Identification of the NF-E2-related Factor-2-dependent Genes Conferring Protection against Oxidative Stress in Primary Cortical Astrocytes Using Oligonucleotide Microarray Analysis*

John-Min Lee‡§, Marcus J. Calkins‡§, Kaimin Chan¶¶, Yuet Wai Kai††, and Jeffrey A. Johnson‡‡§§

The antioxidant responsive element (ARE) mediates transcriptional regulation of phase II detoxification enzymes and antioxidant proteins such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferases, and glutamate-cysteine ligase. In this study, we demonstrate that NF-E2-related factor-2 (Nrf2) plays a major role in transcriptional activation of ARE-driven genes and identify Nrf2-dependent genes by oligonucleotide microarray analysis using primary cortical astrocytes from Nrf2+/+ and Nrf2−/− mice. Nrf2−/− astrocytes had decreased basal NQO1 activity and no induction by tert-butyliydroquinone compared with Nrf2+/+ astrocytes. Similarly, both basal and induced levels of human NQO1-ARE-luciferase expression in Nrf2−/− astrocytes were significantly lower than in Nrf2+/+ astrocytes. Furthermore, human NQO1-ARE-luciferase expression in Nrf2−/− astrocytes was restored by overexpression of Nrf2, whereas ARE activation in Nrf2−/− astrocytes was completely blocked by dominant-negative Nrf2. In addition, we observed that Nrf2-dependent genes protected primary astrocytes from H2O2-or platelet-activating factor-induced apoptosis. In support of these observations, we identified Nrf2-dependent genes encoding detoxification enzymes, glutathione-related proteins, antioxidant proteins, NADPH-producing enzymes, and anti-inflammatory genes using oligonucleotide microarrays. Proteins within these functional categories are vital to the maintenance and responsiveness of a cell defense system, suggesting that an orchestrated change in gene expression via Nrf2 and the ARE gives a synergistic protective effect against oxidative stress.

The antioxidant responsive element (ARE) is a cis-acting regulatory element in promoter regions of several genes encoding phase II detoxification enzymes and antioxidant proteins (1). The ARE plays an important role in transcriptional activation of downstream genes such as NAD(P)H:quinone oxidoreductase; glutathione S-transferase; glutamate-cysteine ligase; thioredoxin reductase-1; thioredoxin; ferritin; Nrf2; tBHQ; tert-butylihydroquinone; PAF; platelet-activating factor; CMV; cytomegalovirus; GCLM; glutamate-cysteine ligase modifier subunit; GCLC; glutamate-cysteine ligase catalytic subunit; TUNEL; terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

* This work was supported by Grants ES08089 and ES10042 (to J. A. J.) and Grant ES08089 (to the Environmental Health Sciences Center) from the NIEHS, National Institutes of Health, by the Burroughs Wellcome New Investigator in Toxicological Sciences award (to J. A. J.), and by Grant DK16666 from the National Institutes of Health (to Y. W. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶¶ To whom correspondence should be addressed: School of Pharmacy, University of Wisconsin, 6125 Rennebohm Hall, 777 Highland Ave., Madison, WI 53705-2222. Tel.: 608-262-2993; Fax: 608-262-5345; E-mail: jajohnson@pharmacy.wisc.edu.

1 The abbreviations used are: ARE, antioxidant responsive element; NQO1, NAD(P)H:quinone oxidoreductase; GST, glutathione S-transferase; HO-1, heme oxygenase-1; TXNRD1, thioredoxin reductase-1; Nrf2, NF-E2-related factor-2; tBHQ, tert-butylihydroquinone; PAF, platelet-activating factor; CMV, cytomegalovirus; GCLM, glutamate-cysteine ligase modifier subunit; GCLC, glutamate-cysteine ligase catalytic subunit; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.
trointestinal tract (39, 40) and promotion of the wound-healing process (41). In addition, many chronic neurodegenerative diseases (i.e. Parkinson’s disease and Alzheimer’s disease) are thought to involve oxidative stress as a component contributing to the progression of the disease. The regulation and cell-specific expression of these genes in cells derived from brain could therefore be important for understanding how to protect neural cells from oxidative stress. One of the Nrf2-dependent ARE-driven genes, NQO1, has been demonstrated to play an important role in protecting cells against oxidative stress (42).

To further understand how Nrf2 contributes to the regulation of ARE-driven genes in astrocytes and how expression of these genes affects the sensitivity of astrocytes to oxidative stress, we compared primary cortical astrocyte cultures derived from transgenic reporter mice (34). To determine differential sensitivity, we performed oligonucleotide microarray analysis.

EXPERIMENTAL PROCEDURES

Nrf2 Knockout Mice—Nrf2 knockout mice were generated by replacing the basic leucine zipper domain with the lacZ reporter construct as described previously (47).

Primary Cortical Astrocyte Culture—Nrf2−/− mice were bred with Nrf2+/− mice, and primary cortical astrocyte cultures were prepared individually. Cerebral cortices from newborn pup littersmates were removed, placed in ice-cold Hanks’ balance salt solution (3 m/l/pup; In Vitrogen), centrifuged at 300 × g for 2 min, and digested individually in

---

**Fig. 1. ARE activation by tBHQ in Nrf2−/− astrocytes.** Nrf2−/− (A) and Nrf2+/+ (B) astrocytes were transfected with hNQO1-ARE-luciferase (80 ng/well) and CMV-β-galactosidase (20 ng/well). After 24 h of transfection, cells were treated with vehicle (V; 0.01% Me2SO), tBHQ, H2O2, and phorbol 12-myristate 13-acetate (PMA) for 24 h. Luciferase and galactosidase activities were measured, and ARE-luciferase gene expression was calculated by the ratio of luciferase to galactosidase activity. Each data bar represents the mean ± S.E. (n = 6). *, significantly different from the vehicle-treated group by Student’s t test (p < 0.05).

**Fig. 2. Nrf2-dependent ARE activation and NQO1 expression.** A, primary astrocytes were transfected with hNQO1-ARE-luciferase (80 ng/well) and CMV-β-galactosidase (20 ng/well). After 24 h of transfection, cells were treated with tBHQ (0–20 μM) for 24 h. Luciferase and galactosidase activities were measured, and ARE-luciferase gene expression was calculated by the ratio of luciferase to galactosidase activity. Each data bar represents the mean ± S.E. (n = 6). B, primary astrocytes were treated with tBHQ (0–50 μM) for 72 h, and NQO1 activity was determined from cell lysates. Each data bar represents the mean ± S.E. (n = 6). C, primary astrocytes were treated with vehicle (0.01% Me2SO) or tBHQ (50 μM) for 72 h, and NQO1 activity was determined by histochemistry using LY 83583 as a substrate. Magnification is ×200.
Identification of Nrf2-dependent ARE-driven Genes

0.5 mg/ml trypsin (Invitrogen) in Hanks’ balance salt solution at 37 °C for 25 min. Tissues were washed twice with Hanks’ balance salt solution and resuspended in minimal essential medium with Earle’s salt (Mediatech) containing heat-inactivated (55 °C for 25 min) fetal bovine serum (10%) and horse serum (10%) (both from Atlanta Biologics, Inc.). Cell suspensions were seeded into well plates (75 µm; Falcon) and plated at a density of 5 × 10^4 cells/ml. The medium was changed after 24 h of initial plating and every 3 days thereafter. Cultures were maintained at 37 °C, changed after 24 h of initial plating and every 3 days thereafter. Cultures were maintained at 37 °C, changed after 24 h of initial plating and every 3 days thereafter. Cultures were maintained at 37 °C, changed after 24 h of initial plating and every 3 days thereafter. Cultures were maintained at 37 °C, changed after 24 h of initial plating and every 3 days thereafter. Cultures were maintained at 37 °C, changed after 24 h of initial plating and every 3 days thereafter. Cultures were maintained at 37 °C, changed after 24 h of initial plating and every 3 days thereafter.}

**Reverse Transcription-PCR**—Total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was synthesized (reverse transcription system, Promega) according to the manufacturer’s protocol. Aliquots of cDNA were used for PCR amplification using Taq DNA polymerase (Promega). PCR primers specific to each gene are as follows: NQO1, 5’-CATTCTGAAAAAGGTGGTTAGA-3’ and 5’-CTAGTCTTATGCTGTGTTG-3’; GSTM1, 5’-CTCCGGACATTGACCGAC-3’ and 5’-GCAAAGTCCCTAGTTTG-3’; GSTP1, 5’-CAAGAGGCTGGTTTGC-3’ and 5’-GAAGACAGCTGGTTG-3’; GCLC, 5’-CCCTGAGGGTGCTGTTG-3’ and 5’-GAAGAGGCTGGTTG-3’; GCLM, 5’-CTCAGTCTTCTGCTGTGCTG-3’ and 5’-GATTGCTGGTGTTGGGT-3’.}

**Cytotoxicity**—Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay (Promega), and apoptotic cell death was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining (Roche Molecular Biochemicals). Primary astrocytes in 96-well plates were pretreated with vehicle (0.01% MeSO) or tBHQ (50 µM) for 48 h. After 48 h, cells were treated with H2O2 (0–300 µM, 4 h) or PAF (0–50 µM, 24 h). For PAF treatment, the medium was changed with serum-free Dulbecco’s modified Eagle’s medium. The media were changed with fresh media, and 3-(4,5-dimethylthiazol-2-yl)-5-carboxymethylphenyl/tetrazolium salt substrate was added. After a 2-h incubation, the absorbance at 490 nm was measured. Percent cell viability was calculated by A0/A1 × 100%. For TUNEL staining, astrocytes in eight-chamber slides were treated with (0.01% MeSO or 50 µM tBHQ, 48 h), treated (phosphate-buffered saline; 150 µM H2O2, 4 h; or 20 µM PAF, 24 h), and stained according to the manufacturer’s protocol.

**Oligonucleotide Microarray Analysis**—Nrf2−/− and Nrf2+/+ primary astrocytes were treated with vehicle (0.01% MeSO) or tBHQ (50 µM) for 24 h. Biopolymerized cRNA was prepared from total RNA, and fragmented cRNA was hybridized to MG U74 Av2 arrays (Affymetrix) (32, 33). Affymetrix Microarray Suite 5.0 was used to scan and analyze the relative abundance of each gene (scaling target signal 2500 and default analysis parameters). Data were analyzed by rank analysis as previously described (32, 33). Briefly, the definition of increase, decrease, or no change of expression for individual genes was based on ranking the difference call from two intergroup comparisons (2 × 2 matrix, t test). No change = 0, marginal increase = 1, marginal decrease = -1, increase = 2, and decrease = -2. The final rank reflects the sum of the four values (2 × 2 matrix) corresponding to the difference calls. The cutoff values for increase/decrease were set as ±1/−1 (2 × 2 matrix). The reproducibility of paired comparisons was based on the coefficient of variation (S.D./mean) for the fold change of the ranked genes. A distribution curve of the coefficient of variation (CV) was used to determine its cutoff value. The cutoff values were CV < 1.0 and ±1.2-fold for increased genes and CV > 1.0 and ±1.2-fold for decreased genes. This method of analysis is critical in generating an accurate list of genes associated with Nrf2 and tBHQ treatment. Because these littermate cultures were derived from mice of mixed background, there is the possibility that some changes in expression may be associated with differences in genetic background. However, this type of matrix analysis selects for consistent reproducible associations associated with the presence of Nrf2 and tBHQ treatment in lieu of random changes due to genetic background (32, 33). Gene categorization was based on the NetAffx Database.2

2 Available at www.NetAffx.com.
Identification of Nrf2-dependent ARE-driven Genes

RESULTS

*tBHQ Selectively Activates the ARE in Nrf2<sup>−/−</sup> Astrocytes*—Initially, to choose an ARE activator for this study, we tested several known ARE activators in other cell types such as tBHQ in IMR-32 human neuroblastoma cells (2, 3), and H<sub>2</sub>O<sub>2</sub> (1) and phorbol 12-myristate 13-acetate in HepG2 human hepatoma cells (31). Nrf2<sup>−/−</sup> and Nrf2<sup>+/+</sup> astrocytes were transfected with hNQO1-ARE-luciferase and treated with vehicle, tBHQ, H<sub>2</sub>O<sub>2</sub>, or phorbol 12-myristate 13-acetate. First, the basal level of hNQO1-ARE-luciferase expression in Nrf2<sup>+/+</sup> astrocytes (3086.5 ± 320.7) (V in Fig. 1B) was significantly higher than in Nrf2<sup>−/−</sup> astrocytes (657 ± 91.6) (V in Fig. 1A). Second, none of the tested chemicals activated the ARE in Nrf2<sup>−/−</sup> astrocytes (Fig. 2A). Third, only tBHQ increased reporter gene expression in Nrf2<sup>−/−</sup> astrocytes (Fig. 2B), suggesting that a tBHQ-specific signaling pathway mediates Nrf2-dependent ARE activation in primary astrocytes.

*Nrf2-dependent ARE Activation*—hNQO1-ARE-luciferase gene expression and endogenous NQO1 activity were determined in tBHQ-treated Nrf2<sup>−/−</sup> and Nrf2<sup>+/+</sup> astrocytes. In Nrf2<sup>−/−</sup> astrocytes, basal ARE-luciferase reporter gene expression was markedly decreased, and there was no induction of reporter gene expression by tBHQ compared with Nrf2<sup>−/−</sup> and Nrf2<sup>+/+</sup> astrocytes (Fig. 2A). Similarly, both basal and induced levels of endogenous NQO1 activity in Nrf2<sup>−/−</sup> astrocytes were significantly lower than in Nrf2<sup>−/−</sup> and Nrf2<sup>+/+</sup> astrocytes (Fig. 2B), implying that Nrf2 plays an important role in both basal and induced ARE-driven gene expression in mouse primary cortical astrocytes. In addition, histochemical detection of NQO1 activity confirmed the Nrf2-dependent NQO1 gene expression. The NQO1 staining of vehicle-treated Nrf2<sup>−/−</sup> astrocytes was significantly higher than that of vehicle-treated Nrf2<sup>−/−</sup> cells (Fig. 2C, upper left panel versus lower left panel), and tBHQ increased NQO1 staining intensity only in Nrf2<sup>−/−</sup> astrocytes (lower left panel versus lower right panel). To further investigate the role of Nrf2 in ARE activation, we transfected Nrf2<sup>−/−</sup> astrocytes with an Nrf2 overexpression vector to restore ARE activation and Nrf2<sup>−/−</sup> astrocytes with dominant-negative Nrf2 to inhibit ARE activation. Dominant-negative Nrf2 (N-terminally truncated Nrf2) inhibits endogenous Nrf2 function by occupying and limiting its binding partners and DNA-binding sites (5). Indeed, overexpression of Nrf2 led to dramatic ARE activation in Nrf2<sup>−/−</sup> astrocytes (Fig. 3A). tBHQ did not activate the ARE in pEF-transfected Nrf2<sup>−/−</sup> astrocytes. However, tBHQ did activate the ARE in Nrf2-overexpressing Nrf2<sup>−/−</sup> astrocytes in a dose-dependent manner (Fig. 3B). Finally, dominant-negative Nrf2 blocked both basal and induced ARE activation by tBHQ in Nrf2<sup>−/−</sup> astrocytes (Fig. 3B).

**Diffrential Sensitivity to H<sub>2</sub>O<sub>2</sub> and PAF-induced Cytotoxicity**—Nrf2 regulates ARE-driven genes involved in detoxification and antioxidant potential. Therefore, we hypothesized that
### Table I
Identification of Nrf2-dependent ARE-driven Genes

The genes changed by Nrf2 were functionally categorized. R, rank; CV, coefficient of variation of -fold; G6PD, glucose-6-phosphate dehydrogenase; MMTV, murine mammary tumor virus. For decreased genes, genes with fold lower than −1.4 are listed.

| Gene | Accession no. | R | -fold ± S.E. | CV |
|------|---------------|---|-------------|----|
| Detoxification | | | | |
| NQO1<sup>a</sup> | U12961 | 4 | 2.1 ± 0.13 | 0.124 |
| GST A4<sup>a</sup> | L06047 | 6 | 1.5 ± 0.10 | 0.126 |
| GST P12<sup>a</sup> | X53451 | 8 | 1.3 ± 0.04 | 0.065 |
| GST M1<sup>a</sup> | J03952 | 8 | 1.8 ± 0.11 | 0.126 |
| GST Mu2<sup>a</sup> | J04696 | 4 | 1.3 ± 0.09 | 0.141 |
| GST Mu<sup>a</sup> | J09893 | 8 | 1.6 ± 0.09 | 0.114 |
| Aldehyde dehydrogenase-1 | M74570 | 6 | 2.4 ± 0.45 | 0.378 |
| Aldehyde dehydrogenase-2 | U07235 | 4 | 1.2 ± 0.07 | 0.113 |
| Cytochrome P450 1B1 | X78445 | 6 | 1.2 ± 0.09 | 0.144 |
| Antioxidant/reducing Potential | | | | |
| GCLM<sup>a</sup> | U95053 | 4 | 1.3 ± 0.08 | 0.126 |
| GCLC<sup>a</sup> | U85414 | 8 | 1.9 ± 0.16 | 0.169 |
| HO-1 (decycling)<sup>b</sup> | X66824 | 8 | 1.5 ± 0.09 | 0.113 |
| TXNRD 1<sup>a</sup> | AB027565 | 8 | 1.7 ± 0.07 | 0.08 |
| Ferritin light chain<sup>a</sup> | L59879 | 6 | 1.2 ± 0.03 | 0.057 |
| Type I peroxiredoxin | AB023564 | 8 | 1.3 ± 0.04 | 0.057 |
| 1-Cys peroxiredoxin protein-2 | AF093853 | 6 | 1.3 ± 0.09 | 0.146 |
| G6PD, X-linked | Z19111 | 8 | 1.3 ± 0.06 | 0.089 |
| G6PD-2 gene | Z5447 | 4 | 1.3 ± 0.06 | 0.089 |
| Transketolase | U05899 | 6 | 1.3 ± 0.10 | 0.156 |
| Malic enzyme, supernatant<sup>a</sup> | J02652 | 6 | 1.5 ± 0.21 | 0.277 |
| Transcription | | | | |
| Nrf2 | U70475 | 8 | 35.6 ± 3.96 | 0.223 |
| Homeobox, MSH-like 2 | X59252 | 6 | 2.3 ± 0.25 | 0.225 |
| Prolactin regulatory element binding | AB362895 | 4 | 1.7 ± 0.12 | 0.144 |
| CCAAT/enhancer-binding protein-β | M61007 | 6 | 1.4 ± 0.16 | 0.226 |
| Zinc finger protein of cerebellum 3 | D70849 | 4 | 1.4 ± 0.14 | 0.193 |
| Growth | | | | |
| Colony-stimulating factor-1 receptor | X06388 | 8 | 1.8 ± 0.08 | 0.089 |
| Ecotropic viral integration site-2 | M34896 | 8 | 1.8 ± 0.11 | 0.126 |
| Insulin-like growth factor-1 | X04480 | 4 | 1.7 ± 0.12 | 0.141 |
| Nerve growth factor-β | M17298 | 4 | 1.3 ± 0.11 | 0.166 |
| Defense/immune/Inflammation | | | | |
| Macrophage C-type lectin | AF061272 | 8 | 6.8 ± 0.91 | 0.268 |
| Cathepsin E | AJ009840 | 4 | 3.0 ± 0.41 | 0.273 |
| Cathepsin S | AJ223208 | 8 | 2.0 ± 0.19 | 0.198 |
| P lysosome structural | X61547 | 6 | 2.2 ± 0.60 | 0.543 |
| Matrix metalloproteinase-12 | M20323 | 8 | 1.8 ± 0.20 | 0.218 |
| Cytotoxic T lymphocyte-associated protein-2α | X15959 | 8 | 1.8 ± 0.18 | 0.198 |
| Cytotoxic T lymphocyte-associated protein-2β | X15992 | 8 | 5.2 ± 2.06 | 0.788 |
| Chemokine (C-C) receptor-5 | AV370035 | 6 | 2.4 ± 0.22 | 0.182 |
| Complement component-1qα | X58861 | 8 | 1.7 ± 0.10 | 0.114 |
| Complement component-1qβ | X52521 | 8 | 1.9 ± 0.22 | 0.223 |
| Complement component-1qε | X66295 | 8 | 1.7 ± 0.08 | 0.089 |
| Small inducible cytokine A9 | U49513 | 8 | 1.7 ± 0.19 | 0.223 |
| Small inducible cytokine A27 | AA672499 | 4 | 1.6 ± 0.32 | 0.403 |
| Small inducible cytokine subfamily D1 | U92565 | 5 | 1.4 ± 0.15 | 0.202 |
| Integrin αL | M31039 | 6 | 1.5 ± 0.08 | 0.109 |
| Lysosomal-associated protein transmembrane-5 | AV356071 | 6 | 1.4 ± 0.07 | 0.103 |
| Prostaglandin-endoperoxide synthase-2 | M82442 | 4 | 1.4 ± 0.07 | 0.103 |
| Lipopolysaccharide-binding protein | X99347 | 4 | 1.3 ± 0.15 | 0.224 |
| PAF acetylhydrolase | U34277 | 6 | 1.2 ± 0.07 | 0.113 |
| Signaling | | | | |
| Protein kinase Cθ | AV33804 | 4 | 6.7 ± 1.57 | 0.469 |
| Thyroid-stimulating hormone receptor | U20602 | 4 | 3.3 ± 0.57 | 0.341 |
| Protein kinase Ca | M25811 | 4 | 1.3 ± 0.07 | 0.105 |
| Others | | | | |
| Retinoid-inducible serine carboxypetidase | AV049787 | 6 | 2.3 ± 0.71 | 0.618 |
| Lipoprotein lipase | M63335 | 8 | 2.1 ± 0.33 | 0.311 |
| Neoplastic progression-3 | Z31362 | 8 | 2.1 ± 0.07 | 0.072 |
| Retinoic acid-inducible E3 protein | U28539 | 8 | 1.7 ± 0.06 | 0.067 |
| Plasticin-2L | D37537 | 8 | 1.6 ± 0.16 | 0.200 |
| Adenylsuccinate synthetase-1, muscle | M74495 | 6 | 1.6 ± 0.14 | 0.170 |
| Esterase-10 | AB025480 | 8 | 1.5 ± 0.03 | 0.040 |
| Low density lipoprotein receptor-related protein-2 | AW299788 | 4 | 1.3 ± 0.07 | 0.103 |
| Decreased | | | | |
| Proteasome-1 | X07625 | 8 | −44.2 ± 4.04 | −0.183 |
| Wingless-related MMTV integration site-4 | M98797 | −4 | −2.2 ± 0.20 | −0.182 |
| Prostaglandin D2 synthase (21 kDa, brain) | AB006361 | −4 | −1.8 ± 0.14 | −0.159 |
| Zinc finger protein of cerebellum-2 | D70848 | −6 | −1.7 ± 0.28 | −0.320 |
| T-cell-specific protein | L598444 | −4 | −1.7 ± 0.19 | −0.222 |
| Insulin-like growth factor-2 | X71922 | −8 | −1.6 ± 0.21 | −0.256 |
| Osteoblast-specific factor-2 (fasciclin I-like) | D13664 | −8 | −1.5 ± 0.00 | −0.000 |
| Cellular retinoic acid-binding protein II | M35523 | −6 | −1.5 ± 0.14 | −0.187 |
Nrf2<sup>-/-</sup> astrocytes would be more sensitive to oxidative stress compared with Nrf2<sup>+/+</sup> astrocytes due to reduced levels of detoxification and antioxidant potential. To investigate this differential sensitivity, we pretreated Nrf2<sup>-/-</sup> and Nrf2<sup>+/+</sup> astrocytes with tBHQ (50 μM, 48 h) to increase ARE-driven gene expression and then with H<sub>2</sub>O<sub>2</sub> to investigate differential sensitivity. Also, we used the potent inflammatory agent PAF (46) to investigate the anti-inflammatory effect of Nrf2. As shown in Fig. 4A, vehicle-pretreated Nrf2<sup>-/-</sup> astrocytes were more sensitive to H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity compared with vehicle-pretreated Nrf2<sup>+/+</sup> astrocytes. Furthermore, tBHQ pretreatment significantly increased cell viability in Nrf2<sup>-/-</sup> (but not Nrf2<sup>+/+</sup>) astrocytes (Fig. 4A). Similarly, Nrf2<sup>-/-</sup> and Nrf2<sup>+/+</sup> astrocytes were more sensitive to PAF compared with Nrf2<sup>-/-</sup> astrocytes, and tBHQ pretreatment protected only Nrf2<sup>-/-</sup> astrocytes (Fig. 4B). TUNEL staining and the corresponding phase-contrast microscope pictures confirmed this differential sensitivity. As shown in Fig. 4C, the numbers of TUNEL-positive cells in H<sub>2</sub>O<sub>2</sub>- or PAF-treated Nrf2<sup>-/-</sup> astrocytes were greater than in the corresponding Nrf2<sup>+/+</sup> astrocytes. Although tBHQ did not increase the number of TUNEL-positive cells in Nrf2<sup>-/-</sup> astrocytes, tBHQ pretreatment decreased TUNEL-positive cells in both H<sub>2</sub>O<sub>2</sub>- and PAF-treated Nrf2<sup>-/-</sup> astrocytes (data not shown). Consistent with the TUNEL data, H<sub>2</sub>O<sub>2</sub> and PAF induced more caspase-3 activation in Nrf2<sup>-/-</sup> astrocytes than in Nrf2<sup>+/+</sup> astrocytes (data not shown). These observations suggest that Nrf2<sup>-/-</sup> astrocytes are more sensitive to oxidative stress and inflammation compared with Nrf2<sup>+/+</sup> astrocytes and that coordinate up-regulation of ARE-driven genes by tBHQ further protects Nrf2<sup>-/-</sup> cells from H<sub>2</sub>O<sub>2</sub>- and PAF-induced cytotoxicity.

Identification of the Nrf2-dependent Genes—To identify the Nrf2-dependent genes that play an important role in protecting astrocytes from H<sub>2</sub>O<sub>2</sub>- and PAF-induced apoptosis, we performed oligonucleotide microarray analysis. The genes changed by Nrf2 and/or tBHQ were identified by four comparisons, as depicted in Fig. 5A. tBHQ increased 16 genes (stromal cell-derived factor, Induced in fatty liver dystrophy-2, histones H2B and H2A, histone H1, TG-interacting factor, Thr-1 glycoprotein, Lumican, cysteine- and histidine-rich-1, ectonucleotide pyrophosphatase/phosphodiesterase-2, proteasome 26 S subunit, and six expressed sequence tags) and decreased 27 genes in Nrf2<sup>-/-</sup> astrocytes (comparison I in Fig. 5A), suggesting that the changes in expression of these genes are Nrf2-independent. Genes changed by Nrf2 in the absence of tBHQ (comparison II) are listed in Table I, and genes changed by tBHQ in the presence (comparison III) or absence (comparison I) of Nrf2 are listed in Table II. Interestingly, the majority of the genes increased by tBHQ in Nrf2<sup>-/-</sup> astrocytes (97.6%) were not changed by tBHQ in Nrf2<sup>+/+</sup> astrocytes (Fig. 5B and Table II), suggesting that most of the tBHQ-induced genes are Nrf2-dependent. Only five genes (Induced in fatty liver dystrophy-2, ectonucleotide pyrophosphatase/phosphodiesterase-2, TG-interacting factor, Thr-1 glycoprotein, and expressed sequence tag AW124185) were increased by tBHQ in both Nrf2<sup>-/-</sup> and Nrf2<sup>+/+</sup> astrocytes. The gene list in comparison IV includes most of the genes changed in comparisons I and III. The major functional categories of Nrf2-dependent genes are 1) detoxification, 2) antioxidant/reducing potential, 3) growth, and 4) defense/immune/inflammation (Tables I and II). Clearly, the oligonucleotide microarray data verify that Nrf2 is important for the expression of NQO1 and other ARE-driven genes such as GSTs. Interestingly, cytochrome P450 1B1 was the only member of the cytochrome P450 family that appeared to be Nrf2-dependent in primary astrocytes (Tables I and II). Another evident Nrf2-dependent gene category is antioxidant/ reducing potential. As shown in Tables I and II, many glutathione-related proteins (GCLM, GCLC, GSTs, glutathione reductase, and glutathione peroxidase), antioxidant proteins (TXNRD1, thioredoxin, peroxiredoxin, HO-1, ferritin, catalase, and superoxide dismutase), and genes involved in NADPH production (glucose-6-phosphate dehydrogenase, malic enzyme, transaldolase, and transketolase) were identified as Nrf2-dependent genes. Furthermore, oligonucleotide microarray analysis revealed that many defense/immune/inflammation-related genes (i.e. cathepsin, complements, lipopolysaccharide-binding protein, and PAF acetylhydrolase), metabolic enzymes (i.e. lipoprotein lipase and esterase), growth factors (i.e. platelet-derived growth factor and nerve growth factor), and signaling proteins (i.e. protein kinase C) were regulated in an Nrf2-dependent manner (Tables I and II). Clearly, oligonucleotide microarray data showed that Nrf2-dependent antioxidant and anti-inflammatory genes play an important role in protecting primary astrocytes from the H<sub>2</sub>O<sub>2</sub>- and PAF-induced apoptosis observed in this study.

Verification of Microarray Data—To verify the oligonucleotide microarray data, we performed reverse transcription-PCR and Western blot analysis for selected genes. As shown in Fig. 6 (A and B), the expression levels of the selected genes observed by reverse transcription-PCR were consistent with the oligonucleotide microarray data. Also, Western blot analysis (Fig. 6C) and GSH quantification data (Fig. 6D) showed that Nrf2 plays an important role in both GCLM/GCLC expression and GSH synthesis, as expected from the reverse transcription-PCR and oligonucleotide microarray data.

DISCUSSION

In this study, we demonstrated that the basic leucine zipper transcription factor Nrf2 plays a critical role in both basal and induced gene expression of NQO1 in primary cortical astrocytes. Overexpression of wild-type Nrf2 restored the basal expression and activation of ARE by tBHQ in Nrf2<sup>-/-</sup> cells, and dominant-negative Nrf2 significantly decreased both basal expression and activation of ARE by tBHQ in Nrf2<sup>-/-</sup> astrocytes. The reduced expression and lack of ARE activation in Nrf2<sup>-/-</sup> astrocytes directly correlate with an increased sensitivity to H<sub>2</sub>O<sub>2</sub>- and PAF-induced cytotoxicity compared with Nrf2<sup>+/+</sup> astrocytes. Finally, the genes responsible for conferring protection against oxidative stress or inflammation were identified by oligonucleotide microarray analysis. The major functional categories are detoxification enzymes, antioxidant proteins, NADPH-producing proteins, growth factors, defense/immune/
Identification of inducible Nrf2-dependent genes in primary cortical astrocytes

The genes changed by tBHQ in the presence or absence of Nrf2 were functionally categorized. R, rank; CV, coefficient of variation of fold; NR, not ranked; G6PD, glucose-6-phosphate dehydrogenase; EST, expressed sequence tag.

| Gene Accession no. | Nrf2+/−/vehicle vs. Nrf2−/−/tBHQ R -Fold CV | Nrf2−/−/vehicle vs. Nrf2−/−/tBHQ R -Fold CV |
|-------------------|------------------------------------------|------------------------------------------|
|                  | R | CV |                  | R | CV |
| Detoxification   |   |    |                  |   |    |
| NQO1a             | U12961 8 | 7.2 | 0.23 | NR |
| GST A4a           | L06047 8 | 4.0 | 0.12 | NR |
| GST Pi2           | X53451 8 | 2.3 | 0.07 | NR |
| GST Mu1           | J03952 8 | 1.5 | 0.07 | NR |
| GST Mu2           | J03953 8 | 1.8 | 0.21 | NR |
| GST Omega1        | A1843119 8 | 1.4 | 0.07 | NR |
| GST microsomal-1b | AW124337 6 | 2.0 | 0.39 | NR |
| UDP glycosyltransferase 1A6b | U16818 6 | 1.9 | 0.31 | NR |
| Epoxide hydrolase-1 | U89491 8 | 2.0 | 0.33 | NR |
| Aldehyde dehydrogenase-2 | U07235 8 | 1.4 | 0.08 | NR |
| Aldehyde dehydrogenase-9 | AW120804 4 | 1.2 | 0.13 | NR |
| Aldehyde oxidase-1 | AB017482 8 | 2.6 | 0.15 | NR |
| Cytochrome P450 | X78445 8 | 1.5 | 0.09 | NR |
| Antioxidant/reducing potential |   |    |                  |   |    |
| GCLM              | U95053 8 | 4.9 | 0.09 | NR |
| GCLC              | U85414 8 | 2.2 | 0.16 | NR |
| HO-1 (decycling)c | X56824 8 | 2.7 | 0.11 | NR |
| TXNRD 1           | AB027565 8 | 3.9 | 0.04 | NR |
| Thioredoxin        | X77555 8 | 1.4 | 0.11 | NR |
| Ferritin light chain-1a | L39879 6 | 1.3 | 0.06 | NR |
| Ferritin H subunitc | X52561 6 | 1.3 | 0.12 | NR |
| Type I peroxidoxin | AB023564 8 | 1.6 | 0.09 | NR |
| 1-Cys peroxidoxin protein-2 | AF092853 8 | 1.7 | 0.14 | NR |
| Transferrin receptor | X57349 4 | 1.4 | 0.21 | NR |
| Cu, Zn superoxide dismutasec | M35725 8 | 1.4 | 0.09 | NR |
| Catalase-1         | M29394 8 | 1.9 | 0.21 | NR |
| Glutathione peroxidase-4 | D87896 6 | 1.2 | 0.07 | NR |
| Glutathione reductase-1 | A1851983 8 | 2.6 | 0.32 | NR |
| G-6PD, X-linked    | Z11911 8 | 2.0 | 0.03 | NR |
| G-6PD-2            | Z84471 8 | 2.2 | 0.09 | NR |
| Transaldolase-1    | U67611 8 | 2.9 | 0.09 | NR |
| Transketolase      | U05809 8 | 1.4 | 0.09 | NR |
| Solute carrier family-1/4 | U75215 6 | 1.3 | 0.09 | NR |
| Glycine transporter-1 | X76056 6 | 1.3 | 0.06 | NR |
| Malic enzyme, supernatantc | J02652 8 | 1.7 | 0.20 | NR |
| Transcription      |   |    |                  |   |    |
| CCAAT/enhancer-binding protein-β | M61007 8 | 1.8 | 0.18 | NR |
| Zinc finger protein of cerebellum-2 | D70848 6 | 1.7 | 0.40 | NR |
| TG-interacting factor | X89749 6 | 1.5 | 0.26 | NR |
| MaF6              | AB009693 8 | 1.3 | 0.04 | NR |
| Activating transcription factor-4 | MN4087 6 | 1.4 | 0.20 | NR |
| Growth             |   |    |                  |   |    |
| Proliferin-1       | X16009 8 | 9.4 | 0.61 | NR |
| Proliferin-2       | K03235 8 | 6.8 | 0.64 | NR |
| Nerve growth factor-β | M17298 8 | 1.6 | 0.18 | NR |
| Platelet-derived growth factor-α | M29464 8 | 1.2 | 0.06 | NR |
| Defense/immune/inflammation |   |    |                  |   |    |
| Macrophage C-type lectin | AF061272 8 | 3.5 | 0.25 | NR |
| EST, similar to dihydrothione-inducible-1 | AA596710 8 | 2.9 | 0.12 | NR |
| PAF acetylhydrolase | U34277 8 | 2.7 | 0.10 | NR |
| P lysozyme structural | X51547 8 | 2.2 | 0.17 | NR |
| Lysozyme M        | M21050 8 | 2.1 | 0.06 | NR |
| Prostaglandin-endoperoxide synthase-2 | M88242 8 | 2.0 | 0.07 | NR |
| Matrix metalloproteinase-12 | M82831 6 | 1.5 | 0.04 | NR |
| Signaling          |   |    |                  |   |    |
| Protein kinase, cAMP-dependent regulatory, type 1β | AW125016 4 | 1.9 | 0.07 | NR |
| Mitogen-activated protein kinase-10 | AV280750 8 | 1.4 | 0.17 | NR |
| Others             |   |    |                  |   |    |
| Neoplastic progression-3 | Z31362 8 | 10.1 | 0.12 | NR |
| Esterase-10        | AB025408 8 | 2.4 | 0.03 | NR |
| Arginase-1, liver  | U51805 6 | 2.3 | 0.32 | NR |
| Lipoprotein lipase | M63335 4 | 1.5 | 0.35 | NR |
| Facilitated glucose transporter | M22998 8 | 1.5 | 0.07 | NR |
| Stearoyl-coenzyme A desaturase-1 | M21285 8 | 1.4 | 0.11 | NR |
| Lysosomal acid lipase-1 | Z31689 4 | 1.4 | 0.08 | NR |
| Decreased          |   |    |                  |   |    |
| Interleukin-2 receptor | M26271 -4 | 3.5 | 0.51 | NR |
| α-Fetoprotein      | V00743 -4 | 2.5 | 0.42 | NR |
| Ts-cell-specific GTPase | L38444 -4 | 1.8 | 0.31 | NR |
| Small inducible cytokine A2 | M19681 -6 | 1.8 | 0.37 | NR |
| Myeloid leukemia factor-1 | AF100171 -6 | 1.6 | 0.13 | NR |
Identification of Nrf2-dependent ARE-driven Genes

### TABLE II—continued

| Gene                        | Accession no. | Nrf2+/+ vs. Nrf2−/− R (Fold) CV | Nrf2+/+ vs. ABHq R (Fold) CV |
|-----------------------------|---------------|---------------------------------|------------------------------|
| Tektin-1                    | AF681947      | -4                              | -1.6                         |
| μ-Crystallin                | AF029391      | -5                              | -1.5                         |
| Elastin                     | J058210       | -6                              | -1.5                         |
| Kinase insert domain protein receptor | XT0842 | -6                              | -1.4                         |
| Ribonuclease reductase M1    | AT29111      | -4                              | -1.5                         |
| Cholesterol 25-hydroxylase   | AF059213      | -4                              | -1.5                         |
| Vascular cell adhesion molecule-1 | U13284 | -6                              | -1.4                         |
| Spot-14                     | X95279        | -6                              | -1.4                         |
| WD repeat domain 6          | AW050287      | -6                              | -1.4                         |
| Hemopoitic cell kinase      | X03023        | -6                              | -1.4                         |
| Tropomyosin-2β              | M81068        | -4                              | -1.4                         |

* Known to contain or to potentially have an ARE sequence.

Inflammation-related proteins, and signaling proteins. Proteins within these functional categories are vital to the maintenance and responsiveness of a cell’s defense system, suggesting that an orchestrated change in expression via Nrf2 and the ARE would give a synergistic protective effect.

Recently, Kwak et al. (49) observed 3H-1,2-dithiole-3-thione-increased Nrf2 gene expression and demonstrated that Nrf2 autoregulates its own expression through an ARE-like element. In the present study, tBHQ did not increase Nrf2 expression levels, but induced nuclear translocation of Nrf2 (data not shown), suggesting that ARE activation by tBHQ is mediated by nuclear translocation of Nrf2, not by induction of Nrf2 gene expression in primary astrocytes. tBHQ did, however, increase expression of binding partners of Nrf2 (i.e. Mad3 and activating transcription factor-4) in Nrf2+/− astrocytes (Table II). Maf proteins have been shown to regulate ARE activation negatively or positively depending on cell types and genes (27, 28, 50, 51), and activating transcription factor-4 has been demonstrated to bind to Nrf2, leading to HO-1 gene expression (21). In addition, CCAAT/enhancer-binding protein-β was increased by Nrf2 and tBHQ in Nrf2+/− astrocytes. CCAAT/enhancer-binding protein-β has been shown to mediate negative regulation of rat GST-Ya expression (52). Finally, in contrast to the increased expression of KIAA0132 (human homolog of Keap1) by tBHQ in IMR-32 cells (32), Keap1 was not changed by either Nrf2 or tBHQ in mouse primary astrocytes (Tables I and II). These observations suggest a possible balancing mechanism between positive and negative regulation in Nrf2-mediated gene expression and that the role and regulation of other binding partners of Nrf2 are dependent on the cell type and/or genes being studied.

A recent study reported Nrf2-regulated genes induced by sulforaphane in the small intestine (53). Interestingly, only nine genes were commonly increased by sulforaphane (small intestine) (53) and by tBHQ (primary astrocytes) (this study) in Nrf2+/− cells (NQO1, epoxide hydrolase, GST A4, GST Mu1, GST Mu3, transaldosase, transketolase, GCLM, and GCLC). In the small intestine, genes coding GSTs and drug-metabolizing enzymes were induced by sulforaphane (53). In primary astrocytes, however, tBHQ increased the expression of many antioxidant and anti-inflammatory genes (i.e. HO-1, TXXNR1D1, thioredoxin, ferritin, peroxiredoxin, glucose-6-phosphate dehydrogenase, superoxide dismutase, catalase, malic enzyme, and PAF acetylhydrolase), suggesting cell type-specific gene expression.

The function of a number of Nrf2-dependent genes is dependent on GSH. GSTs catalyze the nucleophilic addition of GSH to an electrophilic group of a broad spectrum of xenobiotic compounds (54). Other GSH-dependent enzymes (i.e. glutathione peroxidase, peroxiredoxin, and glutathione reductase) were also increased in an Nrf2-dependent manner. Glutathione peroxide and peroxiredoxin metabolize H2O2, generating H2O and oxidized GSH (GSSG), and glutathione reductase regenerates reduced GSH. Ideally, in association with an increased utilization of GSH, there would also be an increased production of GSH. The rate-limiting step in the GSH biosynthesis is mediated by GCLM/GCLC. In this study, solute carrier family-1, glycine transporter, GCLM, and GCLC were shown to be Nrf2-dependent genes. The coordinate regulation of these genes can have a synergistic effect in the maintenance of GSH levels as well as detoxification of reactive intermediates (Fig. 7A).

Another cluster of genes including superoxide dismutase and HO-1 are very important for cellular defense against oxidative stress. Superoxide dismutase detoxifies superoxide, resulting in H2O2, and HO-1 generates a potent radical scavenger, bilirubin. However, superoxide dismutase and HO-1 can induce more oxidative stress because they increase the cellular concentrations of H2O2 and free iron, which together can generate hydroxyl radical through the Fenton reaction. For complete detoxification of superoxide, H2O2 should be further metabolized to H2O by glutathione peroxidase, catalase, or peroxiredoxin. Catalase directly detoxifies H2O2, whereas peroxiredoxin uses GSH (Fig. 7A) and/or thioredoxin as an electron donor for peroxidation of H2O2, resulting in generation of GSSG and oxidized thioredoxin, respectively (Fig. 7B). GSSG and oxidized thioredoxin are converted to their reduced forms by glutathione reductase and TXNRD1. Oligonucleotide microarray data showed that superoxide dismutase, catalase, peroxiredoxin, thioredoxin, and TXNRD1 are transcriptionally regulated through an Nrf2-dependent mechanism. In addition, proper management of free iron is also important for minimizing oxidative stress, and this can be best achieved by ferritin. Ferritin converts Fe2+ to Fe3+ (ferroxidase activity) and sequesters it, thereby preventing Fe3+ from participating in the Fenton reaction. Thus, up-regulation of HO-1 together with ferritin is a way to increase antioxidant potential while minimizing hydroxyl radical formation. Based on these observations, we speculate that increased expression of these genes can dramatically increase the efficiency of detoxification of reactive oxygen species. Also, the genes depicted in Fig. 7B provide a molecular mechanism by which tBHQ-treated Nrf2+/− astrocytes are resistant to H2O2-induced apoptosis.

Finally, NQO1, glutathione reductase, and TXNRD1 are important in detoxifying quinones and maintaining the cellular redox balance. One common feature of these proteins is that they use NADPH as an electron donor. So, for efficient detoxification and maintenance of cellular redox status, it would be beneficial to up-regulate these proteins together with the appropriate reducing potential (NADPH) to support enzymatic reactions. Glucose-6-phosphate dehydrogenase/malic enzyme can directly generate NADPH, and transketolase/transaldolase...
can increase NADPH production by regenerating substrates for glucose-6-phosphate dehydrogenase. Oligonucleotide microarray data showed that NQO1, glutathione reductase, TXNRD1, glucose-6-phosphate dehydrogenase, malic enzyme, transketolase, and transaldolase are Nrf2-dependent genes (Fig. 7). These Nrf2-dependent genes would also contribute significantly to a cell’s detoxification potential and cellular redox balance. Together, these coordinately regulated gene clusters presented in Fig. 7 strongly support the hypothesis that Nrf2-dependent gene expression is central to efficient detoxification of reactive metabolites and reactive oxygen species as well as a cell’s ability to deal with stress such as inflammation.

Can these changes in astrocytes protect neurons from oxidative stress-induced apoptosis? Astrocytes have been suggested to interact with neurons and to confer neuronal protection. It has been demonstrated in numerous neuronal culture systems that the survival of neurons is significantly enhanced by astrocytes (55–57). They can promote neuronal survival by removing excitotoxins (i.e. glutamate) from the synapse, modulating antioxidant levels (i.e. GSH), and secreting trophic factors (i.e. glial-derived neurotrophic factor) (58–60). In support of this idea, Nrf2-dependent detoxification and antioxidant proteins in astrocytes can play a role in protecting neurons. However, genetic changes in neurons associated with increased expression of ARE-driven genes in astrocytes could also contribute to an overall protective mechanism. The extent to which this
intercellular communication is required and the specific genetic remodeling in the neurons versus the astrocytes in a co-culture system remain to be determined. Preliminary data from our laboratory suggest that there are unique changes in both astrocytes and neurons that, when combined, may be responsible for protecting neurons from oxidative stress.3

In summary, oxidative stress and reactive metabolites can induce apoptosis or programmed cell death. Programmed cell death can be prevented in many ways, such as addition of external growth factors, antioxidant supplementation, and inhibition of apoptotic signaling pathways. Here we present an alternative in that the coordinate up-regulation of Nrf2-dependent genes provides a way to protect cells through genetic remodeling, a process referred to as programmed cell life. We hypothesize that increased activation of programmed cell pathways can balance programmed cell death and that, in combination with other techniques known to prevent programmed cell death, may be a powerful tool in controlling progressive neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis. Currently, we are focused on evaluating the neuroprotective role of Nrf2-dependent genes in vivo by crossing Nrf2 knockout mice with established transgenic models representing human neurological disorders.

Acknowledgments—We thank Matthew Slattery and the Molecular Biology Core Facility of the University of Wisconsin Environmental Health Science Center for conducting the gene array hybridizations and Dr. Terrance Kavanagh for providing anti-GCLM and anti-GCLC antibodies. We also thank Delinda Johnson, Jiang Li, Thor Stein, and Andrew Kraft for helpful suggestions.

REFERENCES

1. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) J. Biol. Chem. 266, 11632–11639
2. Lee, J.-M., Moehlenkamp, J. D., Hanson, J. M., and Johnson, J. A. (2001) Biochem. Biophys. Res. Commun. 286, 286–292
3. Lee, J.-M., Hanson, J. M., Chu, W. A., and Johnson, J. A. (2001) J. Biol. Chem. 276, 20011–20016
4. Moehlenkamp, J. D., and Johnson, J. A. (1999) Arch. Biochem. Biophys. 363, 98–106
5. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burrow, M. E., and Tou, J. (2000) J. Biol. Chem. 275, 27694–27702
6. Favreau, L. V., and Pickett, C. B. (1995) J. Biol. Chem. 270, 24468–24474
7. Katayama, N., Masutani, H., Yamaguchi, Y., Itoh, K., Yamamoto, M., and Yodoi, J. (2001) J. Biol. Chem. 276, 18389–18406
8. Mulchay, R. T., Wartman, M. A., Bailey, H. H., and Gipp, J. J. (1997) J. Biol. Chem. 272, 7445–7454
9. Nguyen, T., and Pickett, C. B. (1999) J. Biol. Chem. 267, 15355–15359
10. Orino, K., Lehman, L., Tsuji, Y., Ayaki, H., Torti, S. V., and Torti, F. M. (1991) Biochem. J. 267, 241–247
11. Rushmore, T. H., King, R. G., Paulson, K. E., and Pickett, C. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3826–3830
12. Venugopal, R., and Jaiswal, A. K. (1998) Oncogene 17, 3145–3156
13. Boes, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9926–9930
14. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Genes Dev. 13, 76–86
15. Itoh, K., Ishii, T., Wakabayashi, N., and Yamamoto, M. (1999) Free Radic. Res. 31, 319–324
16. Bloom, D., Dhakshinamoorthy, S., and Jaiswal, A. K. (2002) Oncogene 21, 2191–2200
17. Dhakshinamoorthy, S., and Jaiswal, A. K. (2001) Oncogene 20, 3906–3917
18. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Saito, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) Biochem. Biophys. Res. Commun. 236, 313–322
19. Chen, Y. H., and Ramos, K. S. (2000) J. Biol. Chem. 275, 27366–27376
20. Chen, Y. H., and Ramos, K. S. (2000) J. Biol. Chem. 275, 27366–27376
21. Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kessler, T. W., Yamamoto, M., and Biswal, S. (2002) Cancer Res. 62, 5186–5203
22. Dukarcich, B., Schepens, E., Stoff, J., C. Lancevedel, H. C., and Van Muuswinkel, P. L. (1998) Free Radic. Biol. Med. 25, 217–220
23. Tanaka, J., Toku, K., Zhang, B., Ishihara, K., Sakanaka, M., and Maeda, N. (1999) J. Neurosci. 24, 817–826
24. Aschner, M. (2000) Neurotoxicology 21, 1101–1107
25. Mount, H. T., Dean, D. O., Alberich, J., Dreyfus, C. P., and Black, I. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9092–9096
26. Sonnewald, U., Qu, H., and Aschner, M. (2002) J. Pharmacol. Exp. Ther. 301, 1–6

2 J.-M. Lee and J. A. Johnson, unpublished data.