Nrf3 Negatively Regulates Antioxidant-response Element-mediated Expression and Antioxidant Induction of NAD(P)H:Quinone Oxidoreductase1 Gene*

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Antioxidant-response element (ARE) and nuclear factor Nrf2-mediated expression and coordinated induction of genes encoding chemopreventive proteins, including NQO1, are critical mechanisms in chemoprotection. Recently, Nrf3, a new member of the Nrf family with substantial homology to Nrf2, was identified and cloned. In this report, we have investigated the role of Nrf3 in ARE-mediated gene expression and induction of NQO1 in response to antioxidants. Overexpression of Nrf3 in Hep-G2 cells led to a concentration-dependent decrease in transfected and endogenous NQO1 gene expression and induction in response to antioxidant tert-butylhydroquinone (t-BHQ). Deletion mutation analysis revealed that Nrf3 repression of NQO1 gene expression required heterodimerization and DNA binding domains but not transcriptional activation domain of Nrf3. Bandshift and supershift assays with in vitro transcribed and translated proteins and nuclear extracts from Hep-G2 cells treated with Me2SO and t-BHQ and immunoprecipitation assays demonstrated that Nrf3 associates with small Maf proteins to bind to the ARE. RNA interference specific to Nrf3 reduced intracellular Nrf3 leading to decreased expression and induction of transfected and endogenous NQO1 gene expression in response to t-BHQ. These results combined suggest that Nrf3 is a negative regulator of ARE-mediated gene expression.

Exposure of cells to xenobiotics, drugs, and ionizing radiation is known to generate reactive oxygen species (ROS) and electrophiles that lead to oxidative and electrophilic stress and have a profound impact on the survival of all living organisms (1, 2). The cells respond to oxidative/electrophilic stress. The initial effects are the activation of defense mechanisms that lead to coordinated activation of a battery of defensive genes that protect cells against oxidative/electrophilic stress (3–6). It is because of these mechanisms that most of the cells neutralize the effects of the oxidative/electrophilic stress and survive. However, accumulation of ROS and electrophiles under adverse conditions are known to cause membrane and DNA damage, mutagenicity, degeneration of tissues, premature aging, apoptotic cell death, cellular transformation, and cancer (7–10). Therefore, the protection mechanisms are vital for cell growth, differentiation, survival, and chemoprevention. It is interesting to note that antioxidants also undergo metabolic activation to generate the initial effect of oxidative/electrophilic stress leading to coordinated activation of a similar battery of genes as induced in response to xenobiotics, drugs, and ionizing radiations (3, 4). Therefore, antioxidants have been commonly used in experiments to determine the mechanism of coordinated activation of defensive genes and protection against oxidative and electrophilic stress (3, 4).

The battery of defensive genes activated in response to oxidative/electrophilic stress includes NAD(P)H:quinone oxidoreductase1 (NQO1), NRH-quinone oxidoreductase2 (NQO2), glutathione S-transferase Ya subunit (GST Ya subunit), heme oxygenase-1, and γ-glutamylcysteine synthetase (γ-GCS) better known as glutamate-cysteine ligase. NQO1 is a flavoprotein that catalyzes metabolic reductive detoxification of redox cycling quinones (11). GST Ya catalyzes conjugation of hydrophobic electrophiles and ROS with glutathione leading to their excretion (12, 13). Heme oxygenase-1 catalyzes the first and rate-limiting step in heme catabolism (14). γ-GCS plays a key role in the regulation of glutathione metabolism (15). It may be noteworthy that disruption of the NQO1 gene in mice leads to oxidative stress, myelogenous hyperplasia, and increased sensitivity to chemical carcinogenesis (16–19).

Antioxidant-response element (ARE) and nuclear NF-E2 related factors (Nrf1 and Nrf2) are known to mediate the basal expression and induction of a battery of defensive genes in response to antioxidants (3–6). Among these, Nrf2 is more potent than Nrf1 in inducing the gene expression. The NF-E2-related factors belong to the family of basic leucine zipper proteins. The basic region, just upstream of the leucine zipper region, is responsible for DNA binding. The acidic region is required for transcriptional activation. The mechanism of signal transduction from antioxidants to Nrf2 is the current subject of extensive research. The studies have shown that Nrf2 is retained in the cytosol by a cytosolic inhibitor INrf2/Keap1 (20, 21). The signals from antioxidants lead to phosphorylation of Nrf2 and/or redox modulation of INrf2 (3–6). This results in separation of Nrf2 from INrf2. The Nrf2 accumulates in the nucleus leading to its binding to ARE and activation of ARE-mediated gene expression. A third family member of the NF-E2 related factors, Nrf3 with substantial homology to Nrf2, was cloned and sequenced (22). However, the role of Nrf3 in ARE-mediated gene expression and induction in response to antioxidants remains unknown. More recently, Nrf3-null mice deficient in Nrf3 protein were generated (23). Nrf3-null mice developed normally and revealed no obvious phenotypic differences compared with wild type animals. Nrf3-null mice were...
fertile, and gross anatomy as well as behavior appeared normal. This led to the hypothesis that the role of Nrf3 in vivo may become apparent only after appropriate challenge to the mice.

In this report, we investigate the role of Nrf3 in ARE-mediated expression and induction of the gene encoding chemopreventive protein NQO1. The overexpression of Nrf3 in cells led to repression of transfected and endogenous NQO1 gene expression. This repression required DNA binding but not transcriptional activation domain of Nrf3. Nrf3 associated with small Maf proteins binds to the NQO1 gene ARE. The RNAi-mediated inhibition of the intracellular level of Nrf3 resulted in an increase in basal expression of ARE-mediated NQO1 gene and induction in response to antioxidants. The data also demonstrate that overexpression of Nrf2 relieves Nrf3 repression of ARE-mediated NQO1 gene expression. These results suggest that Nrf3 is a negative regulator of ARE-mediated gene expression.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—The construction of pGL2-NQO1-ARE-Luc was described previously (24). We used forward 5′-CCATGGGATCCCTCGTGGAGATGAACTCCGAGAAG→ and reverse 5′-AAGCTTGG-TACCTGGAGACTTTGATGCTCTGCAC-3′ primers in a PCR to amplify full-length Nrf3 cDNA from mouse Nrf3 cDNA clone purchased from the ATCC (Manassas, VA). The PCR-amplified product was subcloned at a T/A cloning site in vector pcDNA (Invitrogen) to generate expression plasmid pcDNA-Nrf3. After transfection in Hep-G2 cells, this plasmid produced 90 kDa (825 amino acids) of protein. The forward primer as described above and the V5 reverse primer 5′-AAGCTTGG-TACCCTTTCTCCTCTCCTCTTTCCCTTTCCCTTTG-3′ were used in a related PCR to amplify Nrf3 that was subcloned in pcDNA to produce expression plasmid pcDNA-Nrf3-V5. The V5 reverse primer did not contain the Nrf3 translation termination codon. This allowed the translation to continue to add V5 tag to the Nrf3 from the vector. The V5 epitope contains 14 amino acids in the sequence Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr. V5 tagging of Nrf3 protein allowed us to detect Nrf3 in Western blots and band/supershift assays. Good Nrf3 antibodies are unavailable at this time, whereas very good V5 antibodies are available from Invitrogen and Santa Cruz Biotechnology, Santa Cruz, CA. The expression plasmid pcDNA-Nrf3-V5 was sequenced to confirm the continuous reading frame of Nrf3 with V5. The pcDNA-Nrf3-V5 upon transfection in Hep-G2 cells produced V5-tagged Nrf3 that was easily detected by Western analysis and probing with V5 antibody. The wild type pcDNA-Nrf3 was used as a template to generate Nrf3 mutants expressing either a 266-amino acid C-terminal region containing Cap‘N/Collar and basic and leucine zipper domains or a 561-amino acid N-terminal region containing hydrophobic and transcriptional activation domains. The forward primer 5′-GACCCTGGTCGCATGCTGGTTGACTGCATC-3′ and reverse primer 5′-GACTCGAGAGTGAAATTTCATCTACAGAAAAGGG-3′ were used in a PCR to amplify full-length Nrf3 cDNA and the reverse primer 5′-GACTCAGATGAACTTTATTTCTACAGAAGAAAGGG-3′ were used in a PCR to amplify the region between nucleotides +1699 and +2128 that was subcloned in pcDNA to generate mutant plasmid pcDNA-Nrf3Δ1-V5. This plasmid was confirmed by sequencing, and upon transfection in Hep-G2, cells produced a C-terminal 266-amino acid Nrf3 protein tagged with V5. Similarly, the forward primer as described above to amplify full-length Nrf3 cDNA and the reverse primer 5′-GACCCTGGTCGCATGCTGGTTGACTGCATC-3′ and V5 reverse primer 5′-GACTCGAGAGTGAAATTTCATCTACAGAAGAAAGGG-3′ were used in a PCR to amplify the region between nucleotides −4 and +1699 of Nrf3 cDNA to generate an N-terminal mutant lacking the C-terminal domain. The amplified product was subcloned in pcDNA in-frame with V5 to produce the expression plasmid pcDNA-Nrf3Δ2.

**FIG. 1. Effect of overexpression of Nrf3 on ARE-mediated NQO1 gene expression and induction in response to antioxidant.** A, the nucleotide sequences of NQO1 ARE and mutant AREs are shown. The mutated sequences are underlined in NQO1 mutant AREs. B–F, Hep-G2 cells were transfected with reporter plasmids NQO1 ARE-Luc or NQO1 mutant ARE-Luc and expression plasmid pcDNA-Nrf3 and pcDNA-Nrf3-V5. The cells were treated with Me2SO (DMSO) or 50 μM t-BHQ 36 h after transfection for 12 h. The cells were harvested and analyzed for luciferase activity. G, Hep-G2 cells were transfected with concentrations of pcDNA-Nrf3-V5 as shown. The cells were harvested 48 h after transfection and analyzed for the expression of Nrf3-V5 by SDS-PAGE, Western blotting, and probing with V5 and actin antibodies.
This plasmid was also confirmed by sequencing, and upon transfection in Hep-G2 cells, produced a 561-amino acid N-terminal region of Nrf3 protein containing the transcriptional activation domain. The forward 5’-AGCCATGCGCTGCTAGGAGCCCTCTGAGGAGC-3’ and reverse 5’-GCCCTGACCTAGAGCCAA-3’ primers and PCR were used to amplify Nrf3 cDNA from plasmid LNCX-MafG (24). The amplified product was subcloned in pcDNA to produce pcDNA-Nrf3-V5.

Cell Culture, Co-transfection of Expression Plasmids, and Reporter Assays—Human hepatoblastoma (Hep-G2) cells were obtained from the American Type Culture Collection and maintained in α-minimum Eagle’s medium (Invitrogen) with 10% fetal bovine serum. Transient transfections were performed in cells grown to ~50% confluence in 6-well plates, using Effectene Transfection Reagent (Qiagen, Valencia, CA) as described in the manufacturer’s protocol. 0.2 µg of reporter constructs of NQO1 ARE-Luc, GST Ya ARE-Luc, or γGCS ARE-Luc was co-transfected with expression plasmid pcDNA bearing wild type Nrf3 or Nrf3-V5, Nrf3Δ11-V5, and Nrf3Δ2-V5. The total amount of transfected DNA was kept constant at 2 µg/well by the addition of pcDNA3 control vector to the DNA mixture. The cells were incubated for 24 h after transfection and induced with 50 µM t-BHQ in carrier Me2SO, and cells were lysed 12 h later. One hundred micrograms of the lysates from transfected cells were separated on 10% SDS-PAGE and Western-blotted. Western blots were probed with V5 and β-actin antibody to visualize the pcDNA-Nrf3-V5 proteins were incubated with MafG translated in vitro and reanimated for NQO1 ARE-mediated luciferase activity. The cytosolic fractions were analyzed for NQO1 protein (Western) and activity by procedures as described previously (25). Nrf2 relieves the Nrf3 repression of NQO1 ARE-mediated luciferase activity by using TNT T7-coupled reticulocyte lysate systems (Promega, Madison, WI) following the manufacturer’s protocol. Briefly, the plasmids were individually added to the rabbit reticulocyte lysate containing T7 polymerase and [35S]methionine in a reaction volume of 50 µl at 30 °C for 90 min. The translation products were verified by SDS-PAGE and autoradiography. Bandshift and supershift assays with proteins translated in vitro were performed by procedures as reported previously (24) and are described below for the Hep-G2 nuclear extract. In vitro translated wild type and mutant Nrf3 proteins were incubated with MafG translated in vitro for 30 min at 30 °C for heterodimerization, and bandshift reactions were carried out with [35S]labeled ARE. Hep-G2 cells were plated in 150-mm dishes and transfected with 2 µg of pcDNA or pcDNA-Nrf3-V5 per dish and treated with Me2SO and 50 µM t-BHQ for 12 h by procedures described previously (24). Nuclear extracts from Me2SO- and t-BHQ-treated Hep-G2 cells were prepared as described previously (24). Fifty micrograms of nuclear extracts from Hep-G2 cells transfected with pcDNA and pcDNA-Nrf3-V5 were resolved by SDS-PAGE and probed with anti-V5 antibody to visualize the Nrf3-V5 protein expression. Human NQO1 ARE synthetic probes, as described previously (24), were annealed and end-labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP. Five micrograms of nuclear extract was incubated at room temperature in a reaction solution containing 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 5 µg of poly(dI-dC)poly(dI-dC). After 15 min, the [35S]-labeled duplex oligonucleotide (50,000 cpm/reaction) was added to the 20-µl reaction mixture and incubated for another 30 min at room temperature. For competition experiments, a 100–200-fold molar excess of unlabeled probe was included in the preincubation mixture at 25 °C before the addition of the labeled probe. DNA-protein complexes were separated on 6% nondenaturing polyacrylamide gels in Tris borate/EDTA buffer, pH 8.0, at 4 °C at 120 V for 4 h. The gel was dried, and the complexes were revealed by autoradiography. For supershift assay, the total reaction mixture was preincubated with 1 µl of anti-V-5 antibody (0.2 mg/ml) from Invitrogen on ice for 30 min before bandshift analysis. Preimmune serum was incubated with the nuclear extracts for nonspecific interaction with IgGs.

Immunoprecipitation—Hep-G2 cells were grown in 150-mm dishes. The cells were transfected with pcDNA-Nrf3-V5 and scraped into 10 ml
of phosphate-buffered saline and pelleted by centrifugation. Nuclei were prepared as described previously (24). The pelleted nuclei were lysed using RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na3VO4, and 1 μM okadaic acid). Five micrograms of nuclear extract was cleared by centrifugation at 12,000 rpm for 10 min. Nrf3-V5 was immunoprecipitated from the nuclear extract with 2 μl of V5 antibody from Santa Cruz Biotechnology by procedures as described (25). In related experiments, the nuclear lysate was also immunoprecipitated with MafG antibody from Santa Cruz Biotechnology. Preimmune serum was used as control for the immunoprecipitation. Equal amounts of precipitate were run on SDS-PAGE, and Western blots were performed with antibodies specific to MafG or V5.

**Nrf3 RNA Interference (RNAi) Assay—**

Mouse hepatoma (Hepa-1) cells were grown as monolayer cultures in 6-well plates at 37 °C in 95% air and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (40 units/ml), and streptomycin (40 μg/ml). RNAi-mediated mouse Nrf3 gene silencing was employed using pre-designed siRNA with sense 5'ggAgAACgggAUgCUAAgATT-3' and antisense 5'UCUUAgCAUCCCgUUCUCCTg-3' oligonucleotides from Ambion-RNA (Austin, TX). The Hepa-1 cells in 6-well plates were transfected with different amounts of pre-designed mouse Nrf3 siRNA without and with Nrf3-V5 and NQO1 ARE-Luc by procedures as described previously (24). The cells were harvested 48 h after transfection. Whole cell extract was prepared in lysis buffer containing 50 mM Tris, pH 7.5, 250 mM NaCl, 0.5% Triton X-100, 5 mM NaF, 50 mM NaF, 2.5 mM phenylmethylsulfonyl fluoride. Two μg of protein in supernatant from whole cell lysate was concentrated using 30YM Centricon tubes for 5 h at 2000 rpm at 4 °C. The siRNA-mediated Nrf3 knockdown efficiency was analyzed by SDS-PAGE with 50 μg of whole cell extract and Western blotting with V-5 antibodies from Invitrogen. The cell lysates were also analyzed for luciferase activity by described procedures (24). In related experiments, Hepa-1 cells were transfected with RNAi alone, and 100 μg of cytosolic protein was analyzed for NQO1 protein (Western blot) and activity by methods described previously (25).

**RESULTS**

The nucleotide sequence of the human NQO1 gene ARE and mutant AREs are shown (Fig. 1A). Overexpression of Nrf3 led to concentration-dependent repression of NQO1 ARE-mediated luciferase gene expression and induction in response to antioxidant t-BHQ in transfected cells (Fig. 1B). The transfection of Hep-G2 cells with 1.0 μg of pcDNA-Nrf3 resulted in a higher than 2-fold repression of luciferase gene expression (p < 0.001) and similar repression of induction in response to t-BHQ (p < 0.001). The mutations in the AP1 or GC box or AP1-like element of ARE significantly reduced the expression and t-BHQ induction and Nrf3 repression of ARE-mediated NQO1 gene expression and induction (Fig. 1, C–E). The replacement of pcDNA-Nrf3 with pcDNA-Nrf3-V5 showed similar results as pcDNA-Nrf3 (Fig. 1F). The Nrf3-V5 showed similar pattern and magnitude of repression of ARE-mediated luciferase expression, as observed with Nrf3. Western blot analysis also revealed that the transfection of 0.1–2.0 μg of pcDNA-Nrf3-V5 in Hep-G2 resulted in a plasmid concentration-dependent increase in Nrf3-V5 protein (Fig. 1G). These results indicated that tagging of Nrf3 with V5 had no effect on the stability or function of Nrf3. Similar results were also observed with GST Ya ARE and γ-GCS ARE (data not shown). However, the mag-
nitude of Nrf3 repression of GST Ya and γ-GCS ARE was lower than the NQO1 gene ARE. The overexpression of Nrf3 and Nrf3-V5 in Hep-G2 cells also repressed endogenous NQO1 protein and activity (Fig. 2). This repression was also Nrf3 and Nrf3-V5 concentration-dependent.

The transfection of pcDNA-Nrf3-V5, pcDNA-Nrf3Δ1-V5, and pcDNA-Nrf3Δ2-V5 in Hep-G2 cells resulted in a plasmid concentration-dependent increase in expression of Nrf3-V5, Nrf3Δ1-V5, and Nrf3Δ2-V5, respectively (Fig. 3). The overexpression of Nrf3-V5 in Hep-G2 cells resulted in an Nrf3-V5 concentration-dependent decrease in NQO1 ARE-mediated luciferase gene expression (Fig. 3). This was same as observed earlier (Fig. 1). The deletion of the N-terminal region containing transcriptional activation domain from Nrf3-V5 did not affect the capacity of Nrf3 to repress ARE-mediated luciferase gene expression (Fig. 3). However, deletion of the C-terminal region containing heterodimerization and DNA binding domains of Nrf3-V5 resulted in the loss of Nrf3 repression of ARE-mediated gene expression (Fig. 3).

The wild type Nrf3-V5, Nrf3Δ1-V5, Nrf3Δ2-V5, MaF, and Nrf2 were successfully transcribed and translated in vitro from their respective pcDNA expression plasmids (Fig. 4A). Bandshift analysis with proteins translated in vitro showed that Nrf3-V5 alone failed to bind to ARE (Fig. 4B). Nrf3-V5 required MaF for binding to the ARE. The Nrf3-V5-MaF binding increased with increasing Nrf3 concentration in bandshift assays (Fig. 4B). Nrf3-V5-MaF binding was competed with cold ARE but not with mutant ARE (Fig. 4B). The origin of the unspecific band shown in Fig. 4B with ** remains unknown. This band is presumably due to unspecific interaction of rabbit reticulocyte lysate protein to the ARE and has also been observed previously (24). Anti-V5 and not preimmune serum supershifted the Nrf3-V5-MaF band in supershift assays (Fig. 4B). The replacement of wild Nrf3-V5 with Nrf3Δ1-V5 lacking the transcriptional activation domain of Nrf3 showed similar results as observed with wild type Nrf3-V5 (Fig. 4C). However, the band shifted with Nrf3Δ1-V5-MaF moved faster than Nrf3-V5-MaF presumably because of truncated Nrf3-V5 (compare Fig. 4, B with C). Nrf3Δ1-V5-MaF band was competed with cold ARE but not with mutant ARE. The replacement of wild type Nrf3-V5 with Nrf3Δ2-V5 lacking the heterodimerization and DNA binding domains of Nrf3 failed to bind to the NQO1 gene ARE by itself or in association with MaF (Fig. 4D). In similar experiments Nrf2 alone or in combination with Nrf3 also failed to bind to ARE (Fig. 4E).

The transfection of Hep-G2 cells with pcDNA-Nrf3-V5 resulted in overexpression of cDNA-derived Nrf3-V5 in transfected cells (Fig. 5A). The results of the bandshift and supershift assays with the NQO1 gene ARE and nuclear extracts from Hep-G2 cells transfected with pcDNA and pcDNA-Nrf3-V5 and treated with Me2SO and t-BHQ are shown in Fig. 5B. Two distinct complexes of nuclear proteins bound to NQO1 gene ARE from Hep-G2 cells treated with Me2SO and t-BHQ in all cases. However, t-BHQ treatment appeared to increase...
binding of both the complexes to the ARE. An anti-V5 antibody showed a supershifted band in the case of t-BHQ-treated nuclear extracts. The supershifted band in the case of Me2SO was hardly visible presumably because of a lower amount of shifted band. The nuclear extracts from untreated Hep-G2 cells transfected with Nrf3-V5 were immunoprecipitated with either MafG or V5 antibodies and analyzed on SDS-PAGE and Western blotting (Fig. 6, A and B). The results demonstrate that Nrf3-V5 was immunoprecipitated along with MafG with MafG antibody (Fig. 6A). Similarly, MafG was immunoprecipitated along with Nrf3-V5 with V5 antibody (Fig. 6B).

Western analysis revealed that co-transfection of Nrf3 RNAi and pcDNA-Nrf3-V5 in Hepa-1 cells resulted in inhibition of intracellular concentration of Nrf3-V5 (Fig. 7A). This inhibition was Nrf3 RNAi concentration-dependent. The decrease in RNAi-induced Nrf3 level in Hep-G2 cells led to the derepression of NQO1 ARE-mediated luciferase gene expression (Fig. 7B). The derepression was also Nrf3 RNAi concentration-dependent. Most interestingly, the derepression of ARE-mediated gene expression and induction in response to t-BHQ in the case of cells transfected with 100 and 150 nM RNAi was significantly higher than the expression and induction in cells transfected with NQO1 ARE-Luc alone (p > 0.01 with 100 nM RNAi and p > 0.001 with 150 nM RNAi). The transfection of Hepa-1 cells with Nrf3-specific RNAi also activated endogenous NQO1 gene expression leading to an increase in NQO1 protein (Fig. 8A) and NQO1 activity (Fig. 8B). The increase in NQO1 was Nrf3 RNAi concentration-dependent.
The overexpression of Nrf2 with Nrf3-V5 relieved the Nrf3 repression of ARE-mediated gene expression (Fig. 9). Most interestingly, the increase in NQO1 ARE-mediated gene expression with 0.2 μg of Nrf2 was significantly higher than the base line of expression (p < 0.001; Fig. 9, compare 5th lane with 1st lane). In a related experiment, Nrf2 alone increased ARE-mediated gene expression to a similar extent as did Nrf2+Nrf3-V5. In other words, Nrf3-V5 was ineffective in the repression of ARE-mediated gene expression in cells overexpressing Nrf2.

**DISCUSSION**

Nrf1 and Nrf2-mediated gene expression and induction in response to antioxidants and xenobiotics are mechanisms of critical importance in chemoprevention (3–6). Nrf2 is more effective than Nrf1 in ARE-mediated gene expression and induction (5). The mechanism of Nrf2 activation of ARE-mediated gene expression is a matter of extensive research. Both Nrf2 and Nrf1 do not bind to the ARE as homodimers or heterodimers with each other and require other leucine zipper proteins to form heterodimers that bind to the ARE (26–28). Jun and small Maf proteins have been reported to heterodimerize with Nrf2 and bind to the ARE (24, 29–32). c-Jun heterodimers with Nrf2 activated gene expression (29). In contrast, the role of Nrf2-small Maf heterodimers in ARE-mediated gene expression remains controversial (24, 29–32). The role of Nrf2-small...
Maf has been shown in activation and repression of gene expression.

Nrf3 is the third member of the Nrf gene family (22). Nrf3 has substantial homology to Nrf2 and Nrf1. The protein domains including transcriptional activation, Cap’N’Collar, DNA binding, and leucine zipper of Nrf2 and Nrf1 are conserved in Nrf3. Therefore, Nrf3 was expected to bind to ARE and alter ARE-mediated gene expression. The present studies demonstrate that Nrf3, like Nrf1 and Nrf2, did not bind to the ARE by itself. Nrf3 required small Maf proteins to bind to the ARE. This is also in agreement with a previous report showing the binding of Nrf3-small Maf to MARE (22). The overexpression of Nrf3 in transfected cells repressed NQO1 gene expression and induction in response to antioxidants. This was observed with transfected ARE-luciferase gene construct and measurement of endogenous NQO1 gene. The Nrf3-mediated repression of the NQO1 gene was dependent on the intact ARE sequence and required heterodimerization/DNA binding but not transcriptional activation domain of Nrf3.

Several observations revealed that Nrf3 repression contributes to the maintenance of basal expression of endogenous and transfected NQO1 gene. The cells transfected with Nrf3 RNAi showed lower levels of Nrf3 and higher basal expression of the NQO1 gene. Similar results were also observed with Nrf3 RNAi and NQO1 ARE-luciferase gene expression. In addition, the cells expressing RNAi-mediated reduced levels of Nrf3 showed significantly higher induction in response to antioxidants of NQO1 gene expression, as compared with wild type cells expressing normal level of Nrf3.

The studies also raise interesting questions regarding the mechanism of Nrf3 repression of ARE-mediated NQO1 gene expression. The two observations in this report suggest that Nrf3 might interfere with the binding of the potent activator Nrf2 to the ARE leading to repression of gene expression and induction. Nrf3 heterodimerization/DNA binding domain is equally effective as full-length Nrf3 in binding to ARE and repression of ARE-mediated gene expression. Overexpression of Nrf2 relieves Nrf3 repression of ARE-mediated gene expression. Alternatively, it is possible that Nrf3 inhibits transcription by dimerization with activators of ARE. Further studies are required to understand the mechanism of Nrf3 repression of ARE-mediated gene expression.

Recently, the transcription factors c-Jun-c-Fos, MafG-MafG, MafK-MafK, c-Maf-MafG, and Nrf2-MafG and more recently Bach1-small Maf have been shown to bind to ARE and negatively regulate ARE-mediated gene expression (3, 4). This is presumably to rapidly bring down the induced enzymes to their normal level and maintain it at a normal level. The negative regulation is because it is always necessary to have a small amount of ROS and other free radicals present to keep the cellular defenses active. Because activation of defensive/chemopreventive proteins leads to significant reduction in the levels of superoxide and other free radicals, the cell may require negative regulatory factors like the small Mafs and c-Fos to keep the expression of defensive proteins “in check.” Nrf3-MafG in the present report is a new addition to the growing list of factors that contribute to negative regulation of ARE-mediated gene expression.

In summary, the results in this report demonstrate that Nrf3 in association with small Maf proteins binds to the ARE and represses ARE-mediated gene expression. This repression requires heterodimerization/DNA binding domains of Nrf3 but not transcriptional activation domain of Nrf3. The negative regulation by Nrf3 might be one of several factors that control the rapid recovery of ARE-mediated gene expression to a normal level and maintain it at a normal level.

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