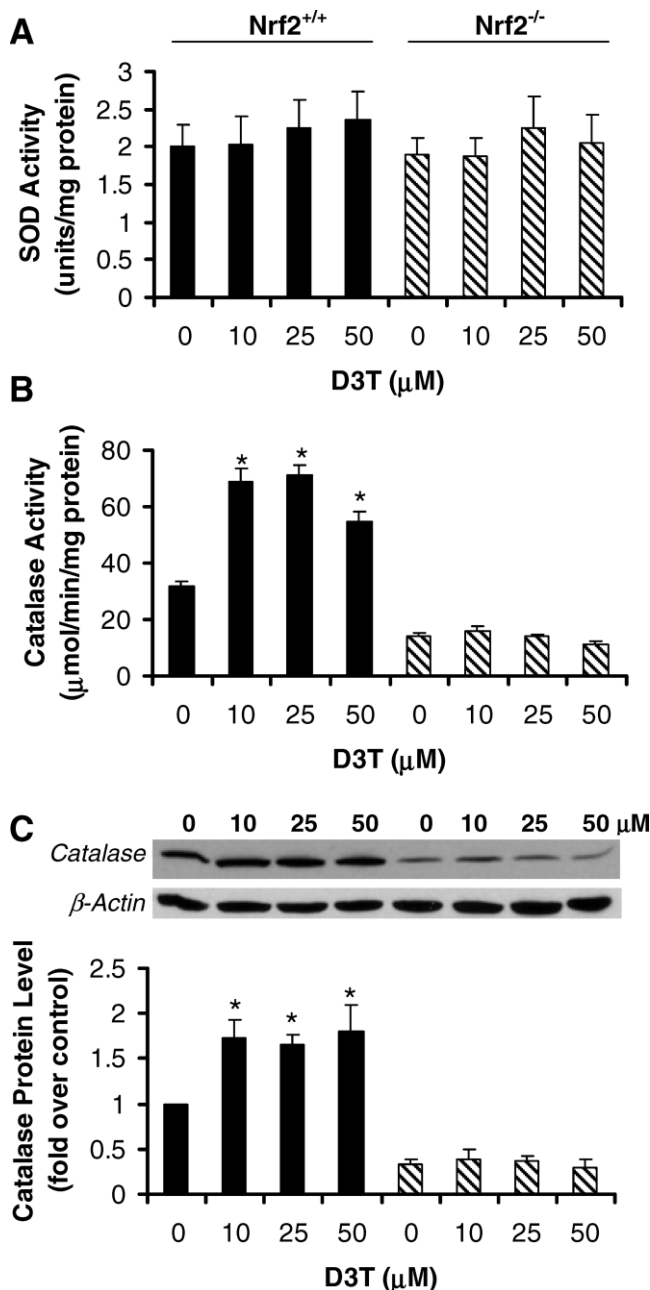


Figure 1. Effects of D3T treatment on SOD (A) and catalase (B) activities, as well as catalase protein expression (C) in Nrf2^{+/+} and Nrf2^{-/-} macrophages. Macrophages were incubated with the indicated concentrations of D3T for 48 h, followed by measurement of cellular SOD and catalase activity, as well as catalase protein expression. Values represent means $\pm$ SEM from three separate experiments. *, significantly different from 0 μ M D3T.

failed to cause any significant induction of GR in Nrf2^{-/-} macrophages (Fig. 3B, 3C). Consistent with the lower basal activity of GR in Nrf2^{-/-} macrophages (Table 2), the basal protein expression of GR was also significantly reduced by targeted disruption of Nrf2 in macrophages (Fig. 3C).

Inducibility of GST and Isozymes by D3T in Wild-Type and Nrf2^{-/-} Macrophages. The total cellular GST activity was highly inducible by D3T (10–50

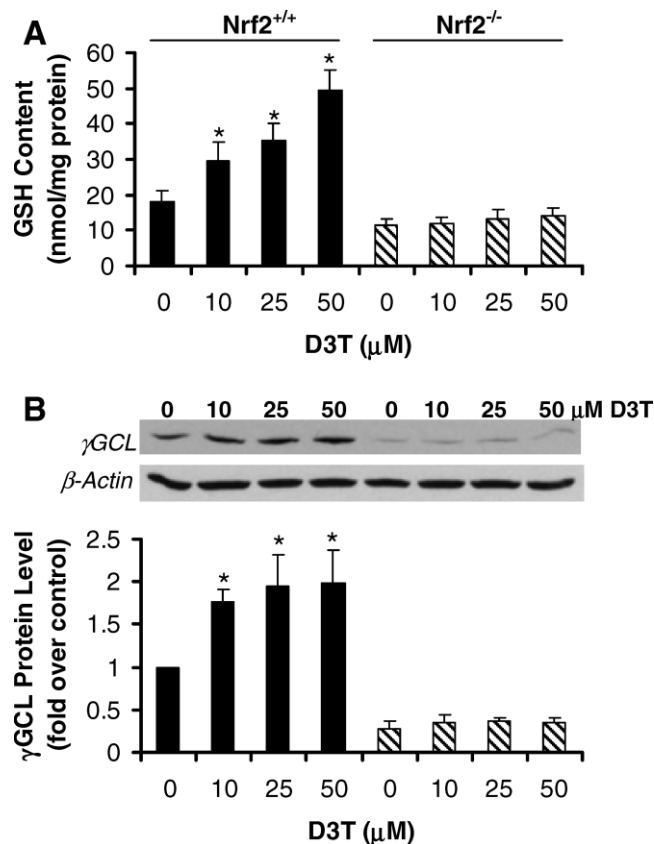


Figure 2. Effects of D3T treatment on GSH content (A) and γ GCL protein expression (B) in Nrf2^{+/+} and Nrf2^{-/-} macrophages. Macrophages were incubated with the indicated concentrations of D3T for 48 h, followed by measurement of cellular GSH content, and γ GCL protein expression. Values represent means $\pm$ SEM from three separate experiments. *, significantly different from 0 μ M D3T.

μ M) in a concentration-dependent fashion in wild-type macrophages, and this D3T-mediated induction of total GST activity was completely abolished in Nrf2^{-/-} macrophages (Fig. 4A). Since GST is a family of various isozymes, we next examined the D3T inducibility of the three major isozymes, GST-A, -M and -P in wild-type and Nrf2^{-/-} macrophages. As shown in Figure 4B and C, incubation of wild-type, but not Nrf2^{-/-} macrophages with D3T (10–50 μ M) led to marked D3T concentration-dependent induction of the protein expression for both GST-A and -M. Notably, the basal protein expression for the above two GST isozymes was also significantly diminished in Nrf2^{-/-} macrophages. In contrast, the same D3T treatment of either wild-type or Nrf2^{-/-} macrophages did not cause any significant induction of GST-P (Fig. 4D). Neither was the basal protein expression of GST-P affected by the status of Nrf2.

Inducibility of NQO1 by D3T in Wild-Type and Nrf2^{-/-} Macrophages. As shown in Figure 5A, although the basal activity of NQO1 is relatively deficient in wild-type macrophages, D3T treatment (10–50 μ M) resulted in remarkable induction of NQO1 activity in wild-type, but not Nrf2^{-/-} macrophages. This D3T-mediated

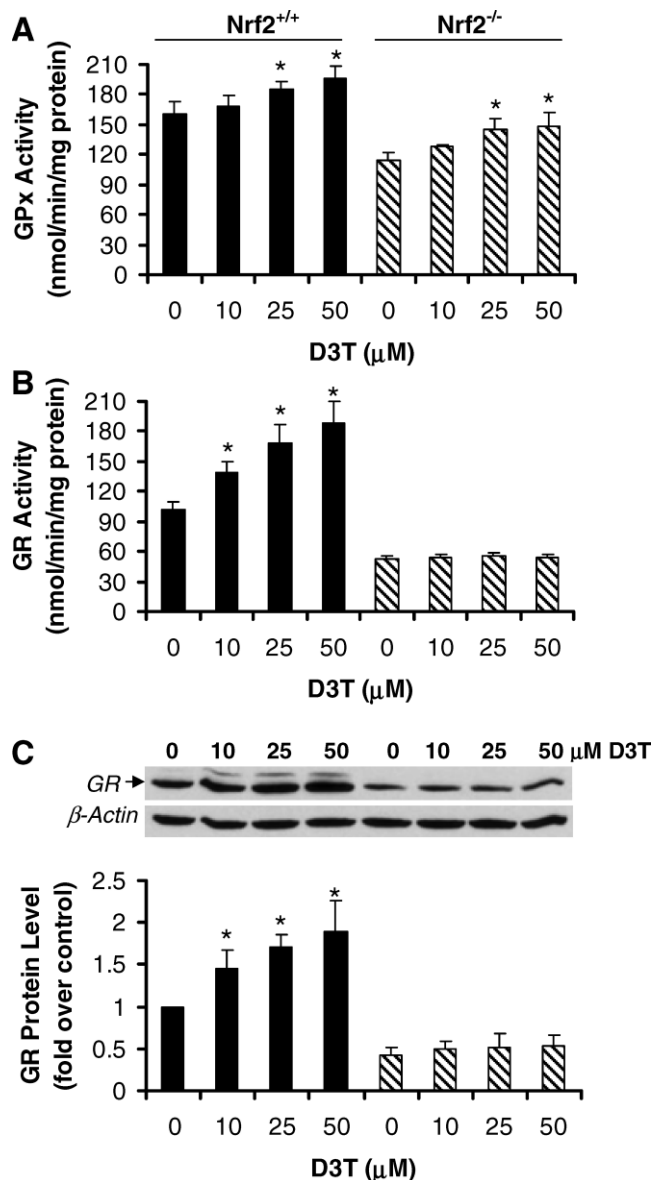


Figure 3. Effects of D3T treatment on GPx (A) and GR (B) activities, as well as GR protein expression (C) in Nrf2^{+/+} and Nrf2^{-/-} macrophages. Macrophages were incubated with the indicated concentrations of D3T for 48 h, followed by measurement of cellular GPx and GR activity, as well as GR protein expression. Values represent means $\pm$ SEM from three separate experiments. *, significantly different from 0 μ M D3T.

induction of NQO1 activity showed a remarkable concentration-dependent relationship. Although the basal protein expression of NQO1 was below the detection limit by immunoblot, D3T treatment caused dramatically increased expression of the NQO1 protein in wild-type, but not Nrf2^{-/-} macrophages (Fig. 5B).

Effects of D3T Treatment on mRNA Levels of Antioxidative and Phase 2 Enzymes in Wild-Type and Nrf2^{-/-} Macrophages. Since D3T-mediated induction of catalase, GSH/GCL, GR, GST-A, GST-M, and NQO1 was dependent on the status of Nrf2 (Figs. 1–5), we

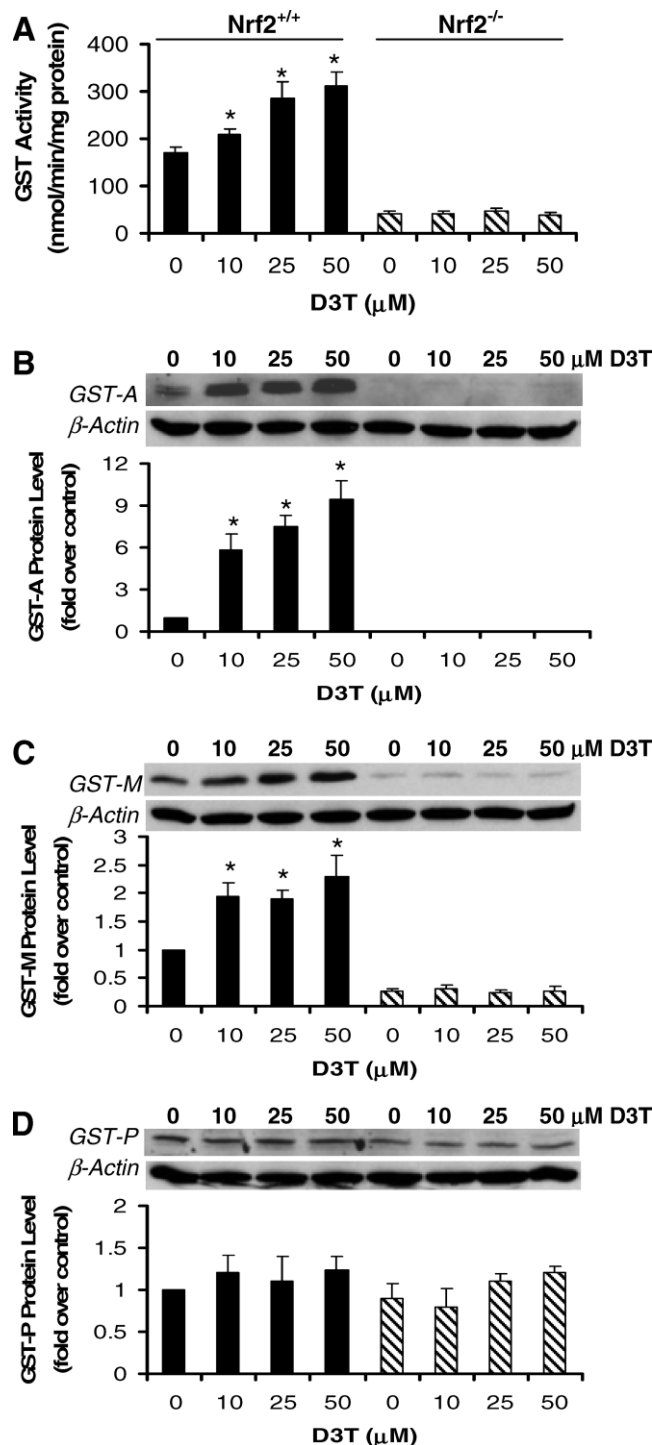


Figure 4. Effects of D3T treatment on total GST activity (A) and the protein expression of GST-A (B), GST-M (C), and GST-P (D) in Nrf2^{+/+} and Nrf2^{-/-} macrophages. Macrophages were incubated with the indicated concentrations of D3T for 48 h, followed by measurement of total cellular GST activity, as well as the protein expression of GST-A, -M, and -P. Values represent means $\pm$ SEM from three separate experiments. *, significantly different from 0 μ M D3T.

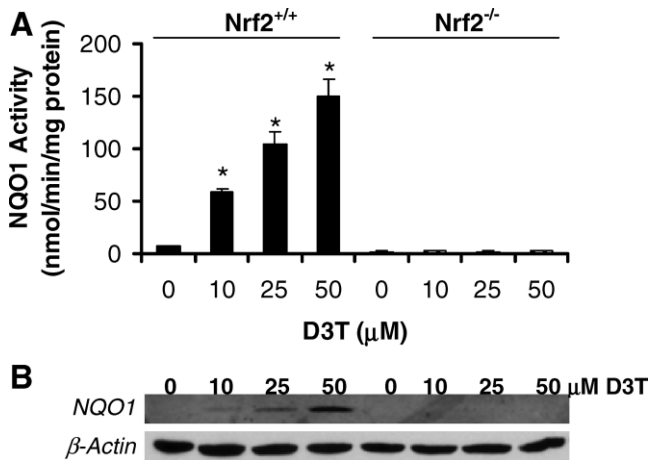


Figure 5. Effects of D3T treatment on NQO1 activity (A) and protein expression (B) in *Nrf2*^{+/+} and *Nrf2*^{-/-} macrophages. Macrophages were incubated with the indicated concentrations of D3T for 48 h, followed by measurement of cellular NQO1 activity and protein expression. Values in panel A represent means $\pm$ SEM from three separate experiments. *, significantly different from 0 μ M D3T. Gel picture in panel B is representative of three separate experiments.

next examined the effects of targeted disruption of Nrf2 on D3T-inducible expression of mRNA for the above antioxidant and phase 2 enzymes. As shown in Figure 6, D3T treatment led to significant time-dependent increases in the levels of mRNA for catalase, γ GCL catalytic subunit (GCLC), GR, GST-A1, GST-M1 and NQO1 in wild-type, but not *Nrf2*^{-/-} macrophages. Notably, the basal levels of mRNA for catalase, GST-A1 and NQO1 were also reduced in *Nrf2*^{-/-} macrophages as compared with the wild-type cells. Interestingly, although the basal activity and/or protein expression of γ GCL, GR, and GST-M were reduced in *Nrf2*^{-/-} macrophages, the basal mRNA levels for GCLC, GR and GST-M1 did not show the same pattern of changes. Unexpectedly, the basal level of mRNA for GST-M1 was higher in *Nrf2*^{-/-} macrophages than in wild-type cells (Fig. 6).

H₂O₂-Mediated Cell Injury in Wild-Type and *Nrf2*^{-/-} Macrophages, and the Cytoprotective Effects of D3T. H₂O₂ is one of the most commonly encountered ROS in causing oxidative biological damage, including macrophage injury. As shown in Figure 7, *Nrf2*^{-/-} macrophages showed increased sensitivity to H₂O₂-induced cell injury, as compared with wild-type cells. Pretreatment of the wild-type macrophages with D3T afforded marked protection against H₂O₂-mediated cell injury. However, the same D3T pretreatment led to no significant cytoprotection against H₂O₂-mediated cell injury in *Nrf2*^{-/-} macrophages.

Peroxynitrite-Mediated Cell Injury in Wild-Type and *Nrf2*^{-/-} Macrophages, and the Cytoprotective Effects of D3T. As compared with wild-type macrophages, *Nrf2*^{-/-} macrophages also exhibited increased susceptibility to cell injury induced by various concentrations of SIN-1, a peroxynitrite generator (Fig. 8). Pretreatment of wild-type macrophages with D3T afforded

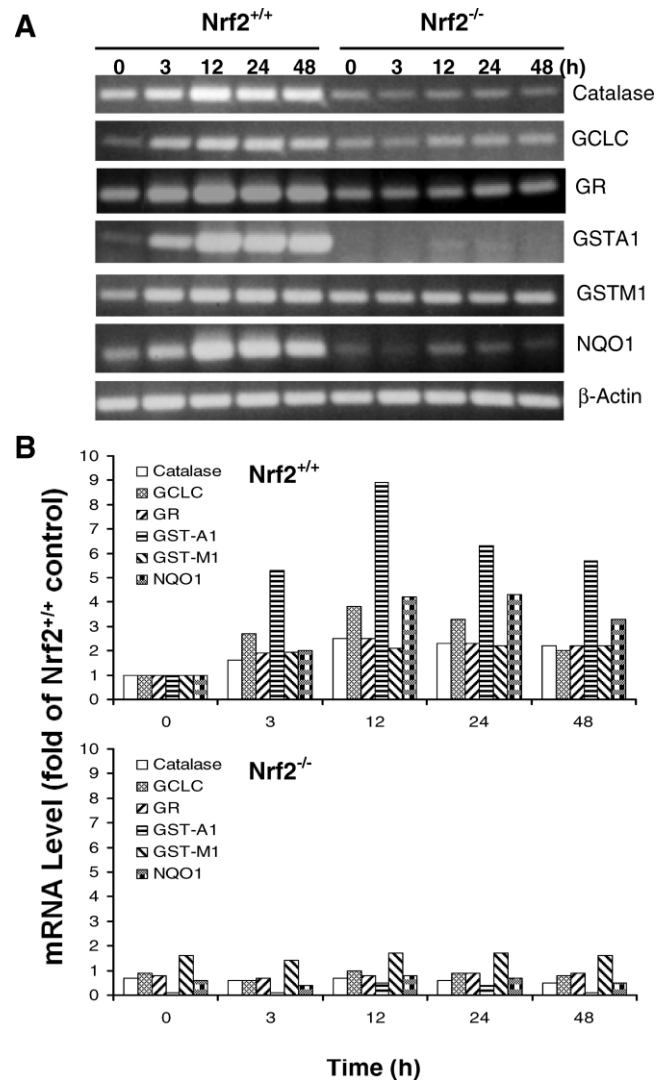


Figure 6. Effects of D3T treatment on mRNA expression of various antioxidant and phase 2 genes in *Nrf2*^{+/+} and *Nrf2*^{-/-} macrophages. Macrophages were incubated with 50 μ M D3T for the indicated time points, followed by detection of cellular mRNA levels for the indicated genes. Gel pictures in panel A are representative of two separate experiments. Values in panel B represent averages from two separate experiments. The data are expressed as relative ratios of density of the gel DNA bands for the respective genes after normalization to that of β -actin.

remarkable cytoprotection against SIN-1-induced cell injury. Notably, remarkable cytoprotection of D3T pretreatment was seen at all of the three toxic concentrations (50, 100 and 200 μ M) of SIN-1. In contrast, pretreatment of *Nrf2*^{-/-} macrophages with the same concentration of D3T only led to little cytoprotection against 50 μ M SIN-1-induced cell injury (Fig. 8).

Acrolein-Mediated Cell Injury in Wild-Type and *Nrf2*^{-/-} Macrophages, and the Cytoprotective Effects of D3T. To investigate the role of Nrf2 in determining the susceptibility of macrophages to electrophilic stress, we exposed to the macrophages to acrolein, a potent electrophilic macrophage toxin. As shown in Figure

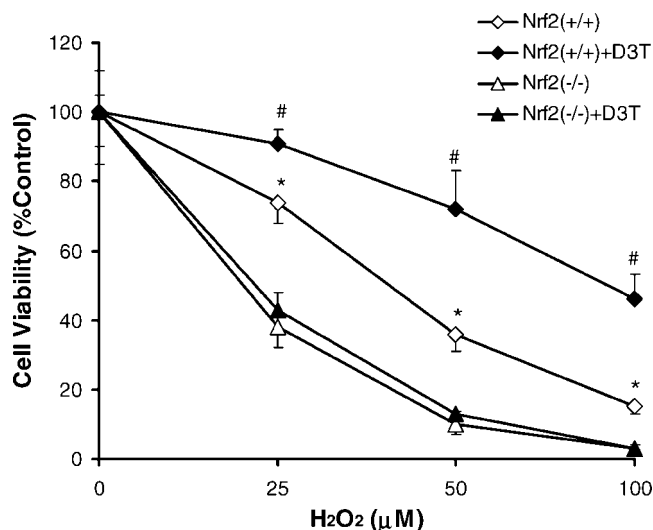


Figure 7. H₂O₂-induced cytotoxicity in Nrf2^{+/+} and Nrf2^{-/-} macrophages, and the cytoprotective effects of D3T pretreatment. Macrophages were incubated with or without 50 μM D3T for 48 h, followed by incubation with various concentrations of H₂O₂ for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means ± SEM from at least three separate experiments. *, significantly different from Nrf2(-/-); #, significantly different from Nrf2(+/-).

9, targeted disruption of Nrf2 dramatically sensitized the macrophages to acrolein-induced cell injury. Exposure of wild-type macrophages to acrolein at 10, 20 and 30 μM also led to significant concentration-dependent cell injury, and the cell injury induced by the above concentrations of acrolein was nearly completely prevented by D3T pretreatment. However, the same D3T pretreatment of Nrf2^{-/-}

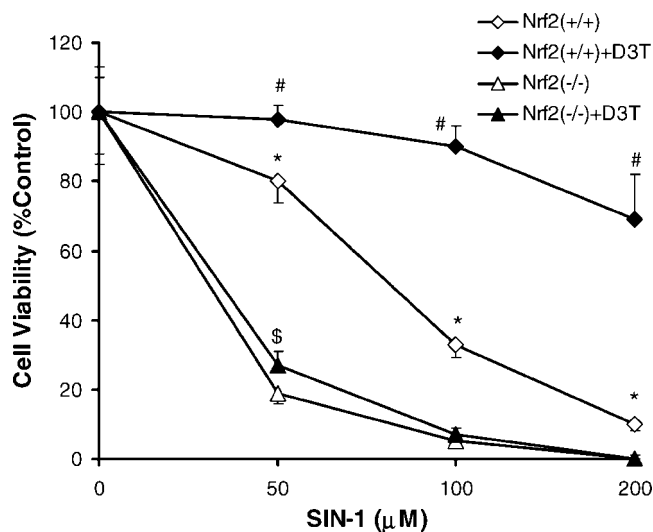


Figure 8. SIN-1-induced cytotoxicity in Nrf2^{+/+} and Nrf2^{-/-} macrophages, and the cytoprotective effects of D3T pretreatment. Macrophages were incubated with or without 50 μM D3T for 48 h, followed by incubation with various concentrations of SIN-1 for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means ± SEM from at least three separate experiments. *, significantly different from Nrf2(-/-); #, significantly different from Nrf2(+/-); \$, significantly different from Nrf2(-/-).

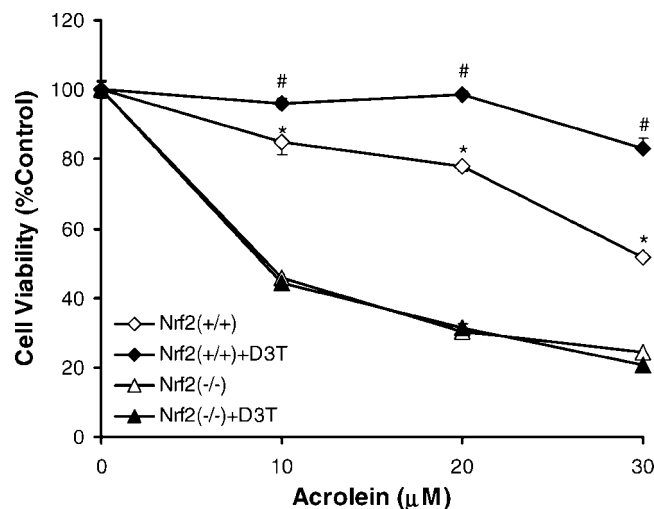


Figure 9. Acrolein-induced cytotoxicity in Nrf2^{+/+} and Nrf2^{-/-} macrophages, and the cytoprotective effects of D3T pretreatment. Macrophages were incubated with or without 50 μM D3T for 48 h, followed by incubation with various concentrations of acrolein for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means ± SEM from at least three separate experiments. *, significantly different from Nrf2(-/-); #, significantly different from Nrf2(+/-).

macrophages led to no significant cytoprotection against acrolein-induced cell injury (Fig. 9).

Cadmium-Mediated Cell Injury in Wild-Type and Nrf2^{-/-} Macrophages, and the Cytoprotective Effects of D3T. Exposure of wild-type macrophages to low micromolar concentrations (5, 10, and 20 μM) of cadmium (CdCl₂), a macrophage toxin, caused significant cell injury in a concentration-dependent manner (Fig. 10). Exposure of

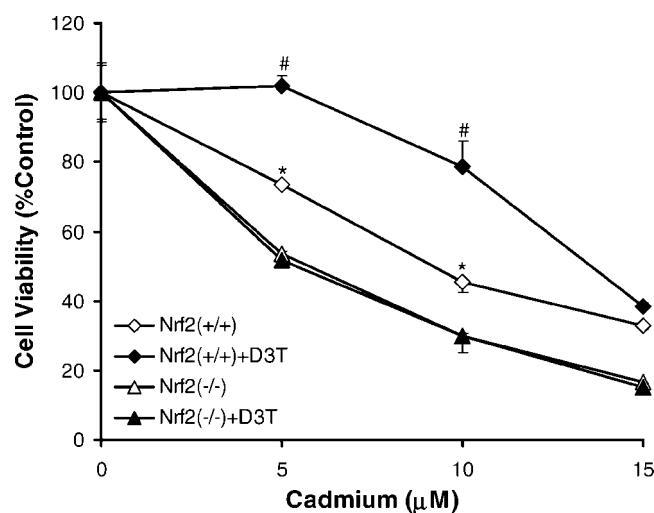


Figure 10. Cadmium-induced cytotoxicity in Nrf2^{+/+} and Nrf2^{-/-} macrophages, and the cytoprotective effects of D3T pretreatment. Macrophages were incubated with or without 50 μM D3T for 48 h, followed by incubation with various concentrations of cadmium chloride for another 24 h. After this incubation, cytotoxicity was determined by MTT reduction assay. Values represent means ± SEM from at least three separate experiments. *, significantly different from Nrf2(-/-); #, significantly different from Nrf2(+/-).

Nrf2^{-/-} macrophages to the same concentrations of cadmium resulted in more pronounced cell injury. The cell injury induced by cadmium (5 and 10 μ M) in wild-type macrophages was significantly protected by D3T pretreatment. In contrast, the same D3T pretreatment afforded no cytoprotection against cadmium-induced cell injury in Nrf2^{-/-} macrophages (Fig. 10).

Discussion

Due to the crucial involvement of macrophages in immunity as well as other physiological processes, injury of macrophages has been implicated in various pathophysiological processes, including increased susceptibility to microorganism infection, tumorigenesis, hematotoxicity, and instability of atherosclerotic plaques (1–5). Macrophages are susceptible to cell injury elicited by various oxidative and electrophilic species, including authentic ROS, RNS, reactive aldehydes, as well as environmental heavy metals, such as cadmium. These reactive species can be formed endogenously from various pathophysiological processes, and/or exist as environmental pollutants (28–30). Although it is known that a number of cellular antioxidants and phase 2 enzymes are involved in the detoxification of various oxidants and electrophiles in mammalian tissues/cells, careful studies on these endogenous antioxidative/phase 2 defenses and their molecular regulation, as well as their inducibility by chemoprotectants in macrophages are lacking. Accordingly, the purpose of the present study was three-fold: (1) to determine the constitutive expression of a series of key antioxidants and phase 2 enzymes, including SOD, catalase, GSH/ γ GCL, GPx, GR, GST, and NQO1 in macrophages and their regulation by Nrf2, a recently identified central regulator of cytoprotective genes in mammalian cells (18, 19); (2) to determine the role of Nrf2 in regulating the inducible expression of the above antioxidants and phase 2 enzymes in macrophages by D3T, a potent chemoprotectant; and (3) to determine the role of Nrf2-regulated antioxidants and phase 2 enzymes in protecting against macrophage injury from oxidative and electrophilic stress.

It is well established that Nrf2 is an indispensable regulator of the constitutive expression of various antioxidative and phase 2 genes in a variety of cell types (18, 19). However, the role of Nrf2 in regulating the constitutive expression of a wide spectrum of different antioxidants and phase 2 enzymes in macrophages has not been reported in literature. The results of the present study demonstrated that wild-type macrophages from mice expressed measurable levels/activities of a number of antioxidants and phase 2 enzymes, including SOD, catalase, GSH, GPx, GR, GST, and NQO1 (Table 2). The basal levels/activities of the above antioxidants and phase 2 enzymes, except for SOD were significantly reduced in Nrf2^{-/-} macrophages, suggesting that Nrf2 is a key regulator of the constitutive expression of various antioxidants and phase 2 enzymes in macrophages.

The basal activity of GPx was also significantly reduced in Nrf2^{-/-} macrophages, which was in contrast to the observations made in other types of cells, including bone marrow stromal cells and cardiac fibroblasts, where the basal activity of GPx was not affected by the status of Nrf2 (20, 31). The exact mechanism involved in this cell-type specific regulation of GPx by Nrf2 remains unclear. GPx exists in various isoforms, which show tissue/cell-specific distribution (32, 33), and the different isoforms might be regulated differently by Nrf2 signaling. Indeed, a gastrointestinal form of GPx (GPx2) was shown to be selectively regulated by Nrf2 (34). It was also worthy of mentioning that the Nrf2 dependence of the basal levels/activities of antioxidants and phase 2 enzymes, as shown in Table 2 was generally consistent with that of the basal protein expression, as detected by immunoblot (Figs. 1–4). For example, the basal levels of protein expression for catalase, γ GCL, GR, GST-A and GST-M were also significantly reduced in Nrf2^{-/-} macrophages as compared with wild-type cells (Figs. 1–4), suggesting that the reduced activities of the antioxidants and phase 2 enzymes in Nrf2^{-/-} macrophages were due to decreased expression of the corresponding proteins.

To determine the role of Nrf2 in controlling the inducible expression of macrophage antioxidants and phase 2 enzymes, both wild-type and Nrf2^{-/-} macrophages were treated with D3T, a potent member of dithiolethiones exhibiting chemoprotective effects (35). D3T potently induced the activity, as well as the protein and mRNA expression of catalase, but not SOD in wild-type macrophages, and such an inducibility of catalase was completely abolished in Nrf2^{-/-} macrophages (Figs. 1, 6), suggesting that Nrf2 not only regulated the basal level/activity of catalase but also its D3T-mediated inducible expression. It remains unknown why SOD in macrophages was not D3T-inducible. In this context, SOD in vascular smooth muscle cells and cardiac fibroblasts was found to be inducible by D3T (31, 36), suggesting that SOD in different cell types might be regulated via distinct signaling mechanisms.

It is known that GSH is inducible by D3T in various types of cells (20, 23, 26). Both the content of GSH, and the level of γ GCL protein and GCLC mRNA were dramatically increased after D3T treatment of wild-type macrophages, but not Nrf2^{-/-} cells (Figs. 2, 6), indicating that Nrf2 is an indispensable factor for determining not only the basal levels but also the D3T inducibility of GSH/ γ GCL. Since γ GCL is the key enzyme of GSH biosynthesis (37), it was likely that the Nrf2-dependent induction of GSH by D3T resulted from the increased expression of the γ GCL in wild-type macrophages. Similar to what was seen with GSH, D3T also potently induced GR activity as well as its protein and mRNA expression in wild-type macrophages, but not in Nrf2^{-/-} cells (Figs. 3, 6), pointing to a critical role for Nrf2 in regulating the inducible expression of GR in macrophages. These observations were also in line with previous studies, demonstrating that γ GCL and GR are two Nrf2-

regulated antioxidant genes (18–20). D3T treatment led to induction of GPx in both wild-type and Nrf2^{-/-} macrophages (Fig. 3), indicating that although Nrf2 appeared to affect the basal activity of GPx (Table 2), it played no role in regulating the D3T-inducible expression of GPx in macrophages. This observation was in contrast to what was observed in bone marrow stromal cells, in which Nrf2 deficiency was found to abolish the D3T-mediated induction of GPx, but not the basal expression of this enzyme (20). Apparently, the regulation of GPx by Nrf2 is complex with regard to cell types, which, as aforementioned, could be due to the differential expression of the GPx isozymes in different cell types.

GST and NQO1 are two most extensively studied Nrf2-regulated phase 2 enzymes in various types of tissues/cells (18, 19, 35). The basal and inducible expression of these two enzymes and their regulation by Nrf2 signaling in macrophages has not been reported in literature. As compared with most other types of cells, wild-type macrophages expressed high basal activity of GST, but the basal activity of NQO1 was relatively deficient (Table 2). The complete abolishment of the D3T-inducibility of the total GST activity in Nrf2^{-/-} macrophages indicated that Nrf2 was also an indispensable factor in regulating not only the basal activity but also the inducible expression of GST in macrophages. It is known that GST exists as a family of various members, with GST-A, -M and -P being the three major isozymes in most types of cells (38). 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 wild-type, but not Nrf2^{-/-} macrophages (Figs. 4, 6). Notably, treatment of either wild-type or Nrf2^{-/-} macrophages with D3T did not result in any changes in the protein expression of GST-P (Fig. 4). Previous studies also suggested that GST-P was not readily inducible by chemoprotectants, including D3T in other types of cells (20, 39). These observations suggested that different isozymes of GST were regulated differently by the Nrf2 signaling. Although the basal expression of NQO1 in wild-type macrophages was relatively deficient, treatment with D3T resulted in remarkable ~9–20-fold induction of NQO1 activity, which was also accompanied by dramatic induction of the NQO1 protein and mRNA expression (Figs. 5, 6). The complete inability of D3T to induce NQO1 in Nrf2^{-/-} macrophages suggested that in macrophages the inducible expression of NQO1 was exclusively regulated by Nrf2 signaling.

As stated above, macrophages are critical target cells of oxidative and electrophilic stress. Since both the constitutive and D3T-inducible expression of a series of key endogenous antioxidants and phase 2 enzymes in macrophages were regulated by Nrf2 signaling, we next determined if Nrf2 signaling also controlled the susceptibility of macrophages to oxidative and electrophilic injury as well as the cytoprotective effects of D3T. To this end, macrophages

were exposed to various oxidative and electrophilic species, including H₂O₂, SIN-1-derived peroxynitrite, acrolein and cadmium. H₂O₂ and peroxynitrite are the most encountered ROS/RNS in biological systems. Indeed, these oxidants have been found to be responsible for macrophage injury/death (40, 41). ROS/RNS are also formed and present in air pollution, which contribute to the injury of alveolar macrophages in animals or humans exposed to air pollutants (8, 42, 43). As expected, cell injury induced by H₂O₂ or SIN-1-derived peroxynitrite was markedly augmented in Nrf2^{-/-} macrophages, and D3T-treatment of the wild-type, but not Nrf2^{-/-} macrophages provided remarkable cytoprotection against these oxidant-induced cell injury (Figs. 7, 8). It is well-known that detoxification of H₂O₂ in mammalian cells relies on several cellular antioxidants, especially GSH (in the presence of GPx) and catalase. GSH (in the presence of GPx) has also been found to be a major cellular factor for detoxification of peroxynitrite (44). In addition, the augmented activity of GR by D3T could also contribute to the increased levels of GSH due to its ability to reduce GSSG to GSH. Indeed, overexpression of GR in macrophages was found to protect against oxidative macrophage injury as well as decrease atherosclerotic lesion formation in LDL receptor-deficient mice (45). Therefore, the reduced constitutive expression of the above antioxidants as well as their inability to be upregulated by D3T in Nrf2^{-/-} macrophages were most likely responsible for the increased susceptibility of these cells to the above ROS/RNS-elicited cell injury. In line with this notion, Wang et al. recently reported that glutathione system acted as a major protective mechanism against oxidized LDL-mediated macrophage injury (40). In view of the crucial role of GSH system in protecting against oxidative macrophage injury as well as in retarding atherosclerosis (40, 45–47), elucidation of the role of Nrf2 signaling in regulating both constitutive and inducible expression of macrophage GSH system is of importance for further understanding the involvement of macrophages in atherogenesis.

Targeted disruption of Nrf2 also resulted in increased susceptibility of macrophages to acrolein- and cadmium-induced cell injury (Figs. 9, 10). Acrolein is a potent electrophilic, α,β -unsaturated aldehyde, which can be formed during lipid peroxidation in biological systems (28). Acrolein is also present in air pollution, and along with cadmium is a component of cigarette smoke (10, 12, 28). Both acrolein and cadmium are known to cause toxicity to macrophages, and have been identified as human respiratory toxins that suppress pulmonary host defense partially due to their toxic effects on alveolar macrophages (9–12). Acrolein and cadmium are also able to cause injury to macrophages in other tissues. For example, acrolein formed from lipid peroxidation of LDL was proposed to contribute to macrophage injury and dysfunction, and acrolein-protein adducts were also detected predominantly in macrophages in human atherosclerotic lesions (48). Similarly, macrophages in various tissues, including bone and liver were

found to be targets of cadmium intoxication (49, 50). Although a number of cellular factors have been proposed to participate in detoxification of both acrolein and cadmium in biological systems, detoxification of these toxins heavily relies on cellular GSH in mammalian cells. In this context, due to its high electrophilic property, acrolein readily reacts with GSH to form a less reactive GSH-conjugate, leading to its detoxification (28). The presence of GST has also been found to promote the conjugation reaction between acrolein and GSH (28, 51). Thus, the decreased level/activity of GSH/GST and their inability to be induced by D3T would account for the augmented sensitivity of the Nrf2^{-/-} macrophages to acrolein-induced cell injury and for the failure of D3T treatment to protect these cells from acrolein toxicity. Similarly, due to the critical role of GSH in detoxification of cadmium, the decreased level of GSH in Nrf2^{-/-} macrophages appeared to be responsible for their increased sensitivity to cadmium-induced cell injury. Recent evidence suggests that cadmium toxicity is also attributed to oxidative stress and lipid peroxidation (52, 53). In this regard, the reduced expression of other antioxidants in Nrf2^{-/-} macrophages might also contribute to the augmented sensitivity of these cells to cadmium-induced injury. On the other hand, the potent induction of GSH as well as other antioxidant enzymes in wild-type, but not Nrf2^{-/-} macrophages by D3T was apparently responsible for the augmented resistance of the D3T-treated wild-type cells to cadmium-induced injury.

In summary, the results of the present study demonstrate that Nrf2 is indispensable for regulation of both constitutive and D3T-inducible expression of a series of key antioxidants and phase 2 enzymes in mouse macrophages. Nrf2 signaling is also an important mechanism in controlling macrophage susceptibility to cell injury elicited by various oxidative and electrophilic species. Due to the crucial involvement of oxidative and electrophilic injury of macrophages in various pathophysiological processes, Nrf2-mediated regulation of antioxidants and phase 2 enzymes in these cells is of importance for protection against the disease processes associated with macrophage injury from oxidative and electrophilic stress.

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