Activation of Nrf2 in Defense against Cadmium-Induced Oxidative Stress†

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Exposure to cadmium (Cd) elicits a range of adverse responses including oxidative damage and cancer. The molecular targets of Cd remain largely unidentified. Here, we analyzed the function and signal transduction of transcription factor Nrf2 in protection against Cd-induced oxidative stress. Wild-type ($Nrf2^{+/+}$) mouse embryonic fibroblasts (MEF) produced reactive oxygen species (ROS) at a low level, whereas treatment with Cd significantly increased the ROS production. On the other hand, Nrf2 knockout ($Nrf2^{-/-}$) MEF cells exhibited an elevated level of ROS under a basal condition, and Cd dramatically increased the ROS production at concentrations as low as 2 μM, resulting in increased sensitivity to Cd-induced cell death. Cd induced the basal and inducible expression of cytoprotective enzymes NQO1 and HO1 in WT MEF cells, but induction was lost in $Nrf2^{-/-}$ MEF cells. Induction of the genes required antioxidant response elements (ARE) as Cd drove ARE-dependent reporter expression and Cd-activated Nrf2 bound to endogenous AREs in mouse hepa1c1c7 cells. Activation of Nrf2 by Cd involved stabilization of the Nrf2 protein, increased formation of Nrf2/Keap1 complex in the cytoplasm, translocation of the complex into the nucleus, and subsequently disruption of the complex. Lastly, Nrf2 was found ubiquitinated in the cytoplasm but deubiquitinated in the nucleus. The study provided a mechanistic transcriptional model in which Cd activates Nrf2 through a metal-activated signaling pathway involving a dynamic interplay between ubiquitination/deubiquitination and complex formation/dissociation of Nrf2 and Keap1.

Introduction

Human bodies have developed elaborate defense mechanisms to counteract various environmental chemical stresses. One such genetic program involves coordinated induction of a battery of cytoprotective genes encoding detoxification enzymes and antioxidant proteins. The cytoprotective proteins promote cellular defense against adverse stimuli, in particular, oxidative damage and electrophilic signals (*1–5*). The coordinated transcriptional response is mediated through the antioxidant response element (ARE)[1] located at the enhancer regions of the genes (*6, 7*).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the cap "n" collar basic region-leucine zipper (CNC bZip) transcription factors. In recent years, Nrf2 has emerged as a key receptor/transcriptional factor in the transcriptional regulation of ARE-dependent gene expression in response to oxidative stimuli and a spectrum of toxic signals. In experimental animals, loss of the expression and function of Nrf2, such as knockout of the gene in mice, increased the susceptibility of the animals to a range of chemical toxicity and diseases including chemical carcinogenesis, inhalation toxic particles, ovarian toxicants, and autoimmune disease (*2, 5, 8–10*). Conversely, induction of Nrf2 target genes enhanced protection against cancer, chemical toxicity, and certain chronic disorders.

In hepatocytes, unactivated Nrf2 is mostly ubiquitinated by the Keap1 and Cul3-dependent E3 and degraded through the 26S proteasome pathway in the cytoplasm with a $t_{1/2}$ of ~20 min (*6, 11–14*). Nrf2 is also present in the nucleus at a low level under basal conditions mediating the constitutive expression of target genes such as the NAD(P)H:quinone oxidoreductase 1 (*Nqo1*) and heme oxygenase 1 (*Ho-1*) (*12*). Chemical signals such as phenolic antioxidant *tert*-butylhydroquinone (tBHQ) activate Nrf2 by binding to Keap1 via interaction with its thiol groups. Activated Nrf2 accumulates in the nucleus to mediate induction of the genes. In this framework, Keap1 serves both as an adaptor protein docking Nrf2 in the E3 complex for ubiquitination and as an initial contact with the chemicals. Several critical issues remain unaddressed in the signal transduction of Nrf2 activation. For instance, treatment with tBHQ or other antioxidants does not dissociate Nrf2 from Keap1; rather, it increases the amount of the Nrf2/Keap1 complex in the cells (*12, 15*). In addition, Nrf2 can be activated by a variety of chemical signals with diverse properties; whether a unified signaling pathway can be found for the activation of Nrf2 by all inducers remains to be examined.

Cadmium (Cd) is a human carcinogen and a major environmental/occupational toxic metal element (*16, 17*). Cd is widely encountered in industries such as electroplating, as color pigments, for nickel−Cd battery production, and as byproducts of zinc and lead mining and smelting. Environmental sources of Cd exposure include tobacco smoking and food consumption

† Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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[1] Abbreviations: ARE, antioxidant response element; As, arsenic; Cd, cadmium; ChIP, chromatin immunoprecipitation; Cr, chromium; DHE, dihydroethium; HO-1, heme oxygenase 1; MEF, mouse embryonic fibroblast; MRE, metal response element; MTF1, metal-activated transcription factor 1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; RNS, reactive nitrogen species; ROS, reactive oxygen species; tBHQ, *tert*-butylhydroquinone.

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since plants readily take up Cd from Cd-contaminated soil. Cd causes a wide range of toxicity and disease in humans, including lung and prostate cancer, obstructive lung disease, kidney dysfunction, bone deformation, hypertension, and cardiovascular disorders. The molecular mechanism(s) accounting for most of the biological effects of Cd are not well-understood but may involve the stimulation of reactive oxygen species (ROS) production, interaction with protein thiol groups, and replacement of essential minerals such as calcium. The molecular targets of Cd toxicity are largely unidentified. In this study, we analyzed the role of Nrf2-mediated gene induction in Cd toxicity and the mechanism by which Cd activates Nrf2.

Our findings revealed that exposure to Cd induced the expression of cytoprotective genes *Nqo1* and *Ho-1*. Induction of the genes required Nrf2 and was mediated through the AREs of the genes. Furthermore, induction of the genes closely correlated with inhibition of Cd-induced ROS production and cell death in an Nrf2-dependent manner. Analysis of the signaling mechanism revealed that Cd stabilized Nrf2 and induced the nuclear accumulation of Nrf2. In the nucleus, the association of Nrf2 and Keap1 was significantly reduced by Cd in comparison to tBHQ treatment but was slightly increased by Cd in comparison to the untreated control. In addition, both Nrf2 and Keap1 were ubiquitinated in the cytoplasm, but ubiquitination of the proteins was largely reduced in the nucleus. Together, the findings suggest a dynamic interplay among the ubiquitination/deubiquitination, nuclear targeting, and complex formation/dissociation of Nrf2 and Keap1 during Nrf2 activation by Cd.

Experimental Procedures

Cell Culture and Treatment. Mouse hepa1c1c7 cells were provided by Dr. J. P. Whitlock, Jr. (Stanford University, Stanford, CA). The cells were grown in α-minimal essential medium with 10% fetal bovine serum (FBS) and 5% CO_2. Mouse $Nrf2^{+/+}$ and $^{-/-}$ embryonic fibroblast cells were derived from wild-type and Nrf2-null mice as described previously (*18*). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 5% CO_2. Penicillin (100 U/mL) and streptomycin (100 μg/mL) were added to the media to prevent contamination. Cells at confluence were treated with reagents as specified in figure legends. Dimethyl sulfoxide (DMSO), tBHQ, and Cd chloride hemi (pentahydrate) were purchased from Sigma-Aldrich (St. Louis, MO). MG132 was from BioMol (Plymouth, PA).

Plasmid Construction and Transfection. The full-length coding cDNA of mouse Nrf2 was obtained by reverse transcription polymerase chain reaction (PCR) and was verified by sequencing. The plasmid pCMV-HA-Nrf2 was constructed as reported before (*12*). The *Ho-1* ARE reporter was kindly provided by Drs. Itoh and Yamamoto (University of Tsukuba, Tsukuba, Japan). The reporter plasmid, pCMV-HA-Nrf2, and the Renilla construct (as an internal transfection control, Promega) were cotransfected into hepa1c1c7 cells using the lipofectamine plus reagents from Invitrogen according to the manufacturer's instructions, and the activities were measured using the dual luciferase assay protocol (Promega) as described previously (*12, 19*). The activities were corrected for both the protein content and the internal control plasmid transfection efficiency.

Cell Fractionation, Immunoprecipitation (IP), and Immunoblotting (IB). Nuclear and cytoplasmic fractions were prepared using the Nuclei EZ PREP reagents from Sigma. Briefly, cells at 90% confluence in 10 cm dishes were washed with ice-cold PBS and were lysed with an ice-cold Nuclei EZ PREP lysis buffer containing protease and phosphatase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/mL of each aprotinin, leupeptin, and pepstatin A; all added immediately before use). The cell lysate was centrifuged at 500*g* for 5 min at 4 °C to give rise to nuclei (pellet fraction) and cytosol (supernatant fraction). The nuclei pellets were washed once with the lysis buffer and resuspended in a RIPA buffer (50 mM Tris-HCl, pH 7.4, with 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA; protease and phosphatase inhibitors were added as described above). The nuclear and cytoplasmic fractions were stored at −80 °C till use.

IB was performed as follows. Cells were washed twice with ice-cold PBS and lysed on ice with the RIPA buffer with protease and phosphatase inhibitors for 30 min. The cell lysate was sonicated briefly and was centrifuged at 14000*g* for 20 min to remove cell debris. Cell lystate (10 μg each), nuclear (5−10 μg), or cytoplasmic (30 μg) proteins were fractionated on 10% SDS-PAGE, transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA), and blotted with primary antibodies at 4 °C overnight with shaking, followed by incubation with horseradish peroxidase-conjugated second antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents from Amersham Biosciences (Piscataway, NJ). Actin was blotted to ensure equal loading.

For IP, cell lysates or cell fractions were precleared with protein G-agarose (Invitrogen) for 1 h at 4 °C, followed by incubation with IP antibodies at 4 °C overnight with shaking. Immune complexes were precipitated by incubation with protein-G agarose at 4 °C for 1 h and a brief centrifugation. The precipitates were washed extensively with PBST and were subjected to fractionation by SDS-PAGE. Protein bands were detected by immunoblotting with specific antibodies as specified in figure legends. Data shown were representatives from two to three separate experiments. Antibodies against Nrf2, Keap1, and HO-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against HA was obtained from Babco (Berkeley Antibody Co., Richmond, CA). Antiubiquitin antibody was from Zymed Laboratories Inc. (South San Francisco, CA).

Chromatin Immunoprecipitation (ChIP) Assay. Hepa1c1c7 cells were treated with Cd or tBHQ for 5 h. The ChIP assay was performed as described previously (*20*). Briefly, DNA proteins were cross-linked by incubating cells with 1% formaldehyde at 37 °C for 10 min. Excess formaldehyde was quenched with 0.125 M glycine at room temperature for 5 min. Cells were collected in 1 mL of a lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, and 0.5% IGEPAL CA-630) with protease inhibitors and were centrifuged to pellet nuclei. The nuclei were resuspended in the lysis buffer, repelleted, and resuspended in a nuclei lysis buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA, and 1% SDS) with protease inhibitors. Chromatin was sonicated to an average size of 200−1000 bp using a tapered microtip at 40% power output (10 times, 10 s each). Cell debris was removed by centrifugation at 14000*g* for 10 min at 4 °C. Sheared chromatin was diluted in an IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.0, and 167 mM NaCl), precleared with protein G containing salmon sperm DNA (240 μg of sperm DNA in 1 mL of solution), and immunoprecipitated with anti-Nrf2 antibody. DNA−protein complexes were eluted from the protein G-agarose beads with 200 μL of an elution buffer (50 mM $NaHCO_2$ and 1% SDS) and were reverse cross-linked by incubating with 8 μL of 5 M NaCl at 65 °C overnight. The DNA samples were purified and were analyzed by real-time PCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) performed on a Bio-Rad iCycler (Bio-Rad, Hercules, CA) following standard procedures. Real-time PCR results were normalized using 1% input as an internal control. Relative DNA amounts were calculated from C_T values for each sample by interpolating into a standard curve obtained using a series dilution of standard DNA samples run under the same conditions. Primer sets used for real-time PCR were as reported before (*12*).

ROS Detection. Intracellular reactive oxygen species (ROS) production was detected by a method described by Carter et al. (*21*). Mouse $Nrf2^{+/+}$ and $^{-/-}$ embryonic fibroblast cells were cultured in eight-well chamber slides to reach 90% confluence and were treated as indicated in the figure legends. Thirty minutes prior to the end of treatment, dihydroethium (hydroethidine or DHE, Invitrogen) was added at 5 μM as a fluorescent indicator of ROS

generation, and the cells were protected from the light. The cells were fixed with ice-cold 4% paraformaldehyde PBS solution for 20 min on ice, and the slides were mounted with mounting solution with DAPI (Vector Laboratories, Burigame, CA) counterstaining to visualize the nucleus. The images were observed with Zeiss LSM510 confocal microscope using a Rhodamin-DAPI setting. The pictures were taken with fixed exposure times. Fluorescence intensity was quantitated using the Optimus Version 6.51 Software (Media Cybernetics, MD). Quantitation data represent means and standard deviations from five to seven separate fields for each treatment.

NQO1 Enzyme Assay. The NQO1 activity was performed as described previously (*22*). Briefly, cells were seeded in 96-well plates at a density of 2×10^4 cells/mL and cultured overnight. The culture was replaced with fresh medium with different treatments as described in figure legends. After an additional 48 h of culture, the medium was decanted and the cells were incubated with 50 μL of 0.8% digitonin and 2 mM EDTA solution for 10 min at 37 °C. A reaction mixture was prepared as follows: 0.375 mL of 1 M Tris-HCl (pH7.4), 10 mg of BSA, 0.1 mL of 1.5% Tween-20, 10 μL of 7.5 mM FAD^+ (Sigma), 0.1 mL of 150 mM glucose-6-phosphatase (Sigma), 9 μL of 50 mM $NADP^+$ (Sigma), 30 units of yeast glucose-6-phosphate dehydrogenasse (Sigma), 4.4 mg of MTT (Sigma), 15 μL of 50 mM menadione (Sigma, added just before use), and distilled water to a volume of 15 mL. Two hundred microliters of the reaction mixture was added to each well for 5 min, and the reaction was stopped by adding a stop solution containing 0.3 mM dicumarol in 0.5% DMSO and 5 mM potassium phosphate, pH 7.4. Absorbance at 595 nm was measured using a 96-well plate reader.

Cytotoxicity Assay. Mouse $Nrf2^{+/+}$ and $^{-/-}$ cells were seeded into a 96-well plate at 5×10^4 cells per well and were cultured overnight. The cells were treated with Cd at concentrations of 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 μM for 6 h. Twenty microliters of Cell Titer 96 AQ_{ueous} One solution (Promega, Madison, WI) was added into each well per 100 μL of culture medium, and the plate was incubated at 37 °C for 3 h in a humidified, 5% CO_2 incubator. Absorbance at 490 nm was recorded in a 96-well plate reader.

Data Quantification and Statistical Analysis. Quantification of RNA or protein bands was performed using the ImageQuant program (Molecular Dynamics, San Jose, CA). Statistical analysis was performed with one-way ANOVA followed by *t* tests using the the GraphPad PRISM program.

Results

Ablation of the Nrf2 Gene Renders the Cell Sensitive to Cd-Induced Oxidative Stress and Cell Death. A hallmark of Cd toxicity is the elevated production of ROS inducing oxidative stress in cells. To analyze the role of Nrf2 in the toxic response to Cd, mouse embryonic fibroblast cells (MEF) derived from Nrf2 wild-type ($Nrf2^{+/+}$) and knockout ($Nrf2^{-/-}$) mice were treated with Cd, and the production of superoxide anion radical was measured. Cd induced oxidative stress in both wild-type and Nrf2-null cells dose dependently (Figure 1). However, the Nrf2-null cells exhibited significantly elevated levels of both basal production and induction of ROS as compared with the wild type. Induction of ROS required 50 μM Cd in the wild type, whereas markedly elevated ROS production was observed in Nrf2-null cells treated with as low as 2 μM Cd, indicating that loss of Nrf2 function increased oxidative stress in cells both under basal conditions and in the presence of Cd. Concomitant to elevated ROS production, the $Nrf2^{-/-}$ cells showed significantly higher levels of cell death as compared with the wild type in a concentration-dependent manner (Figure 2). Increased sensitivity of Nrf2-null cells to Cd toxicity is particularly apparent at low doses of Cd; elevated cell death was observed at concentrations between 1 and 2 μM Cd in Nrf2-null cells ($EC_{20} = \sim 1$ μM), whereas similar levels of toxicity required a magnitude higher concentration of Cd in wild-type cells ($EC_{20} = \sim 20$ μM). The EC_{50} of cell death in $Nrf2^{-/-}$ cells was 50 μM, whereas that of $Nrf2^{+/+}$ cells was 80 μM. Similar results were obtained in cells treated with increasing concentrations of Cd for 12 h (data not shown). Taken together, the findings revealed a protective role of Nrf2 in defense against Cd-induced ROS production and cell toxicity.

Cd Induced Cytoprotective Genes *Ho-1* and *Nqo1* Via the Nrf2/ARE Pathway. The remarkable protective effect of Nrf2 against Cd toxicity prompted us to analyze the target genes of Nrf2 and the signaling pathway of induction of the genes by Nrf2. Screening a panel of cytoprotective genes revealed that Cd induces the mRNA expression of *Nqo1*, a major cytoplasmic quinone reductase critical in quinone metabolism and toxicity, and *Ho-1*, a stress-inducible enzyme involved in the metabolic breakdown of heme products (data not shown). In Hepa1c1c7 cells, the NQO1 enzyme activity was significantly increased by treatment with tBHQ or Cd (Figure 3A). The basal expression and induction of NQO1 enzyme activity required the presence of functional Nrf2, as both activities were lost in Nrf2-null cells (Figure 3B). In Figure 3C, Cd increased the protein level of Nrf2 (110 kDa) at concentrations as low as 2 μM and dramatically increased the level at 10 and 50 μM in wild-type MEF cells; the protein was absent in the Nrf2 knockout cells as expected. The protein of HO-1 was induced by Cd concentration dependently and parallel to the increase of the Nrf2 protein, consistent with the notion that Cd induces HO-1 through Nrf2. However, induction of HO-1 was not totally dependent upon Nrf2, as induction was diminished to a large extent but was not totally abolished in the $Nrf2^{-/-}$ MEF cells (Figure 3C).

Nrf2 controls the transcription of target genes by binding to the AREs located in the enhancer regions of the genes. We tested if induction of *Ho-1* and *Nqo1* by Cd was mediated through ARE using both ARE-driven reporters and an in vivo ARE-binding (ChIP) assay. As shown in Figure 4, Cd induced the induction of a luciferase reporter that was under the control of *Ho-1* ARE. Similar results were obtained with the *Nqo1* ARE-controlled reporter construct (data not shown). In Figure 5, Cd induced the binding of Nrf2 to the ARE of the endogenous *Ho-1* (Figure 5A,B) and *Nqo1* (Figure 5C,D) genes as measured in the ChIP assay. Nrf2 was found to bind the ARE of both genes constitutively (Figure 5A,C, lane 4). Treatment with Cd increased the binding significantly that was comparable to treatment with tBHQ (compare lanes 6 with 5, Figure 5A,C). Normal rabbit IgG was used as a negative control for anti-Nrf2 IgG (lanes 1−3, Figure 5A,C). Figure 5B,D represented quantification of ARE-binding coimmunoprecipitated with Nrf2 in the ChIP assay by real-time PCR. The findings demonstrated that Cd induced *Nqo1* and *Ho-1* by activating Nrf2 and inducing the binding of Nrf2 to AREs of the genes.

Activation of Nrf2 by Cd Involves Disruption of the Nrf2/Keap1 Complex and Inhibition of Nrf2 Ubiquitination in the Nucleus. Unactivated Nrf2 is rapidly degraded through the ubiquitin-26S proteasome pathway in the cytoplasm under a normal circumstance with a $t_{1/2}$ value of ~20 min (*12*). Activation of Nrf2 involves stabilization of the Nrf2 protein; however, the mechanism by which Nrf2 is stabilized remains controversial. To analyze how Nrf2 was activated by Cd, cytoplasmic and nuclear fractions were prepared. The purities of the cell fractions were confirmed by immunoblotting GAPDH (marker of the cytoplasmic fraction) and lamin A (marker of the nuclear fraction), respectively (Figure 6A, the third and fourth panels). The Nrf2 protein was undetectable in the

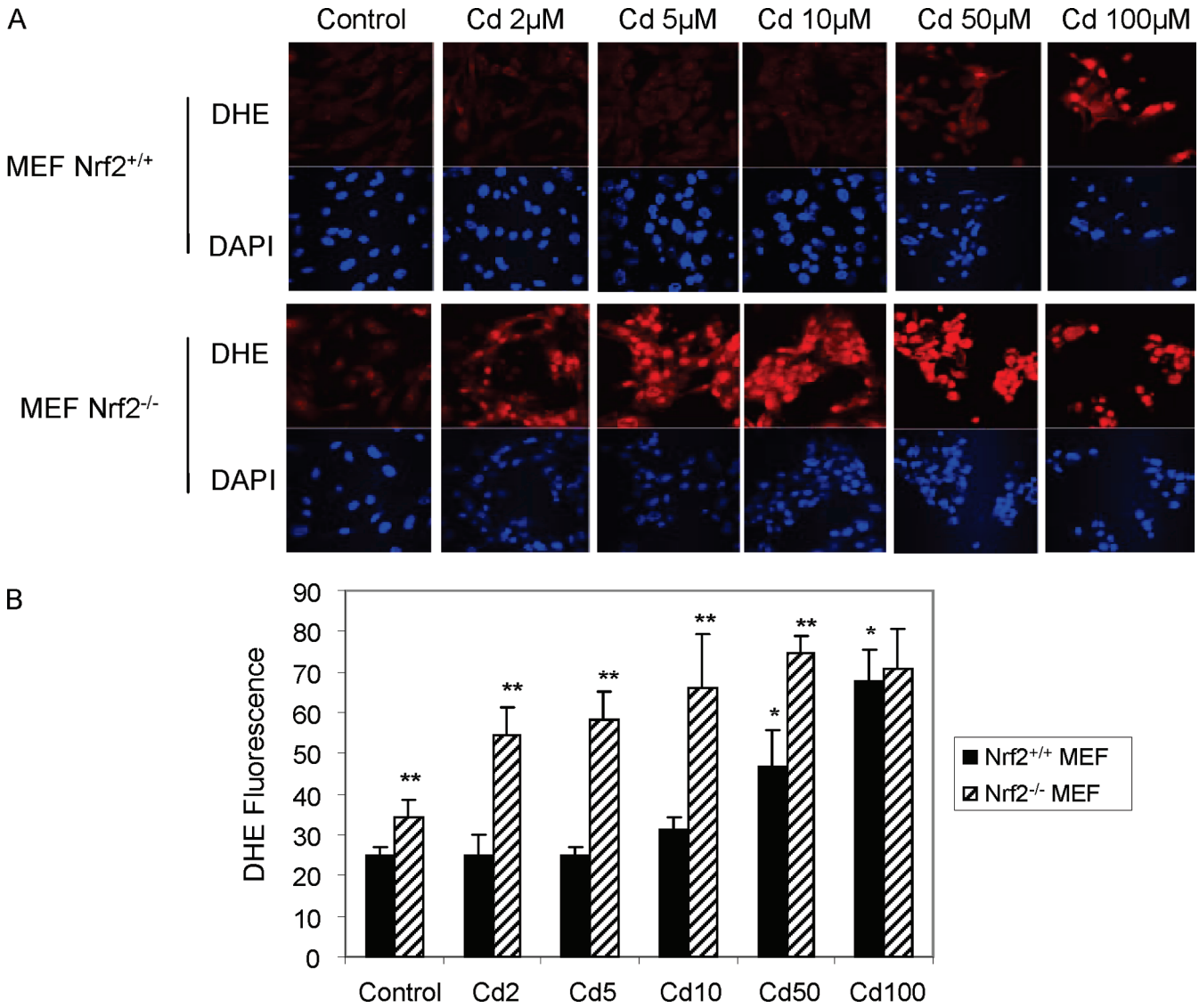

Figure 1. Nrf2 protects against Cd-induced oxidative stress. (A) Nrf2$^{+/+}$ and $^{-/-}$ MEF cells were treated with Cd at 2, 5, 10, 50, and 100 μM for 5 h. DHE, a fluorescent dye specific for superoxide anion radical, was added 30 min prior to the end of the treatment. DHE, which is blue in the cytoplasm, is oxidized to ethidium by ROS that intercalates with DNA and stains the nucleus in a bright red color. Cells were also stained with DAPI for the nucleus (blue). Fluorescence was examined under a fluorescent confocal microscope. (B) Quantification of DHE fluorescence. Data represent means and standard deviations from 5 to 10 separate view fields. *, Nrf2$^{+/+}$ MEF treated with Cd vs control, $p < 0.05$; **, Nrf2$^{-/-}$ MEF vs Nrf2$^{+/+}$ MEF, $p < 0.05$.

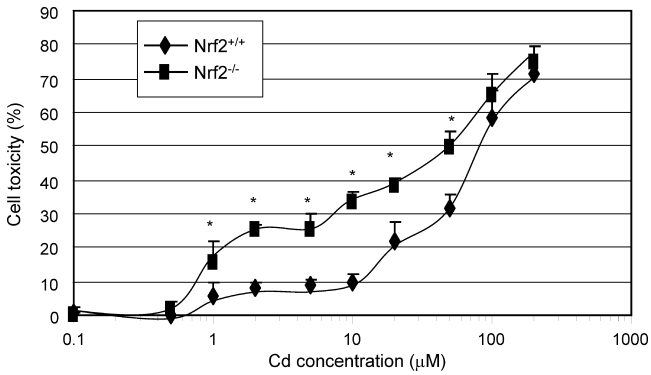

Figure 2. Nrf2$^{+/+}$ and $^{-/-}$ MEF cells were treated with Cd at 0, 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 μM for 6 h. Cell toxicity was detected by adding 20 μL of CellTiter 96 Aqueous One Solution Reagent and measuring the absorbance at 490 nm. Data represent means plus standard deviations from three separate samples; statistical treatment was performed as described in the Experimental Procedures. *, $p < 0.05$.

cytoplasmic extract from untreated cells (left panel, lane 1); treatment with tBHQ increased the protein to detectable level (lane 2), but treatment with Cd did not increase the amount of the Nrf2 protein in the cytoplasmic preparation (lane 3). The Nrf2 protein was enriched in the nucleus by tBHQ and Cd (right panel, compare lanes 2 and 3 with 1). In the presence of MG132, the Nrf2 protein was further increased in both the cytoplasmic and the nuclear fractions (left and right panels, lanes 4−6), consistent with a strong inhibition of proteasomal degradation of Nrf2 by MG132. On the contrary, the Keap1 protein level was not affected by the treatments in both fractions (2nd panels), supporting the notion that Nrf2 activators regulate the protein level of Nrf2 but not of Keap1.

The association of Nrf2 with Keap1 was detected in both the cytoplasm and the nucleus at low levels (Figure 6B, lane 1) constitutively by coimmunoprecipitation of the proteins. Treatment with tBHQ did not inhibit but increased the association in both fractions (Figure 6B, lane 2), in particular in the nucleus (right panel, lane 2) (*12*). Treatment with Cd moderately increased the

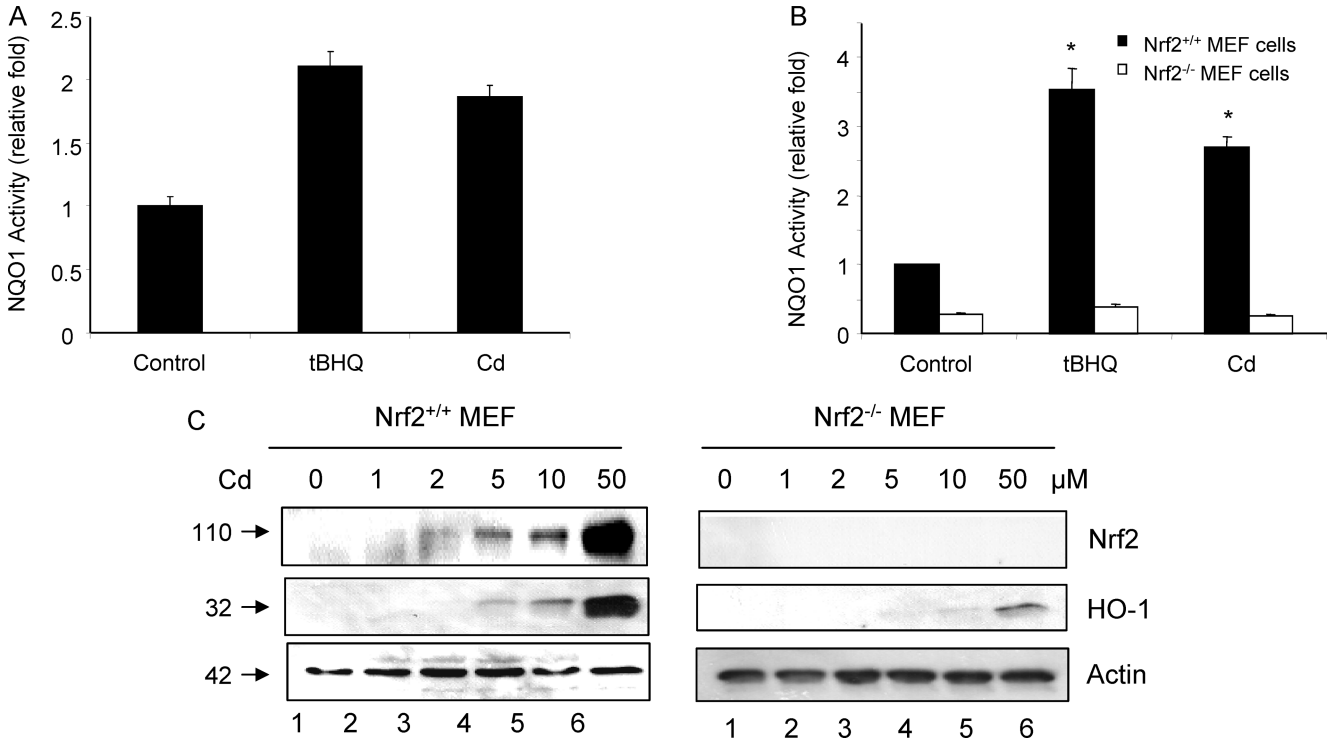

Figure 3. Nrf2 dependence of induction of HO1 and NQO1 by Cd. (A) Induction of NQO1 activity. Heapa1c1c7 cells were treated with 30 μM tBHQ or 10 μM Cd for 48 h. NQO1 activity was determined as described in the Experimental Procedures. *, $p < 0.05$. (B) NQO1 activity. Nrf2$^{+/+}$ and $^{-/-}$ MEF cells were treated with tBHQ or Cd for 48 h, and the NQO1 activity was measured. (C) IB: Nrf2$^{+/+}$ and $^{-/-}$ MEF cells were treated with Cd at 0, 1, 2, 5, 10, and 50 μM for 5 h. Total cell lysates were immunoblotted with anti-Nrf2 or anti-HO-1 antibodies. Actin was blotted as a loading control.

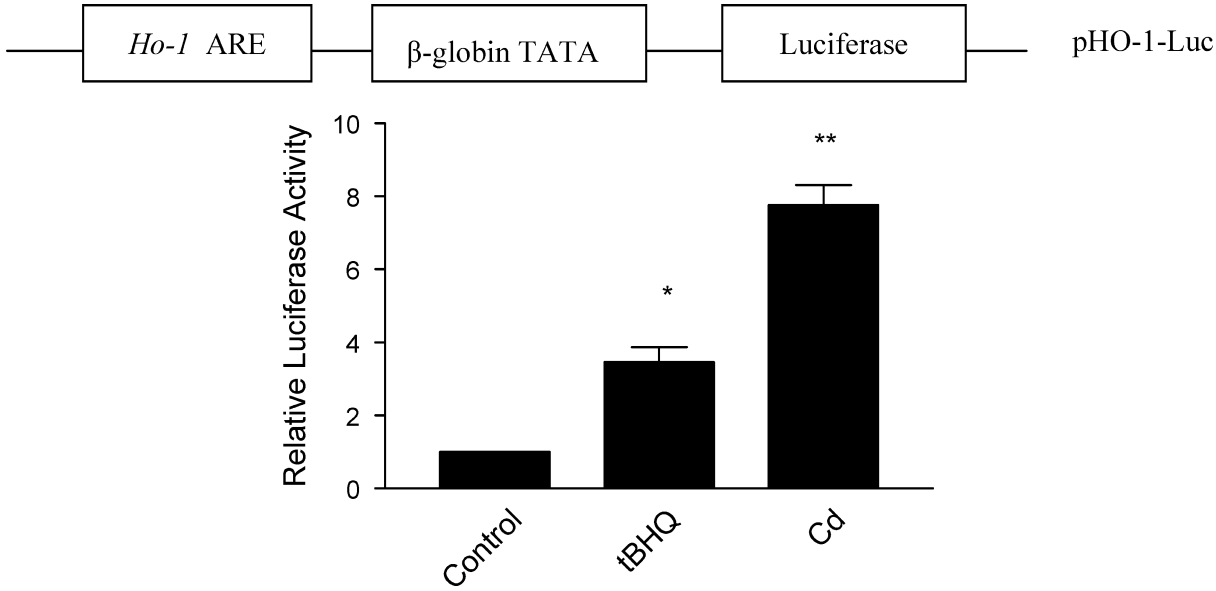

Figure 4. Induction of ARE reporter gene. Hepa1c1c7 cells were transfected with pHO-1 ARE together with pCMV-HA-Nrf2 and the Rinilla construct (as an internal control, Promega), followed by induction with 30 μM tBHQ or 10 μM Cd for 5 h. Cell lysates were prepared, and the luciferase activity was measured using the dual luciferase assay reagents from Promega. Data represent means plus standard deviations from three separate samples; statistical treatment was performed as described in the Experimental Procedures. *, $p < 0.05$.

association in the cytoplasm (left panel, compare lane 3 with 1), consistent with a slightly increased protein level of Nrf2 in the cytoplasm by Cd. However, the association between Nrf2 and Keap1 was significantly reduced in the nucleus in the presence of Cd as compared with tBHQ (right panel, compare lane 3 with 2). The results revealed differential effects of tBHQ and Cd on the interaction between Nrf2 and Keap1 in the nucleus: Cd dissociates Nrf2 from Keap1, whereas tBHQ does not. To further analyze the inhibitory effect of Cd on the association between Nrf2 and Keap1, the cells were treated with MG132, MG132 plus tBHQ, or MG132 plus Cd. As expected, Nrf2 and Keap1 were associated with each other at high levels in both fractions from cells treated with MG132 alone or MG132 plus tBHQ (Figure 6B, lanes 4 and 5 in both panels), whereas, the association was significantly reduced—but not totally eliminated—in the nucleus from MG132 plus Cd treated cells (Figure 6B, right panel, compare lane 6 with lanes 4 and 5), suggesting that Cd disrupts the existing complex of the two proteins in the nucleus in the presence of MG132 to a certain extent.

Ubiquitination of Nrf2 in the cytoplasm and nucleus was examined by IP of the protein using anti-Nrf2 antibodies

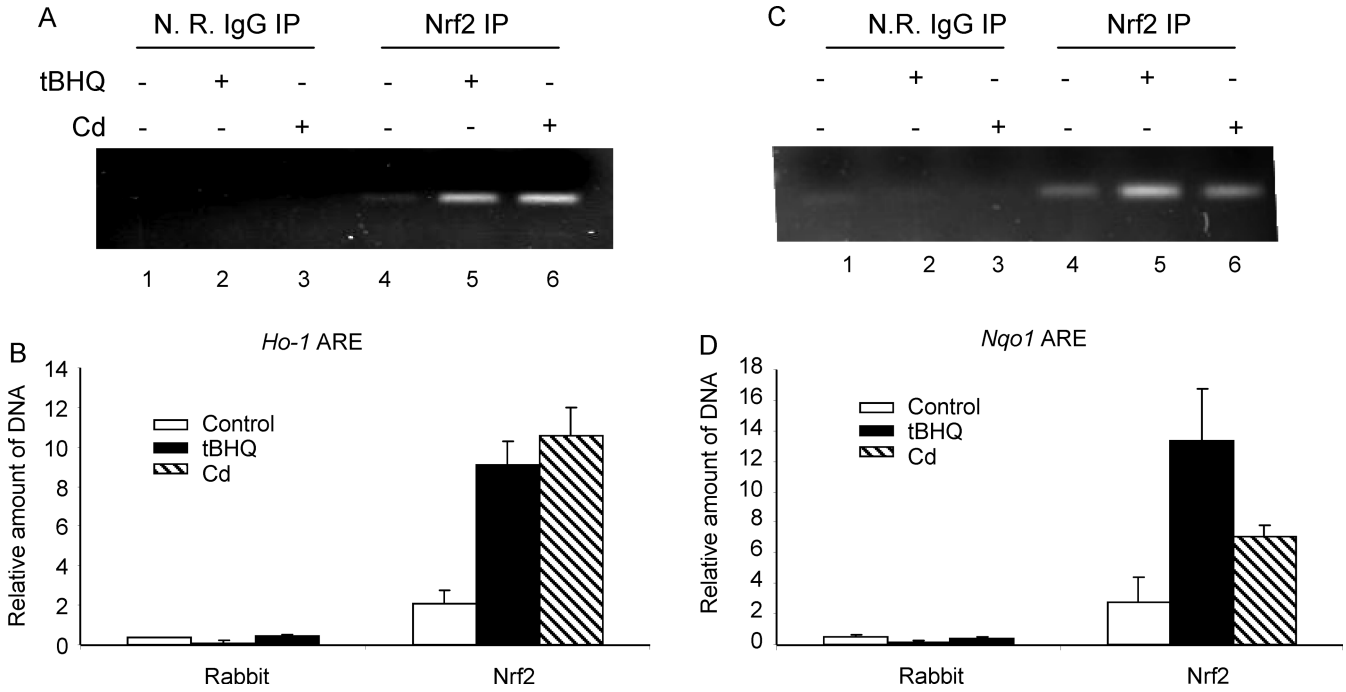

Figure 5. Recruitment of Nrf2 to endogenous ARE of *Nqo1* and *Ho-1* by Cd. Hepa1c1c7 cells were treated with 30 μM tBHQ or 50 μM Cd for 5 h. Binding of Nrf2 to ARE was examined by ChIP. Cross-linked DNA−protein complexes were coimmunoprecipitated with Nrf2 antibody or normal rabbit IgG. DNA fragments were amplified by real-time PCR with primers specific to mouse *Nqo1*ARE or *Ho-1* ARE. PCR products were run on agarose gel of 1.2% to visualize the PCR products. (A) ChIP assay on *Ho-1* ARE. (B) Quantification of *Ho-1* ARE DNA bound to Nrf2 normalized by 1% input DNA. (C) ChIP assay on *Nqo1* ARE. (D) Quantification of *Nqo1* ARE DNA bound to Nrf2 normalized by 1% input DNA. Quantifications were performed with real-time PCR. Data represent means and standard deviations from three separate experiments.

followed by immunoblotting of polyubiquitins (Figure 6C). Ubiquitination of Nrf2 was detected in the cytoplasmic fraction in the absence or presence of Cd or tBHQ (left panels, lanes 1−3); the ubiquitination was largely increased by cotreatment with MG132 (lanes 4−6). On the contrary, the levels of the ubiquitinated Nrf2 protein were dramatically reduced in the nucleus even in the presence of MG132, which is in sharp contrast to the markedly increased levels of the Nrf2 protein of the same samples (compare parts A and C, lanes 4−6 in both left and right panels); the lack or reduced levels of ubiquitinated Nrf2 in the nucleus as compared with those in the cytoplasm suggest deubiquitination of the Nrf2 protein upon nuclear translocation of the activated Nrf2 protein. Ubiquitination of Keap1 in the cytoplasm and reduced ubiquitination of the protein in the nucleus in parallel to those observed in Nrf2 were also observed previously and in the present study (*12, 19*)(data not shown).

Discussion

Nrf2-mediated gene transcription plays a pivotal role in both the basal and the inducible expressions of a range of cytoprotective genes through the AREs of the genes (*6, 23, 24*). Moreover, both genetic and biochemical evidence reveal that the Nrf2/ARE system serves as a genetic program of cellular defense against oxidative, electrophilic, and a range of other toxic signals as well as certain disease processes. In this regard, the cytoplasmic Nrf2-binding protein Keap1 serves as an initial contact with chemical signals via chemical−protein thiol group interactions (*25*), leading to the activation of Nrf2, followed by transcription of ARE-dependent xenobiotic-metabolizing enzymes and antioxidant proteins/enzymes. Induction of enzymes/proteins enhances metabolic elimination of the chemicals, reduces production of ROS, and improves repair of tissue damage. Induction is reduced as the chemical stimuli are removed during the process. Thus, this coupled chemical sensing and gene induction mechanism forms a transcriptional loop that allows cells to adapt to the ever changing and often toxic chemical environment (*12*).

Toxic metals represent a major class of environmental and occupational sources of chemical toxicity. Moreover, many metal elements are human carcinogens (*26*). The molecular targets for most of the biological effects of metals remain largely unclear. Cd has been shown to be a potent inducer of metallothioneins I and II; induction of MTs is mediated through metal-activated transcription factor 1 (MTF1) and requires metal response element (MRE) (*27, 28*). MT I and II protect against Cd toxicity by chelating free Cd and by serving as a scavenger of ROS and reactive nitrogen species (RNS) induced by Cd and other metals (*29, 30*). In this study, we analyzed the role of Nrf2 in protection against Cd toxicity by exploiting the availability of Nrf2 knockout cells. The findings revealed that loss of Nrf2 remarkably increased the sensitivity of cells to Cd-induced cell killing and oxidative stress, thus establishing the activation of Nrf2/ARE-dependent transcription as a key adaptive response in cellular defense against Cd-induced oxidative damage and cell death. In this respect, the activation of the Nrf2•Keap1•ARE pathway and the MTF1•MRE pathway by Cd launches dual protective responses that complement each other through controlling the induction of different sets of target genes in the defense against Cd and Cd-induced oxidative damage. Whether the Nrf2 and MTF-1 pathways directly cross-interact with each other in the responses to Cd remains to be examined.

Stewart et al. have previously reported that Cd at 50 μM stabilizes the Nrf2 protein (a 88 kDa protein) in mouse Hepa cells that correlated with induction of *Ho-1* mRNA, induced Nrf2•StRE mobility shift, and induction of a mouse *Ho-1* enhancer-controlled reporter gene expression (*31*). In addition to demonstrating a critical role of Nrf2 in protection against Cd-induced ROS production genetically, our current study

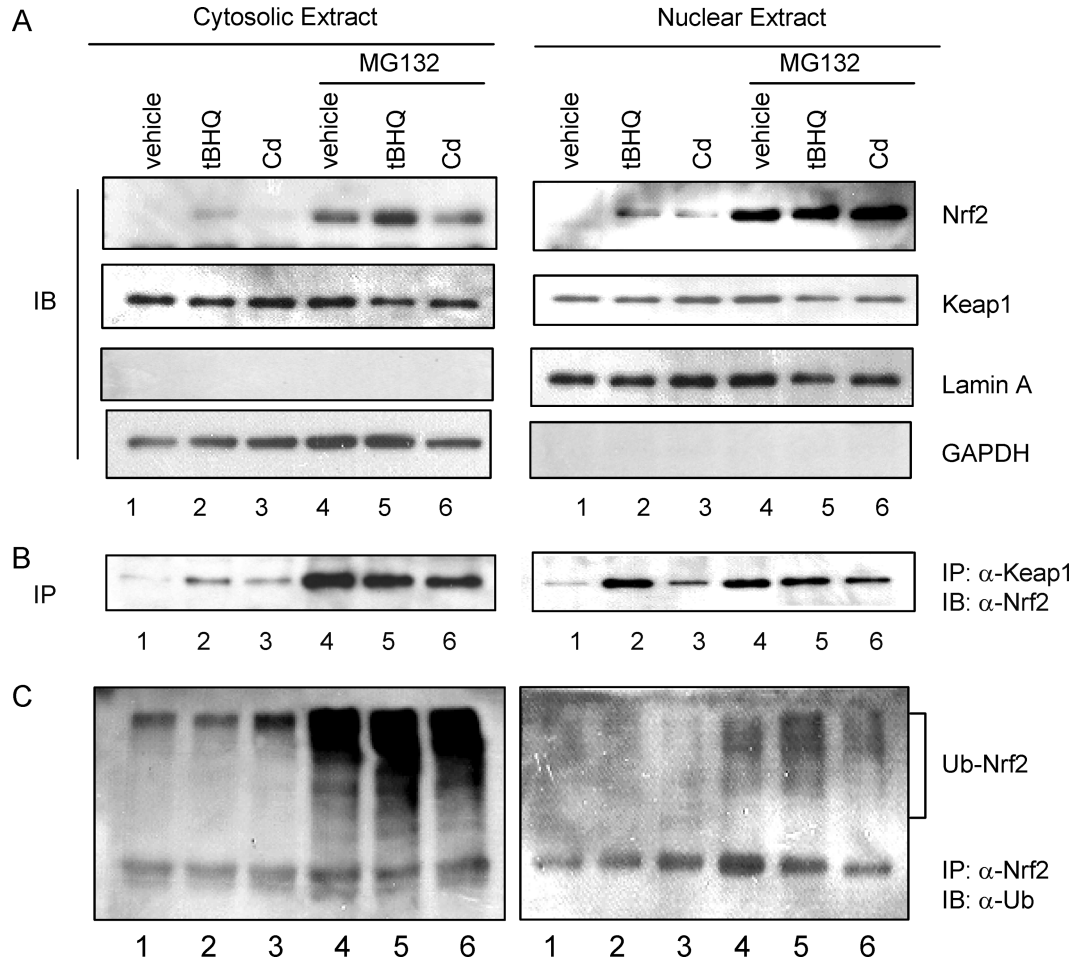

Figure 6. Dissociation of Nrf2 with Keap1 and inhibition of ubiquitination of Nrf2 in the nucleus by Cd. Hepa1c1c7 cells were treated with tBHQ (30 μM), Cd (10 μM), MG132 (15 μM), MG132 plus tBHQ, or MG132 plus Cd for 5 h. Cytoplasmic and nuclear fractions were prepared. Lamin A (nuclear marker) or GAPDH (cytoplasmic marker) was used to assess the purity of the cytoplasmic and nuclear fractions as well as loading variations. (A) IB of Nrf2 and Keap1. (B) Coimmunoprecipitation of Keap1 and Nrf2. The cytoplasmic and nuclear fractions were immunoprecipitated with anti-Keap1 antibody and immunoblotted with anti-Nrf2 antibody. (C) Inhibition of ubiquitination by Cd. The cytoplasmic and nuclear fractions were immunoprecipitated with anti-Nrf2 and immunoblotted with antiubiquitin antibody.

revealed a number of novel aspects in the activation of Nrf2 by Cd as compared with the report by Stewart et al. Our data showed that the Nrf2 protein (110 kDa) level was dramatically increased by Cd at concentrations between 5 and 50 μM and the protein was absent in Nrf2-null cells; the apparent molecular mass of 110 kDa of Nrf2 is consistent with previous reports from our group and other laboratories that showed the stabilization of the Nrf2 protein of 110 kDa by tBHQ, arsenic (As), chromium (Cr), and other inducers in cells and the expression of the 110 kDa Nrf2 protein from Nrf2 cDNA by in vitro transcription and translation and by transient transfection into cells (*12, 19, 32*). Activated Nrf2 was shown to be enriched in the nucleus and to mediate the induction of *Nqo1* and *Ho-1* by binding to the endogenous AREs of the genes—as shown by the ChIP assays. Importantly, Cd was shown to activate Nrf2 via a mechanism that apparently deviates from that by the phenonic antioxidant, tBHQ, in that tBHQ increases the formation of the immunoprecipitable Nrf2•Keap1 complex, whereas Cd disrupts or reduces such a complex in the nucleus as compared with tBHQ. Moreover, Nrf2 was found to be ubiquitinated in the cytoplasm in the presence of the inducers, but the ubiquitination was largely reduced in the nucleus (see below for more discussions).

Previous studies identified Keap1 as the cytoplasmic binding protein of Nrf2 and as a key component of a Cul3-based E3 enzyme catalyzing the ubiquitination of Nrf2, which leads to the proteasomal degradation of Nrf2 (*11–14*). These findings established a mechanistic framework for the regulation of Nrf2 under basal conditions, in which Nrf2 is rapidly turned over via the 26S proteasomal degradation pathway with a $t_{1/2}$ value of only ~20 min. However, the key events leading to the activation of Nrf2 by many inducing signals of diverse properties remain controversial. In the above-mentioned model, Keap1 was predicted to serve as a negative regulator of Nrf2 by sequestering Nrf2 in the cytoplasm and by catalyzing the ubiquitination of Nrf2 for the proteasomal degradation of Nrf2; binding of inducers with Keap1 triggers the dissociation of Nrf2 from Keap1, and thereby, Nrf2 escapes the ubiquitination and proteasomal degradation in the cytoplasm resulting in increased stability and nuclear translocation of Nrf2. However, it was found that treatment with antioxidants does not decrease but increases the amount of the immunoprecipitable Nrf2•Keap1 complex in both the cytoplasm and the nucleus (Figure 6B, lanes 2 and 5) (*12, 15*). This observation is contradictory to the prediction from the model. In the present study, several new findings provided insights into the interactions between Nrf2 and Keap1 during Nrf2 activation.

First, treatment with Cd increased the amount of the cytoplasmic Nrf2•Keap1 complex similarly to tBHQ (Figure 6B, left panel, lanes 2 and 3); however, in contrast to tBHQ, the level of the complex in the nucleus was significantly reduced despite the fact that both Nrf2 and Keap1 were present in this

cellular compartment at similar levels (Figure 6B, right panel, compare lane 3 with 2). Reduced binding was even observed in the nucleus in the presence of MG132 in which high levels of Nrf2 were detectable, suggesting that Cd prevented the formation of new complexes and disrupted the existing complexes. Previous studies revealed that As and Cr also disrupt the Nrf2•Keap1 association in the nucleus; the potencies of the three metals in dissociating the nuclear Nrf2 from Keap1 in the absence or presence of MG132 are in the order of As > Cr > Cd, which correlates with the potencies of the metals for the induction of Nrf2 target genes (*12, 19*). Second, the observation that the Nrf2 and Keap1 complex was detected in a large amount in the nucleus of tBHQ-treated cells suggests that the two proteins translocate into the nucleus in a complex. Third, Nrf2 (and Keap1) was found to be ubiquitinated in the cytoplasm in the presence of either Cd or tBHQ; however, ubiquitinated Nrf2 (and Keap1) was largely reduced in the nucleus even though the proteins were present in the nucleus in large amounts, especially in the presence of MG132, suggesting that a deubiquitination process exists and is responsible for the deubiquitination of Nrf2 (and Keap1) in the nucleus. Deubiquitination for the regulation of transcription factors in the nucleus such as p53 and NF-κB has been reported previously (*33, 34*). Lastly, the detection of the Keap1 protein in both the cytoplasm and the nucleus at similar levels in the absence and presence of the inducers suggests that Keap1 undergoes a cytoplasmic/nuclear cycling to assist Nrf2 activation and nuclear translocation. We have previously observed similar findings in cells treated with As or Cr (*12, 19*). Taken together, our findings provided evidence for a model of Nrf2 activation by toxic metals through a signaling pathway that is distinctively different from that of phenolic antioxidants. In this regard, studies are underway to dissect the molecular interactions of toxic metals with the Keap1 and Nrf2 proteins. Such analysis may aid in the elucidation of different courses of Nrf2 activation by toxic metals and phenolic antioxidants in future. Because antioxidants and toxic metals cause very different biological responses in cells and in whole animals, it can be predicted that the different pathways of Nrf2 activation by tBHQ and toxic metals are functionally related to biological effects of the inducers to certain extents. If so, the differential effects of the antioxidants and toxic metals on the Nrf2 pathways may be exploited for developing effective and safe preventive/therapeutic drugs against oxidative damage.

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