Nrf2 is a member of the “cap ‘n’ collar” family of transcription factors. These transcription factors bind to the NF-E2 binding sites (GCTGAGTCA) that are essential for the regulation of erythroid-specific genes. Nrf2 is expressed in a wide range of tissues, many of which are sites of expression for phase 2 detoxification genes. Nrf2−/− mice are viable and have a normal phenotype under normal laboratory conditions. The NF-E2 binding site is a subset of the antioxidant response elements that have the sequence GCCNNGTCA. The antioxidant response elements are regulatory sequences found on promoters of several phase 2 detoxification genes that are inducible by xenobiotics and antioxidants. We report here that Nrf2−/− mice are extremely susceptible to the administration of the antioxidant butylated hydroxytoluene. With doses of butylated hydroxytoluene that are tolerated by wild-type mice, the Nrf2−/− mice succumb from acute respiratory distress syndrome. Gene expression studies show that the expression of several detoxification enzymes is altered in the Nrf2−/− mice. The Nrf2−/− mice may prove to be a good in vivo model for toxicological studies. As oxidative damage causes DNA breakage, these mice may also be useful for testing carcinogenic agents.

The study of human β-globin gene expression identified a number of regulatory DNA sequences found at the globin gene promoters and the locus control region (1–3). One of these sequences is the NFE2-AP1 motif that has the sequence of GCTGAGTCA (4–6). This sequence plays an important part in regulating globin gene expression as determined by in vitro and in vivo experiments (4, 5, 7, 8).

Three transcription factors, p45-Nfe2, Nrf1, and Nrf2, have been found to bind to this motif (9–13). These three proteins are members of the “cap ‘n’ collar” (CNC) subfamily of basic region-leucine zipper transcription factors. CNC is a homotypic gene required for the development of the head and neck structures in Drosophila (14). These CNC proteins have been shown to heterodimerize with another family of basic region-leucine zipper proteins, the p18-Mafs (15–18). A fourth member of this CNC subfamily recently has been cloned by sequence homology (19). Of these four CNC genes, only p45-Nfe2 is hematopoietic specific; the other three are ubiquitously expressed. Targeted disruption of p45-Nfe2, Nrf1, and Nrf2 was carried out to determine their function. Disruption of p45-Nfe2 results in a defect in megakaryocyte maturation with minimal effect on globin gene expression (20, 21). Loss of Nrf1 results in embryonic lethality (22, 23), whereas Nrf2−/− mice are viable and show no obvious phenotype when cared for in a normal laboratory setting (24–26).

The AP1-NFE2 motif was later found to be a subset of the antioxidant response element (27–30). Nrf1 and Nrf2 have been shown to transactivate reporter genes linked to the antioxidant response element (31, 32). Also, the sites of expression for Nrf2 coincide with the sites of expression for many of the phase 2 detoxifying genes (24). Itoh et al. (33) showed that Nrf2 plays a role in the induction of two phase 2 detoxifying genes by the phenolic antioxidant butylated hydroxyanisole (BHA).

BHA and butylated hydroxytoluene (BHT) are phenolic antioxidants that are widely used as food additives to prevent rancidity caused by oxidation of lipids. Because of their widespread use as food preservatives, the biochemical properties of BHA and BHT have been studied extensively (34–37). When administered to mice, BHA was well tolerated, whereas BHT was shown to be toxic. The toxicity of BHT and its metabolites have been studied in mice by systemic or oral administration. BHT treatment by either means causes transient lung damage because of the destruction of alveolar type I epithelial cells. Lungs from BHT-treated mice show damage to the alveoli with massive edema and hemorrhage. However, dramatic these injuries are, they are reversible within 7 days of BHT treatment. Subsequently, the lungs recover with the proliferation of type II cells, which differentiate into type I cells (38).

Because BHT has been shown previously to be toxic when administered to mice and because Nrf2 has been shown to regulate several phase 2 detoxifying enzymes, we administered BHT to Nrf2−/− mice. At oral doses that are well tolerated by wild-type mice, Nrf2−/− mice showed a high mortality rate. On i.p. administration, the Nrf2−/− mice have an appreciably lower LD50. These Nrf2−/− mice may therefore serve as a good animal model for drug-toxicity studies.

Materials and Methods

Chemicals. BHT was purchased from Sigma. BHT crystals were ground to a fine powder and incorporated into the powdered feed at a concentration of 0.5–1.0% by weight. For i.p. injection, BHT was dissolved in corn oil (Mazola) at concentrations of 40–120 mg/ml to deliver a dose ranging from 400 mg/kg to 1,200 mg/kg by injecting 0.1 ml per 10 g of body weight.

Animals, Diets, and Treatments. Wild-type and Nrf2−/− mice were obtained by breeding from our stock of C57B/SV129 mice. Mice were maintained on CellSorb bedding (Fangman, Cincinnati, OH) and were fed a regular diet. For the BHT feeding experiment, control and Nrf2−/− mice were switched to a powdered feed (feed 8604M, Harlan Teklad, Madison, WI) for an adaptation period of 3 days and then fed the amended feed. In a typical experiment, age-matched mice were segregated into two groups, one of wild-type and the other of Nrf2−/− mice, with a minimum of six per group. Both groups were either fed the same amended feed or injected with the same BHT solution. For RNA isolation, mice were injected with BHT at a dose of 400 mg per kg body weight then killed 48 h later for tissue harvesting. For lung damage assessment, mice were killed on day 5 of 1% dietary

Abbreviations: CNC, cap ‘n’ collar transcription factor; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

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BHT treatment by cervical dislocation; whole lungs were excised and weighed immediately.

**Histology and in Situ Hybridization.** Mice were killed by cervical dislocation on day 5 of BHT feeding, and the tissues were collected, rinsed in PBS, fixed in a buffered formalin solution (Accustain, Sigma), and paraffin embedded. Sections of 7 μm were stained with eosin and hematoxylin. In situ hybridization was performed as described (24).

**Northern Blots.** Northern blot analyses were performed according to standard procedures (39). Total RNAs were extracted from lung by using Ultraspec RNA (Biotex Laboratories, Edmonton, Canada). The radioactivities of the Northern blots were quantified with Fuji imaging plates and an FLA-2000 image analyzer (Fuji). The activities of the Nrf2−/− bands and the wild-type bands were normalized with those of the actin bands and expressed as Nrf2−/−/wild type.

**cDNAs Probes Used for Northern Blots.** Total RNA was used as template for the first-strand synthesis by using Superscript II reverse transcriptase (GIBCO/BRL) and random hexamers. Primer pairs were designed from sequences from GenBank. The sequences of the primers used to amplify the cDNAs are shown in Table 1. Sequence analyses were used to confirm the identities of the cDNAs fragments. The cDNAs produced were then labeled for Northern blot analyses.

**Results**

Both wild-type and Nrf2−/− mice, when administered BHT orally through feeding, showed evidence of respiratory distress by the second day of the experiment. They had an exhausted appearance characterized by shallow rapid breathing, ruffled fur, and hunched postures. These conditions worsened for the Nrf2−/− mice, the majority of which died. The conditions of the mice that survived stabilized by day 5, and the mice recovered by day 9. At the concentrations of 0.5% and 1% (wt/wt) BHT

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**Table 1. Primers used for amplification of cDNA fragments for probes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>CAGCTCCGCAATCTACACC</td>
<td>CAGCGTTGATTACAGGTGATCC</td>
</tr>
<tr>
<td>NQO1</td>
<td>TTCAGGGTGTCACAGGCCC</td>
<td>ACTTCTCTATTGTTGTTATG</td>
</tr>
<tr>
<td>SOD1</td>
<td>AAGCATGGCGTGAAGGGCCG</td>
<td>ATTACGGTTAATGTTGAGG</td>
</tr>
<tr>
<td>GCS1c</td>
<td>ATGTTTGGGACTACACCT</td>
<td>TGAGCTGGATTGAAAGCC</td>
</tr>
<tr>
<td>UGT1a6</td>
<td>CTTCTGCGAGGGTTTCCTTCC</td>
<td>CAACGATGGATGCTCCC</td>
</tr>
<tr>
<td>HO1</td>
<td>TACACATCAAGCCAGGATGC</td>
<td>GCGGTGTCCTGGATGAGGTGC</td>
</tr>
</tbody>
</table>

NQO1, NAD(P)H: quinone oxidoreductase; SOD1, superoxide dismutase; GCS1c, glutamate cysteine ligase regulatory subunit; UGT1a6, UDP glycosyl transferase 1a6; HO1, heme oxygenase 1.
in the diet, there were 59% and 80% lethalitys, respectively, for Nrf2−/− mice; all between day 5 and day 12. None of the wild-type mice died (Fig. 1 A and B). Autopsies of the dead Nrf2−/− mice revealed diffuse and extensive lung injury. Their lungs were grossly enlarged and hemorrhagic, with pulmonary infiltrates and destruction of the alveolar architecture. In contrast to these mice, the lungs of the wild-type mice showed minimal morphological changes (Fig. 2 A–D).

Relative lung weight used as a lung-damage index showed that Nrf2−/− mice were injured to a much greater extent than wild-type mice when fed BHT (Table 2). No significant differences in relative lung weight were found between the untreated wild-type and Nrf2−/− mice or between the untreated and BHT-treated wild-type mice. However, the lungs of the BHT-treated Nrf2−/− mice were significantly heavier than those of similarly treated wild-type mice (P = 0.006; Student’s t test) as well as those of their untreated siblings (P = 0.04). The lung toxicity affecting the mice was age dependent; younger mice (aged 8–24 weeks) were affected more severely, whereas older mice (aged 35–50 weeks) seemed to cope better and accounted for most of the Nrf2−/− mice that survived the BHT treatment (data not shown).

i.p. administration of greater than 400 mg of BHT per kg of body weight caused death in both groups. However, the Nrf2−/− mice are more susceptible than the wild-type mice (Fig. 1 C and D). At a dose of 600 mg/kg, 67% of wild-type mice survived, whereas only 23% of null mice survived. At doses of 700 mg/kg, 800 mg/kg, or 1,200 mg/kg, 50% of the wild-type mice survived, whereas none of the null mice survived. The dose-mortality curve for Nrf2−/− mice was very steep and yields the i.p. LD50 and LD100 of 530 mg/kg and 660 mg/kg, respectively (Fig. 3). The LD50 and LD100 values of the wild-type mice could not be determined.

Table 2. Weight of lungs expressed as percentage of body weight in mice fed 1% BHT

<table>
<thead>
<tr>
<th>Geneotype</th>
<th>No.</th>
<th>Untreated</th>
<th>Treated, day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6</td>
<td>0.63 ± 0.07</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>Nrf2−/−</td>
<td>6</td>
<td>0.61 ± 0.13</td>
<td>0.82 ± 0.12**</td>
</tr>
</tbody>
</table>

*, P = 0.006 versus treated wild-type mice. †, P = 0.04 versus untreated Nrf2−/− mice.

Fig. 2. Photomicrographs of lungs at day 5 of 1% dietary BHT. (A and B) No difference is found between the lungs of untreated wild-type (Nrf2+/+) and Nrf2−/− mice. (C) The lung of a wild-type mouse fed 1% BHT shows thickening of the alveolar septa and some congestion. (D) In the lung of a mouse fed BHT, the alveolar architecture is mostly destroyed; extensive capillary damage was evident, and erythrocytes are found in the alveolar space. (Bars = 50 μm.)

Fig. 3. LD50 and LD100 values for Nrf2−/− injected i.p. with BHT. Solid line, wild-type mice; broken line, Nrf2−/− mice. The LD50 and LD100 of Nrf2−/− are 530 mg/kg and 660 mg/kg, respectively.
assessed, because 50% of the mice survived i.p. administration of BHT from 700 mg/kg to 1,200 mg/kg.

Although Nrf2 is expressed ubiquitously, we have shown previously by in situ hybridization in mouse embryos that a high level of expression was present in the tracheobronchial tree, the alveoli, the stomach, the intestine, and the kidney (24). We performed in situ hybridization on the stomach and intestine of the adult animals. Using antisense Nrf2 riboprobe, we detected a high level of expression of Nrf2 in the luminal epithelia covering the villi and the intestinal crypts (Fig. 4) as well as in the lining of the stomach (data not shown), as was previously found in the mouse embryos.

The cause of death by BHT pulmonary toxicity for the Nrf2−/− mice could be the loss of Nrf2, which may be required for the up-regulation of detoxifying enzymes. Because the lung was the organ affected by BHT, Northern blot analyses of lung RNA were performed. They revealed that gene expression for several detoxification enzymes is altered by the ablation of nrf2. Expression of catalase, glutamate cysteine ligase regulatory subunit (GCSlc), heme oxygenase 1 (HO1), NAD(P)H:quinone oxidoreductase (NQO1), superoxide dismutase 1 (SOD1), and UDP glycosyl transferase 1A6 (Ugt1a6)—identified by their accession numbers L25069, U95053, X13536, S75951, X06683, and U09930, respectively—was decreased in the Nrf2−/− mice (Fig. 5).

Discussion

These studies show that Nrf2 plays an important role in the defense against oxidative stress in mice, because its ablation renders mice extremely sensitive to the food preservative BHT. Nrf2−/− mice died when fed BHT at concentrations that were well tolerated by wild-type mice, and when BHT was administered i.p., Nrf2−/− mice had a higher mortality rate than did wild-type mice. Nrf2−/− mice treated with BHT died of asphyxia brought on by lung failure; their lungs were grossly edematous, and microscopy showed loss of alveolar architecture with pulmonary infiltration and hemorrhage.
When fed 1% dietary BHT, 80% of the Nrf2−/− mice died. Wild-type mice have been shown to be extremely resilient to BHT in their diet. Although they go through a difficult period for 5 days, characterized by reduced food intake, loss of body weight, and pulmonary distress, they eventually recover. When mice were housed in cages with bedding, up to 5% (wt/wt) dietary BHT for 1 month was tolerated (40). No other reports of lethality caused by acute toxicity from dietary BHT were found in the literature for mice similarly housed. Because we used a powdered feed, we did not quantitate the amount of BHT ingested, and hence, we did not compute the values for oral LD50 or LD100. One study reported the LD50 of 2,000 mg/kg and LD100 of 2,500 mg/kg for BHT administered orally to mice (36). The acute toxicity affecting the mice fed BHT apparently began with the BHT ingested on day 1, because by day 2, they got sick and showed a lack of appetite. Most of the Nrf2−/− mice surviving the 1% dietary BHT tended to be older and may have acquired some degree of adaptive resistance. How they acquired this resistance is not known, but with wild-type mice, it has also been reported that older mice were less susceptible to BHT-induced lung injury (41).

When BHT was administered i.p., Nrf2−/− mice had a higher mortality rate than did wild-type mice. Although both groups tolerated doses up to 400 mg/kg, at higher doses, the wild-type mice began to die but at a lower rate than the Nrf2−/− mice. All of the Nrf2−/− mice succumbed at 700 mg/kg, whereas half of the wild-type mice treated survived, even up to a dose of 1,200 mg/kg (Fig. 3).

Why are the Nrf2−/− mice so much less tolerant of oral administration of BHT compared with the wild type? We have shown by in situ hybridization that Nrf2 expression is high in the gastrointestinal epithelial cells and the liver. Because Nrf2 controls the expression of a number of antioxidant genes, the lack of this transcription factor in the gastrointestinal tract may allow BHT and its metabolites to escape detoxification before they enter the general circulation. When BHT is administered by i.p. injection, the phase 2 enzymes that are regulated in the gastrointestinal tract by Nrf2 are bypassed. Two studies reported the accumulation of BHT in several organs of wild-type mice after different two methods of administration. In the plasma, the lung, the liver, and the kidney, the relative BHT concentrations were consistently higher when BHT was injected into the peritoneal cavity than when BHT was administered orally. This higher concentration persisted from day 1 throughout day 4 after treatment (42, 43). This higher BHT level in the circulation may have caused the death of some of the i.p. treated wild-type mice, whereas none of these wild-type mice died when fed BHT because of the detoxification enzymes regulated by Nrf2 in the gut and the liver. As Nrf2 expression is also high in the kidney, the detoxification and excretion may also be impaired in the Nrf2−/− mice. The greater pulmonary toxicity leading to death may also be attributed to the lack of Nrf2, which is normally highly expressed in the lung.

There is growing evidence that the metabolites BHT-BuOH and the corresponding quinone methide are more potent pneumotoxins than BHT per se (44, 45). In vitro data suggest that putative toxic metabolites are formed readily in Clara cells (46), which are sites of high expression for cytochrome P450 enzymes. However, it is not clear whether the pulmonary toxicity of BHT in the lungs is solely caused by its bioactivation in situ or by one or more of its metabolites that are produced in other organs such as the liver and then transported to the lungs. Also, it is still unknown how BHT and its metabolites cause the cellular damage seen in the lung.

We have shown that, in the lung, several phase 2 detoxification genes are differentially expressed between the wild-type and the Nrf2−/− mice. Future work will have to identify additional target genes that are regulated by Nrf2 and test the toxicity of other drugs. Because of the enhanced toxicity that we found in these mice, they may provide a valuable model to screen for drug toxicity. Because oxidative damage causes DNA breakage, these mice may also be useful for testing compounds for their potential to cause cancer. As Nrf2 potentially regulates a wide spectrum of detoxification genes, it would also be of interest to investigate whether Nrf2 mutations exist in humans and whether carriers of Nrf2 mutations are predisposed to higher risks for respiratory disease or cancer.

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