Electrophiles and reactive oxygen species have been implicated in the pathogenesis of many diseases. Transcription factor Nrf2 was recently identified as a general regulator of one defense mechanism against such havoc. Nrf2 regulates the inducible expression of a group of detoxication enzymes, such as glutathione S-transferase and NAD(P)H:quinone oxidoreductase, via antioxidant response elements. Using peritoneal macrophages from Nrf2-deficient mice, we show here that Nrf2 also controls the expression of a group of electrophile- and oxidative stress-inducible proteins and activities, which includes heme oxygenase-1, A170, peroxiredoxin MSP23, and cystine membrane transport (system $\chi_c$) activity. The response to electrophilic and reactive oxygen species-producing agents was profoundly impaired in Nrf2-deficient cells. The lack of induction of system $\chi_c$ activity resulted in the minimal level of intracellular glutathione, and Nrf2-deficient cells were more sensitive to toxic electrophiles. Several stress agents induced the DNA binding activity of Nrf2 in the nucleus without increasing its mRNA level. Thus Nrf2 regulates a wide-ranging metabolic response to oxidative stress.

Oxidative stress conditions or enhanced production of reactive oxygen species (ROS) result from a variety of stimuli including ionizing radiation, exposure to xenobiotics, inflammation, and phagocytosis (1). Treatment of mammalian cells with electrophilic agents usually provokes cellular responses, including transcriptional activation of genes encoding proteins that partake in the defense against oxidative stress. This process is referred to as the electrophile counterattack response (2).

Through analyses of mouse and rat glutathione S-transferase (GST) Ya subunit genes and the rat NAD(P)H:quinone oxidoreductase (NQO1) subunit gene, the cis-acting element responsible for the induction by electrophiles was independently identified as an electrophile-responsive element (EpRE) (3) or antioxidant-responsive element (ARE) (4). The consensus ARE sequence has been extensively characterized (5).

The consensus binding sequence of erythroid transcription factor NF-E2 shows high similarity to the ARE/EpRE sequence. Also, the expression profile of Nrf2, one of the NF-E2 subunit factors, overlaps with those of drug-metabolizing enzymes such as GST and NQO1. Based on these facts, we recently demonstrated that transcription factor Nrf2 (6–8) is essential for the coordinated transcriptional activation of genes encoding the drug-metabolizing enzymes, such as GST and NQO1, via AREs/EpREs (9). Nrf2-deficient mice fed with butylated hydroxyanisole, which normally leads to a pronounced up-regulation of Alpha, Pi, and Mu classes of GSTs and NQO1, failed to induce either of these detoxication enzymes in the liver or intestine (9). Since these detoxication enzymes decrease the level of oxidative stress by removing compounds capable of generating ROS or other highly reactive substances, they thereby constitute part of the defense mechanism against oxidative stress (10). Because ARE-type cis-acting sequences are frequently found in the regulatory regions of a number of other oxidative stress-inducible genes (5, 11–13), we hypothesized that Nrf2 might also serve as the key transcription factor activating these genes.

A number of defense proteins and activities in murine peritoneal macrophages are markedly induced upon exposure to electrophilic agents or other oxidative stresses. These proteins include heme oxygenase-1 (HO-1) (14–16), peroxiredoxin MSP23 (17), the cystine membrane transporter (system $\chi_c$) (18) and 60-kDa stress protein A170 (19). HO-1 is prominently induced under various oxidative stress conditions in many different cell types (14). HO-1-deficient embryonic fibroblasts are hypersensitive to the cytotoxicity of both hemin and hydrogen peroxide (15). Induction of system $\chi_c$ activity increases the intracellular cysteine pool, which consequently augments the synthesis of GSH (20), a potent antioxidant with a short half-life. MSP23 is the murine peroxiredoxin I with antioxidative activity (21). It was recently shown that a mammalian peroxiredoxin isofrom reduces the intracellular hydrogen peroxide level utilizing thioredoxin as an immediate electron donor (22) and protects cells from apoptosis by oxidative stress (23). A170 has a structural domain that interacts with ubiquitin (24) and PKC-ζ (25). Electrophilic agents, such as diethylmaleate (DEM), and other oxidative stress agents have been reported to induce the proteins HO-1, A170, MSP23, and system $\chi_c$ activity in peritoneal macrophages (20) and fibroblasts (26). To determine whether these antioxidant stress proteins are also under the regulation of Nrf2, we examined in this study the electrophilic induction of this group of genes in peritoneal macrophages from the nrf2-null mutant mouse.
**EXPERIMENTAL PROCEDURES**

**Culture of Macrophages**—Female wild type ICR and nrf2 mutant mice (9) weighing 20–25 g received an intraperitoneal injection of 2 ml of 4% thioglycollate broth. Four days later, macrophages were collected by peritoneal lavage (17). The cells were resuspended at 7.5 × 10⁶ cells/ml and cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum as described previously (17). For the 3-(4,5-dimethyl-2-3-furazan)-7-(2-2,6-p-diphenyl-2,4-dinitroretrazolium bromide (MTT) (27) assay, the cells were resuspended at 2 × 10⁶ cells/ml and cultured in medium without fetal bovine serum. After 1 h of culture, stress agents were added to the medium. Final concentrations of the agents in the medium were 100 μM for DEM, paraquat, and hydrogen peroxide (H₂O₂); 80 μM for catechol; 20 milliunits/ml for glucose oxidase (GO); 5 μM for CdCl₂ and menadione (2-methyl-1,4-naphthoquinone); 10 μM for 1-chloro-2,4-dinitrobenzene (CDNB); 20 μM for iodoacetic acid; 2.5 μM for t-butylhydroquinone (t-BHQ) and sodium arsenite (NaAsO₂); and 1 ng/ml for lipopolysaccharide (LPS). The macrophages were harvested at the times indicated in the figure legends. Cell viability was measured by the MTT assay (27) and the trypan blue dye exclusion test.

**RNA Blot Hybridization Analysis**—Total cellular RNA was extracted from macrophages by RNAzol™ B (TEL-TEST, Inc., Friendswood, TX). The RNA samples (10 μg) were electrophoresed and transferred to Zeta-Probe GT membranes (Bio-Rad). The membranes were probed with [³²P]-labeled cDNA probes as indicated in the figure legends. β-Actin cDNA was used as a positive control.

**Immunoblotting**—Macrophages were solubilized with SDS-sample buffer (without dye or 2-mercaptoethanol), and protein concentrations were estimated by the BCA protein assay (Pierce). The proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and electrotransferred onto Immobilon membrane (Millipore Corp., Bedford, MA). To detect immunoreactive proteins, we used horseradish peroxidase-conjugated anti-rabbit IgG and ECL blotting reagents (Amersham Pharmacia Biotech). Polyclonal rabbit antiserum raised against Nrf2 by immunizing rabbits with recombinant Nrf2 protein (amino acids 140–318 fused with E. coli maltose-binding protein) was purchased from Santa Cruz Biotechnology. In competition experiments, a 100-fold excess of unlabeled double-stranded oligonucleotides was included in the binding reaction at 1:10 to 1:100 dilutions. The anti-chicken MafK antibody was described previously (9), and that against human Nrf2 was raised against Nrf2 by immunizing rabbits with recombinant Nrf2 protein (amino acids 140–318 fused with E. coli maltose-binding protein). An anti-actin antisera was purchased from Santa Cruz Biotechnology. For immunoblots, proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and electrotransferred onto Immobilon membrane (Millipore Corp., Bedford, MA). To detect immunoreactive proteins, we used horseradish peroxidase-conjugated anti-rabbit IgG and ECL blotting reagents (Amersham Pharmacia Biotech). Polyclonal rabbit antiserum raised against Nrf2 by immunizing rabbits with recombinant Nrf2 protein (amino acids 140–318 fused with E. coli maltose-binding protein) was purchased from Santa Cruz Biotechnology. In competition experiments, a 100-fold excess of unlabeled double-stranded oligonucleotides was included in the binding reaction at 1:10 to 1:100 dilutions. The anti-chicken MafK antibody was described previously (9), and that against human Nrf2 was raised against Nrf2 by immunizing rabbits with recombinant Nrf2 protein (amino acids 140–318 fused with E. coli maltose-binding protein). An anti-actin antisera was purchased from Santa Cruz Biotechnology.

**RESULTS**

**Impaired Induction of Antioxidative Stress Proteins in Nrf2-deficient Macrophages**—A number of proteins or activities are induced by electrophilic agents in murine peritoneal macrophages. To test whether the electrophilic induction of this group of genes shares a common regulatory mechanism with that of the drug-metabolizing enzymes, we examined their expression in Nrf2-deficient macrophages. Peritoneal macrophages were harvested from nrf2-homozygous and -heterozygous (wild type) female ICR siblings. The macrophages were then independently challenged with DEM (a drug-metabolizing enzyme) agent, paraquat (an O₂ generator), GO (an H₂O₂ generator), or CdCl₂ (heavy metal). After challenging macrophages with these agents, we examined expression levels of three oxidative stress-inducible proteins (below) by immunoblotting and RNA blotting analyses. Since wild type and heterozygous mutant mice did not show large differences in induction of the antioxidative proteins, we used both types of macrophages as positive controls.

We first measured the levels of HO-1, MSP23, and A170 genes in in vitro culture and found that the basal levels of these proteins in the heterozygous macrophages were similar to those of nrf2-homozygous mutant macrophages (Fig. 1A, lanes 1 and 7). After transfer of the nrf2-deficient cells to in vitro culture, these stress-inducible proteins were gradually induced by unknown mechanisms (compare lanes 1 and 2). The gradual induction of HO-1 and MSP23 expression was not seen in Nrf2-deficient cells (lanes 2 and 8). The important finding here was that whereas all of the stress agents...
tested induced HO-1, MSP23, and A170 in nrf2-heterozygous cells, induction was largely canceled in Nrf2-deficient cells. Closer examination revealed that in nrf2-null mutant cells (*lanes 9–12*) induction of HO-1 and A170 by DEM and GO was severely affected, but induction by paraquat and CdCl₂ was less impaired. In contrast, while MSP23 was markedly induced by these agents in *nrf2*-heterozygous cells, induction was largely absent in *nrf2*-null mutant cells.

**Quantitative Analysis of Antioxidative Stress Protein Induction by Various Stress Agents**—We also examined induction of these proteins by other stress agents: catechol, CDNB, H₂O₂, iodoacetic acid, sodium arsenite, menadione, and t-BHQ. Quantitative analysis by densitometry of the stained bands is shown in Table I. Menadione and catechol induced HO-1 in the *nrf2*-deficient cells at levels comparable with those in *nrf2*-heterozygous mutant cells, suggesting the involvement of signal-transduction pathway(s) other than the Nrf2 system. Apparently, induction of HO-1 by sodium arsenite, t-BHQ, CDNB, and iodoacetic acid is largely, if not exclusively, dependent on the presence of Nrf2. A significant increase of MSP23 by all stress agents except sodium arsenite, H₂O₂, and iodoacetic acid was also observed in the heterozygous mutant cells but not in homozygous mutant cells (Table I). These results thus indicate that inducible expression of MSP23 is regulated mainly through the ARE/Nrf2 system.

To assess the induction process at the level of transcription, RNA was extracted from macrophages that had been treated with various stress agents and analyzed by the RNA blot analysis. Treatment with stress agents significantly increased the levels of HO-1 and MSP23 mRNA in *nrf2*-heterozygous cells (Fig. 1B, *lanes 1–6*), but the induction was markedly impaired in *nrf2*-null mutant cells (*lanes 7–12*). In contrast, whereas the lack of the induction of A170 mRNA in *nrf2*-null mutant cells was evident when DEM was used as the stress agent, the induction was only partially affected in *nrf2*-deficient cells when paraquat, GO, or CdCl₂ was used as an inducer. Marked induction of the genes was also observed in the *nrf2*-heterozygous cells treated with menadione, t-BHQ, catechol, or CDNB (data not shown). The induction of HO-1 mRNA by menadione and catechol was diminished in Nrf2-null mutant cells, while that by t-BHQ and CDNB was largely abolished. Induction of MSP23 mRNA by all of these agents was markedly impaired in *nrf2*-null mutant cells. The three oxidative stress-inducible genes we addressed here showed roughly comparable variation in the mRNA and protein levels in response to various stress agents (Fig. 1, compare A and B, and data not shown). These results thus indicate that Nrf2 regulates the stress agent-mediated induction of HO-1, MSP23, and A170 gene expression. The results also clearly show that the contribution of Nrf2 to the transcriptional activation of these genes differed based upon the stress-inducing agent.

**Induction of the Cystine Transporter x_c⁻活性 System Is Defective in Nrf2-deficient Cells**—Because oxidative stress agents transcriptionally induce system x_c⁻活性 activity in macrophages (20, 26), the stress induction of system x_c⁻活性 activity in *nrf2*-null mutant cells was examined next (Fig. 2A). Whereas *nrf2*-heterozygous cells show system x_c⁻活性 activity comparable with that of wild type cells under both basal and induced conditions (data not shown), the oxidative stress agents DEM, paraquat, GO, and CdCl₂ barely induced system x_c⁻活性 activity in *nrf2*-null mutant cells (Fig. 2A). In contrast, LPS, a well known inducer of system x_c⁻活性 activity (30), significantly induced the system x_c⁻活性 activity even in *nrf2*-null mutant cells, indicating that LPS induction is mediated through an alternative regulatory pathway rather than the Nrf2 pathway. These results argue that the transcription of the cystine transporter gene may be under the regulatory influence of Nrf2.

It should be noted that system x_c⁻活性 activity is necessary to maintain a high GSH level in cultured macrophages, since cysteine is easily oxidized to cystine upon exposure to air (18). While the addition of 500 μM to 1 mM of DEM to the culture medium depletes the intracellular stores of GSH significantly, the addition of 100 μM DEM only diminishes the GSH level minimally, and then its level increases as a result of induced system x_c⁻活性 activity (26). As expected, the defect in inducible

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**TABLE I**

<table>
<thead>
<tr>
<th>Agents</th>
<th>HO-1</th>
<th>MSP23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nrf2 (+/+−)</td>
<td>Nrf2 (−/−)</td>
</tr>
<tr>
<td>relative absorbance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (1 h)</td>
<td>0.58 ± 0.02</td>
<td>0.50 ± 0.19</td>
</tr>
<tr>
<td>None (9 h)</td>
<td>1.00</td>
<td>0.57 ± 0.12</td>
</tr>
<tr>
<td>DEM</td>
<td>1.67 ± 0.17</td>
<td>0.69 ± 0.04</td>
</tr>
<tr>
<td>CDNB</td>
<td>2.94 ± 0.21</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>GO</td>
<td>1.22 ± 0.02</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>1.26 ± 0.10</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>t-BHQ</td>
<td>1.91 ± 0.77</td>
<td>0.86 ± 0.47</td>
</tr>
<tr>
<td>Menadione</td>
<td>6.38 ± 3.95</td>
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</tr>
<tr>
<td>Catechol</td>
<td>3.70 ± 0.02</td>
<td>1.77 ± 0.06</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>1.57 ± 0.14</td>
<td>1.21 ± 0.08</td>
</tr>
<tr>
<td>Paraquat</td>
<td>1.34 ± 0.03</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td>NaAsO₄</td>
<td>3.70 ± 1.30</td>
<td>1.08 ± 0.51</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>2.05 ± 0.10</td>
<td>0.98 ± 0.27</td>
</tr>
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</table>

*Fig. 2. Oxidative stress agents do not induce system x_c⁻活性 or GSH in Nrf2-deficient macrophages.* A, inability to induce the system x_c⁻活性 cystine transport activity in the *nrf2*-deficient macrophages. Macrophages were incubated for 12 h with DEM, paraquat, GO, CdCl₂, and LPS as described in the legend to Fig. 1A. B, decreased cellular GSH level in *nrf2*-deficient macrophages treated with 100 μM DEM. The values in A and B represent mean ± S.E. of three independent experiments, each carried out in duplicate.
expression of system xᵣ activity in nrf2-null mutant cells resulted in a decrease in cellular GSH content; after a 24-h incubation with DEM, the GSH level dropped to less than half its original level (Fig. 2B).

Overexpression of Nrf2 Up-regulates the Activities of the Distal Enhancer of the ho-1 Gene—The AB1 and SX2 enhancers of the ho-1 gene (31) contain three and two copies, respectively, of the Maf recognition element (MARE), which largely overlaps with ARE (32). Due to their responsiveness to a wide variety of stress agents including oxidative stress, the MAREs were also named stress-responsive elements (StREs) (31–34). To ask whether Nrf2 can regulate the expression of this antioxidative stress gene via the ho-1 AB1 enhancer, we co-transfected a Nrf2 expression plasmid and a ho-1 AB1 enhancer-luciferase construct into QT6 fibroblasts (Fig. 3A). DEM (Fig. 3B) and Nrf2 overexpression (Fig. 3C) both activated HO-1-Luc reporter gene expression, with the highest concentrations of chicken Nrf2 was transfected with pRBGP2 or pHO-1-Luc. Experiments were performed as in B.

**Fig. 3.** Overexpression of Nrf2 is sufficient to activate reporter gene expression driven by the AB1 enhancer of the ho-1 gene. A, schematic representation of the luciferase reporter construct, a 161-base pair fragment of the AB1 enhancer of the ho-1 gene is placed upstream of the rabbit β-globin TATA box and luciferase reporter gene. The pRBGP2 construct is similar, but three copies of the NF-E2 binding sequence from the chicken β-globin enhancer precede the TATA box and luciferase gene (7). B, pRBGP2 or pHO-1-Luc reporter construct was transfected into QT6 fibroblasts in the presence or absence of the electrophilic agent DEM. Luciferase activity in the absence of DEM was set at 100%, and results of three independent experiments each carried out in duplicate are shown with S.E. C, an increasing amount of chicken Nrf2 was transfected with pRBGP2 or pHO-1-Luc. Experiments were performed as in B.

the mRNA level was not changed significantly by any of the oxidative stress agents tested (Fig. 4A). In contrast, we found that these same stress agents significantly enhanced the DNA binding activity of Nrf2 to the StRE of HO-1 AB1 enhancer.

StRE binding activity in nuclear extracts was strongly induced by DEM treatment of macrophages, as revealed by the retarded band (arrow in Fig. 4B, lanes 1 and 2). The induction of binding activity was not observed in nuclear extracts prepared from nrf2-deficient cells (Fig. 4B, lanes 3 and 4). The complex was effectively competed by the addition of an excess of unlabeled StRE, mouse GST Ya gene ARE, or chicken β-globin enhancer NF-E2 binding site but not by a yeast Gal4 binding consensus sequence (Fig. 4D). This DNA-protein complex was markedly diminished by treatment with antibodies against mouse Nrf2 (Fig. 4C, lane 3) and chicken MafK (lane 4), which are partner molecules together comprising the heterodimeric transcription factor complex (34), but the decrease was not obvious using a normal rabbit IgG (lane 5). These results indicate that the DNA-protein complex contains both Nrf2 and small Maf proteins. The same binding activity was also induced in nuclear extracts of macrophages treated with GO, paraquat, or CdCl₂ (Fig. 4E). The Nrf2 expression level was also examined by immunoblotting analyses of nuclear extracts prepared from the macrophages treated with these stress agents. We found that DEM, GO, paraquat, CdCl₂, and CDNB all increased the Nrf2 level 1.5–3-fold (Fig. 4F). We therefore concluded that post-transcriptional regulation might be involved in the activation of Nrf2 by electrophilic agents and ROS.

**Lack of Nrf2 Renders Macrophages Sensitive to Oxidative Stress**—The analysis thus far clearly indicates that macrophages invoke an electrophile-inducible response upon exposure to oxidative stress agents and that the response is mediated by Nrf2. To ask whether this response has a major impact on cell viability, we incubated both nrf2-null and heterozygous control macrophages with 5–20 μM CDNB for 12 h and measured cell viability by the colorimetric MTT assay. While cell
Nrf2 Regulates a Group of Oxidative Stress-inducible Genes

We demonstrate in this study that, in addition to the drug metabolizing enzymes that have already been shown to be regulated by the Nrf2 pathway (9), a group of oxidative stress-inducible genes is also under the immediate transcriptional influence of Nrf2-small Maf heterodimer regulatory proteins. The fact that Nrf2 regulates a group of stress-inducible protein genes via ARE/EpRE, as schematically illustrated in Fig. 6, is intriguing in the context of the physiological origin of these defense mechanisms. In an evolutionary sense, the acquisition of the ARE regulatory mechanism by genes that protect against oxidative stress seems to confer a significant advantage on the survival of living creatures. It should also be noted that, viability was decreased in both nrf2-null mutant and control macrophages treated with 20 μM CDNB, with 10 μM CDNB, nrf2-null mutant cells were more sensitive to the CDNB treatment than the heterozygous control cells (Table II). Notably, 10 μM CDNB treatment resulted in an approximately 2-fold difference in the MTT assay, and this difference is statistically significant (p < 0.05). The difference in the sensitivity to CDNB between the nrf2-null and heterozygous cells was much clear when we measured the cell viability by the trypan blue dye exclusion test after 12 h of the CDNB treatment. The viability of the cells was 77 and 16% (mean of two independent experiments) for nrf2-heterozygous and nrf2-null mutant cells, respectively. Nrf2 thus appears to contribute significantly to cellular defense mechanisms against toxic electrophiles.

To highlight the Nrf2 contribution to cellular defense mechanisms, we pretreated macrophages for 36 h with 100 μM DEM, a concentration that potently induces the antioxidative stress response but with low cytotoxicity. The macrophages were subsequently treated with 5 or 10 μM CDNB for an additional 12 h. After incubation, we measured cell viability by trypan blue dye exclusion. The Nrf2-deficient macrophages were more sensitive to treatment with CDNB than the heterozygous control cells. After CDNB treatment, less than 20% of the Nrf2-deficient macrophages were viable (Fig. 5B), whereas more than 95% of the heterozygous cells were viable (Fig. 5A). These results unequivocally demonstrate that there are electrophile-inducible responses mediated by ARE/EpRE and Nrf2 and that the response machinery protects cells against toxic electrophiles and ROS stresses.

**DISCUSSION**

Fig. 4. Increase in DNA binding activity of Nrf2-small Maf heterodimers upon exposure to stress agents. A, RNA blot analysis of Nrf2 mRNA in macrophages treated with various stress agents. The nrf2-heterozygous mutant cells were incubated for 4 h with the stress agents as in Fig. 1B. Total RNAs extracted from the macrophages were analyzed with Nrf2 and β-actin cDNA probes. B, EMSA analysis of Nrf2-small Maf DNA binding activity in macrophage nuclear extracts. Nuclear extracts were prepared from wild type (lanes 1 and 2) or nrf2-deficient peritoneal macrophages (lanes 3 and 4). These macrophages were either treated with DEM for 4 h (lanes 2 and 4) or not treated (lanes 1 and 3). A double-stranded oligonucleotide of the SRE sequence in the ho-1 gene AB1 enhancer (ho-1 probe) was used as a probe. The complex containing Nrf2 is indicated by an arrow. C, Nrf2-small Maf heterodimer binds to SRE. The ho-1 probe was incubated with the nuclear extract from control macrophages (lane 1) or the nuclear extract of macrophages treated with DEM (lane 2). Anti-Nrf2 (lane 3), anti-MafK (lane 4), or normal rabbit IgG (lane 5) was included in the reaction. The complex containing Nrf2 is indicated by an arrow. D, the binding specificity of the Nrf2-small Maf heterodimer was confirmed by an EMSA competition experiment. The ho-1 probe was incubated with the nuclear extract of macrophages treated with DEM (lane 1). An excess of unlabeled probe oligonucleotide (lane 2) or double-stranded oligonucleotides from mouse GST Ya gene ARE (lane 3), chicken β-globin enhancer NF-E2 binding site (lane 4), or yeast transcription factor Gal4 cognate site (lane 5) was included in the binding reaction. The complex containing Nrf2 is indicated by an arrow. E, increase in the DNA binding activity of Nrf2-small Maf in stress agent-treated macrophages. Nuclear extracts were prepared from the peritoneal macrophages treated with DEM (lane 2), GO (lane 3), paraquat (lane 4), or CdCl₂ (lane 5) and examined by EMSA. An arrow indicates the complex containing Nrf2. F, increase of Nrf2 protein in the nucleus of the stress agent-treated macrophages. Immunoblotting analysis was performed with macrophage nuclear extracts.

**TABLE II**

<table>
<thead>
<tr>
<th>CDNB (mM)</th>
<th>Nrf2 (+/-)</th>
<th>Nrf2 (---)</th>
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<tr>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>5</td>
<td>0.85 ± 0.09</td>
<td>0.86 ± 0.15</td>
</tr>
<tr>
<td>10</td>
<td>0.74 ± 0.06*</td>
<td>0.46 ± 0.13*</td>
</tr>
<tr>
<td>20</td>
<td>0.19 ± 0.02</td>
<td>0.22 ± 0.05</td>
</tr>
</tbody>
</table>

*Np < 0.05.*
Figure 5. Nrf2-null mutant macrophages are sensitive to stress agents. Both nrf2-heterozygous (+/-) (A) and homozygous (-/-) (B) mutant macrophages were first incubated with DEM for 36 h. After changing the medium, the cells were incubated with 10 μM CDNB for 12 h. Cell viability was examined with the trypan blue dye exclusion test.

Figure 6. Schematic presentation of the antioxidative stress response through Nrf2 and ARE/EpRE. The oxidative stress agents liberate Nrf2 from the cytoskeleton into the nucleus (40). Nrf2 then forms a heterodimer with a small Maf protein, interacts with ARE, and induces transcription of a set of genes that encode antioxidant functions. Nrf2 is a general regulator of the defense genes against oxidative stress, which include HO-1, MSP23, and system xc⁻, and also of a group of detoxication enzyme genes including GSTs and NQO1 (9).

There are three levels of regulation should be important for ARE/EpRE-mediated regulation of gene expression. First, the ARE/EpRE cognate sequence shares a high degree of similarity to the consensus MARE sequence (32, 35), so that AREs are competitively bound by a number of bZip transcription factors in the Maf, Jun, Fos, and Cap’n’Collar families. Second, heterodimer formation between these bZip factors creates another level of diversity of regulation (for a review, see Ref. 32). Third, transactivation through an ARE may be regulated coordinately during oxidative stress conditions. For example, the DNA binding activity of transcription factor AP-1 was inhibited when AP-1 was exposed to oxidative stress in vitro (36). This inhibition of AP-1 binding activity may allow the binding of other activating transcription factors, such as an Nrf2-small Maf heterodimer, to ARE and hence induce genes with protective properties against oxidative stress. An interesting extension of this speculation on the physiological roles of the bZip transcription factor network and its perturbation is that the oncogenic property of the bZip (i.e. Maf, Jun, and Fos family) factors may be in part due to the lack of a proper response to electrophiles or ROS, leaving the cells in a rather prooxidative state.

The AB1 and SX2 enhancers of the ho-1 gene mediate the inducible expression of HO-1 by various stimuli (31, 33, 34). Multiple StREs reside in both ho-1 enhancers and have been shown to play important roles in the regulation of ho-1 gene expression by various electrophiles, ROS, heavy metals, and LPS (31, 33, 34). The StRE consensus sequence shares a high degree of similarity to those of ARE/EpRE and MARE (for a review, see Ref. 32), suggesting that StRE is also competitively bound by various bZip transcription factors. In this regard, we previously showed through gene targeting analysis that Nrf2 plays a central role in the regulation of GST and NQO1 gene expression through ARE (9), and the present study has extended the finding. We found that the inducible expression of the ho-1 gene by electrophiles and ROS was severely affected in the Nrf2-null mutant macrophages. We also showed that Nrf2 could activate transcription of the reporter gene through the AB1 enhancer of the ho-1 gene in a transfection assay. These results thus demonstrate that Nrf2 is one of the essential regulators of antioxidative stress genes acting through ARE/EpRE and StRE.

It should also be noted that Nrf2-dependence of stress-inducible gene expression appeared to differ from gene to gene. For instance, none of the electrophilic agents tested induced the expression of MSP23 in the nrf2-null mutant macrophages, whereas three of the agents, menadione, CdCl₂, and catechol, induced ho-1 gene expression to a substantial level even in the nrf2-null mutant macrophages. We envisage that this variation in Nrf2 dependence reflects differences in the structures of the enhancers that mediate the stress signals to gene expression, since integration of signals from several stress-sensing pathways should be executed at the level of enhancer sequence. Indeed, accumulating evidence suggests that in addition to Nrf2, transcription factors NF-κB, AP-1, and heat shock factor are also activated in response to various oxidative stresses. Three lines of evidence further support this hypothesis. First, CAAT boxes, metal-responsive elements and NF-κB binding sequences are found in the ho-1 enhancers (33, 37). Second, menadione is known to effectively activate NF-κB, perhaps by generating ROS (38). Third, NF-κB is also known to mediate signals from LPS (39). Based on these lines of evidence, we speculate that menadione most likely utilized the NF-κB pathway for ho-1 gene induction in nrf2-null mutant cells. LPS induction of system xc⁻ activity in Nrf2-null mutant cells might also be mediated by the NF-κB pathway.

Our present and previous studies uncovered the importance of Nrf2 in cellular protection against oxidative stress. This thesis was supported by the fact that induction of antioxidative stress genes by electrophilic agents was practically absent in the Nrf2-deficient macrophages. An important observation here is that the activation of Nrf2 by electrophilic agents and ROS did not accompany transcriptional induction of the nrf2 gene (Fig. 4A). Based on this observation, we recently identified Keap1, a new factor that binds to the N-terminal Neh2 domain of Nrf2 and negatively regulates Nrf2 activity (40). Electrophilic agents liberate Nrf2 from Keap1 repression. The results
shown in Fig. 4 suggest a possibility that the stress agents facilitate translocation of Nrf2 from the cytosol to the nucleus. We are now investigating the precise molecular mechanism(s) whereby electrophilic agents and ROS affect interaction between the Neh2 domain of Nrf2 and Keap1.

Elevation of the cellular GSH level is one of the most important events in the electrophile-inducible defense response (2). The increase in GSH has been shown to be achieved by inducible trans-transport system for anionic cystine in exchange for glutamate. Interestingly, hepatic γ-glutamylcysteine synthetase, which catalyzes the rate-limiting step of de novo GSH synthesis, is also induced in mice fed on butylated hydroxyanisole (41) and in HepG2 cells treated with t-BHQ (42). Recently, multiple AREs in the distal enhancer of the g were also shown to be responsive to H2O2. Based on these broad observations, we speculate that many genes that function against oxidative stress are also regulated by Nrf2 via AREs. Thus, the Nrf2-centered gene expression regulatory system mediates cellular defense against a wide range of electrophilic compounds and ROS. Coordinated and inducible expression of these defense genes should be important in preventing various free radical-related diseases, such as carcinogenesis, atherosclerosis, ischemia, and neurodegenerative disorders.

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