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Identification of Nrf2-regulated Genes Induced by the Chemopreventive Agent Sulforaphane by Oligonucleotide Microarray[1]

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ABSTRACT

Electrophiles formed during metabolic activation of chemical carcinogens and reactive oxygen species generated from endogenous and exogenous sources play a significant role in carcinogenesis. Cancer chemoprevention by induction of phase 2 proteins to counteract the insults of these reactive intermediates has gained considerable attention. Nuclear factor E2 p45-related factor 2 (Nrf2), a bZIP transcription factor, plays a central role in the regulation (basal and or inducible expression) of phase 2 genes by binding to the "antioxidant response element" in their promoters. Identification of novel Nrf2-regulated genes is likely to provide insight into cellular defense systems against the toxicities of electrophiles and oxidants and may define effective targets for achieving cancer chemoprevention. Sulforaphane is a promising chemopreventive agent that exerts its effect by strong induction of phase 2 enzymes via activation of Nrf2. In the present study, a transcriptional profile of small intestine of wild-type (*nrf2* +/+) and knock out (*nrf2* −/−) mice treated with vehicle or sulforaphane (9 μmol/day for 1 week, p.o.) was generated using the Murine Genome U74Av2 oligonucleotide array (representing ~6000 well-characterized genes and nearly 6000 expressed sequence tags). Comparative analysis of gene expression changes between different treatment groups of wild-type and *nrf2*-deficient mice facilitated identification of numerous genes regulated by Nrf2 including previously reported Nrf2-regulated genes such as *NAD(P)H:quinone reductase* (*NQO1*), *glutathione S-transferase* (*GST*), *γ-glutamylcysteine synthetase* (*GCS*), *UDP-glucuronosyltransferases* (*UGT*), *epoxide hydrolase,* as well as a number of new genes. Also identified were genes encoding for cellular NADPH regenerating enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme), various xenobiotic metabolizing enzymes, antioxidants (glutathione peroxidase, glutathione reductase, ferritin, and haptaglobin), and biosynthetic enzymes of the glutathione and glucuronidation conjugation pathways. The data were validated by Northern blot analysis and enzyme assays of selected genes. This investigation expands the horizon of Nrf2-regulated genes, highlights the cross-talk between various metabolic pathways, and divulges the pivotal role played by Nrf2 in regulating cellular defenses against carcinogens and other toxins.

INTRODUCTION

There is considerable epidemiological evidence suggesting an association of consumption of fruits and vegetables with reduced incidence of cancer, especially in the gastrointestinal tract (1). Induction of phase 2 enzymes by phytochemicals represents an important mechanism for achieving protection against cancer (2). Although phase 2 enzymes have been traditionally recognized as those catalyzing the conjugation of endogenous ligands, glutathione and glucuronic acid, to endo- and xenobiotic substrates, this classification is expanding to include proteins that catalyze a wide variety of reactions that confer cytoprotection against the toxicity of electrophiles and reactive oxygen species (3). The growing list of phase 2 proteins includes NAD(P)H:quinone reductase (NQO1), epoxide hydrolase, dihydrodiol dehydrogenase, γ-GCS,[3] heme oxygenase-1, leukotriene B_4 dehydrogenase, aflatoxin B_1 dehydrogenase, and ferritin (3). These proteins, which enhance resistance to different toxicants, are regulated by a common element (5′-$A^{/G}$ TGA $C^{/T}$ NNN GC $A^{/G}$-3′) in their promoter region that is termed the ARE (4). The mechanisms that regulate the expression of phase 2 genes through the ARE are the subject of intensive investigation (5).

It has been demonstrated clearly that Nrf2, a bZIP transcription factor, translocates into the nucleus and binds to the ARE in conjugation with small Maf proteins after activation by chemopreventive agents and that Nrf2 plays a central role in the constitutive and inducible expression of several phase 2 proteins (6–11). Higher sensitivity of *nrf2*-deficient mice to liver damage by acetaminophen (12), pulmonary injury by butylated hydroxytoluene (13), increased DNA adducts in lungs after exposure to diesel exhaust (14), and gastric tumors by benzo(*a*)pyrene (7) have been attributed to decreased basal expression of phase 2 enzymes in various organs (8, 10, 15). The induction of several phase 2 genes (*e.g., GST* and *NQO1*) by butylated hydroxyanisole, oltipraz, and sulforaphane is also dependent on Nrf2 (7, 10). Collectively, these studies clearly indicate the pivotal role of this transcription factor in the regulation of phase 2 proteins and, thus, chemoprevention against xenobiotic toxicities.

Molecular epidemiological studies have indicated that consumption of dietary isothiocyanates are effective in decreasing the relative risk of colorectal cancer (16) and lung cancer (17, 18). Among the isothiocyanates, sulforaphane (*R*-1-isothiocyanato-4-methylsulfinylbutane) is the most potent inducer of phase 2 proteins (19–21) and functions to activate Nrf2 (10, 11). Sulforaphane effectively reduces colonic aberrant crypt foci formation in carcinogen-treated rats (22). Identification of cytoprotective genes, which are directly or indirectly dependent on Nrf2 for transcriptional activation in response to promising chemopreventive agents such as sulforaphane, will facilitate the understanding of molecular downstream effectors of chemoprevention. By feeding sulforaphane p.o. to wild type and *nrf2*-deficient mice, we have identified several genes in a target organ, the small intestine, using oligonucleotide arrays that are dependent on Nrf2 for their basal and/or inducible expression. This study expands the horizon of Nrf2-regulated protective proteins and identifies novel downstream mediators for chemoprevention by sulforaphane and, presumably, other classes of enzyme inducers.

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[3] The abbreviations used are: γ-GCS, γ-glutamylcysteine synthetase; γ-GCS(h), heavy catalytic subunit of γ-GCS; EST, expressed sequence tag; ALDH, aldehyde dehydrogenase; ARE, antioxidant response element; HSP, heat shock protein; G6PDH, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; NQO1, NAD(P)H:quinone reductase; Nrf2, nuclear factor E2 p45-related factor 2; 6PGDH, 6-phsophogluconate dehydrogenase; UGT, UDP-glucuronosyltransferase.

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MATERIALS AND METHODS

Reagents. L-Sulforaphane was purchased from LKT Laboratories (St. Paul, MN). A nucleotide removal kit was procured from Qiagen (Valencia, CA). NADPH, malate, glucose-6-phosphate, 1-chloro-2,4-dinitrobenzene, *p*-nitrophenylacetate, NAD^+, and menadione were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts, set forth in NIH guidelines and the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee. *Nrf2*-deficient ICR mice were generated as described (8). Genotypes of homozygous wild-type and *nrf2*-deficient mice (10 weeks of age) were confirmed by PCR amplification of genomic DNA extracted from blood or liver. PCR amplification was carried out by using three different primers, 5′-TGGACGGGACTATTGAAGGCTG-3′ (sense for both genotypes), 5′-CGCCTTTTCAGTAGATGGAGG-3′ (antisense for wild type), and 5′-GCGGATTGACCGTAATGGGATAGG-3′ (antisense for LacZ), as described previously (7).

Female mice [wild-type *nrf2* (+/+) and *nrf2* (−/−) deficient], 10 weeks of age, were maintained on an AIN 76A diet and water *ad libitum* and housed at a temperature range of 20–23°C under 12-h light/dark cycles. The mice were grouped into four groups ($n = 3$): I, control (*nrf2* +/+) wild type; II, treatment (*nrf2* +/+) wild type; III, control knock out (*nrf2* −/−); and IV, treatment knock out (*nrf2* −/−). The control and treatment groups were administered either vehicle (corn oil) alone or sulforaphane (9 μmol/mouse/day) by gavage (0.2 ml), respectively, for 7 consecutive days. Body weights were recorded to monitor the health of animals. Animals were sacrificed by cervical dislocation 24 h after the last dose. The small intestine was removed and washed thoroughly with ice-cold PBS to remove the fecal material and frozen in liquid nitrogen before storage at −80°C until further use.

Northern Blotting. Total RNA from the intestine was extracted with TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. Total RNA (10 μg) was separated on 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Nytran Super Charge; Schleicher and Schuell, Dassel, Germany), and UV-cross-linked. Probes for NAD(P)H:quinone oxidoreductase (NQO1), GST Ya, γ-GCS (regulatory subunit), UDP-glucuronosyltransferases (UGT1A6), malic enzyme, glutathione reductase, and β-actin were generated by PCR from the cDNA of murine liver. These PCR products were radiolabeled with [α-^{32}P]dCTP using a random primers DNA labeling kit (Invitrogen, San Diego, CA). Northern hybridization was done using QuickHyb (Stratagene, Carlsbad, CA) as per the manufacturer's protocol. After hybridization, the membranes were washed twice in 0.2% SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS at room temperature for 15 min and finally in 0.1% SSC/0.1% SDS at 60°C for 45 min. The membranes were exposed to a phosphorimager screen, and radioactivity was visualized and quantified with a BAS1000 Bioimaging system (Fuji Photo Film, Tokyo, Japan). Levels of RNA were quantified and normalized for RNA loading by stripping and reprobing the blots with a probe for β-actin.

Transcriptional Profiling by Oligonucleotide Microarray. Total RNA was purified with the RNeasy Mini kit (Qiagen, Valencia, CA) after isolation with TRIzol reagent as described above and was used for experiments with Murine Genome U74A version 2 GeneChip arrays (Affymetrix, Santa Clara, CA), which contain probes for detecting ~6000 well-characterized genes and 6000 expressed sequence tags (ESTs). Briefly, double-stranded cDNA was synthesized from 15 μg of total RNA with SuperScript Choice System (Invitrogen) by using oligo$(dT)_{24}$ primers with a T7 RNA polymerase promoter site added to its 3′ end (Genset Corp., La Jolla, CA). The isolated cDNA was then labeled to generate biotinylated cRNA *in vitro* and amplified using the BioArray T7 RNA polymerase labeling kit (Enzo, Farmingdale, NY). After purification of the cRNA by RNeasy Mini kit, 20 μg of cRNA were fragmented at 94°C for 35 min. Approximately 12.5 μg of fragmented cRNA was used in a 250-μl hybridization mixture containing herring sperm DNA (0.1 mg/ml; Promega Corp., Madison, WI), plus bacterial and phage cRNA controls (1.5 pmol of BioB, 5 pmol of BioC, 25 pmol of BioD, and 100 pmol of Cre) to serve as internal controls for hybridization efficiency as directed by the manufacturer (Affymetrix). Aliquots (200 μl) of the mixture were hybridized onto the array for 18 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix). Each array was washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and amplified with biotinylated anti-streptavidin antibody (Vector Laboratory, Burlingame, CA) on the GeneChip Fluidics Station 400 (Affymetrix). Each array was scanned with the GeneArray scanner (Agilent Technologies, Palo Alto, CA) to obtain image and signal intensities.

Data Analysis Using Affymetrix Software. Scanned output files were analyzed with the Affymetrix Microarray Suite 5.0 and normalized to an average intensity of 500 independently, before comparison. To identify differentially expressed transcripts, pairwise comparison analysis were carried out with Data Mining Tool 3.0 (Affymetrix). The analysis compares the differences in values of perfect match to mismatch of each probe pair in the baseline array to its matching probe pair on the experimental array. *P*s were determined by the Wilcoxon's signed rank test and denoted as increase, decrease, or no change. Analysis using Data Mining Tool also provides the signal log ratio, which estimates the magnitude and direction of change of a transcript when two arrays are compared (experimental *versus* baseline). We have converted the signal log ratio output into fold change for convenience using the formula recommended by Affymetrix:

$$\text{Fold change} = \begin{cases} 2^{\text{Signal Log Ratio}}, \text{Signal Log Ratio} > 0 \\ (-)\,2^{-(\text{Signal Log Ratio})}, \text{Signal Log Ratio} < 0 \end{cases}$$

In the present study, we performed nine pairwise comparisons for each group (experimental, $n = 3$ *versus* baseline, $n = 3$). Only those altered genes that appeared in at least seven of the nine comparisons were selected. This conservative analytical approach was used to limit the number of false positives. In addition, we also performed a Mann-Whitney pairwise comparison test in Data Mining Tool to rank the results by concordance as a calculation of significance (*P*) of each identified change in gene expression. The ESTs obtained in the data were searched for their recent annotation using the "Analysis Center" at the Affymetrix site (www.netaffx.com).

Enzyme Activity Assays. Total GST activity was measured in cytosolic fractions (100,000 × *g*) in the presence of 0.1% BSA with 1-chloro-2,4-dinitrobenzene as a substrate (23), whereas NQO1 activity was determined using menadione as substrate (24). Activity of G6PDH was determined from the rate of glucose 6-phosphate-dependent reduction of $NADP^+$ (25). Malic enzyme activity was measured from the rate of malate-dependent $NADP^+$ reduction (26). Carboxylesterase activity was determined by measuring the hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol at 410 nm (27). UDP-glucose dehydrogenase activity was measured by the reduction of NAD^+ in the presence of UDP-glucose at 340 nm (28). Protein concentration was determined by using the Bio-Rad DC reagent and BSA as the standard.

Statistics. The values for enzyme-specific activities are mean ± SE, and the Student *t* test was used to analyze the statistical significance.

RESULTS

Microarray Analysis. The transcriptional profile of a target organ, the small intestine of *Nrf2* wild-type and -deficient mice with or without sulforaphane treatment was generated using the Murine Genome U74Av2 chip. The obtained transcriptional profile was logically analyzed to derive the set of genes regulated by Nrf2. We performed three categories of comparisons (Fig. 1) to accomplish the objective. Because Nrf2 is well established in the positive regulation of genes (15), we have concentrated only on up-regulated genes.[4] The gene profile that emerged after comparative analysis was further filtered. The genes that appeared in at least seven of the nine comparisons that showed fold changes >1.5 and had *P*s of ≤0.05 were selected. These cutoff values gave a conservative estimate of the number of genes in each category, and only those genes complying with the criteria were considered further.

Wild-Type Control and *Nrf2*-deficient Control. In this category, the altered gene profile reflects the effect of disruption of Nrf2. Overall, there were 45 genes and 27 ESTs with higher expression in

[4] The comprehensive lists of all up-regulated and down-regulated genes in the comparisons are reported in http://commprojects.jhsph.edu/ehs/biswal_data.xls.

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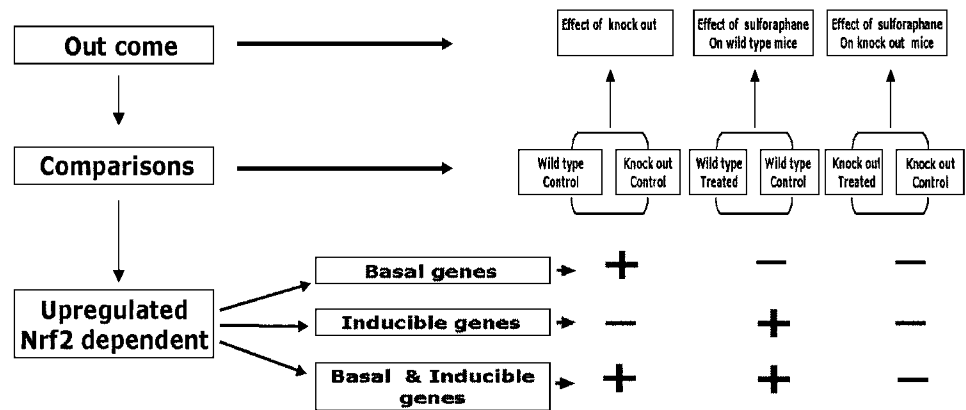

Fig. 1. Schematic depicting the strategy for comparison and analysis of transcriptional profiles obtained from microarray using the Affymetrix Murine Genome U74A version 2 arrays that contain probes for detecting ~6000 well-characterized genes and 6000 expressed sequence tags. *Wild type, nrf2* +/+ mice; *knock out, nrf2* −/− mice.

the wild-type mice. Prominent genes showing large fold changes were *carboxyl esterase* (15-fold), *epoxide hydrolase* (12-fold), various GSTs (3–6-fold), *malic enzyme* (3.5-fold), *UGT* (8-fold), *aldehyde dehydrogenase II* (4.6-fold), and *NQO1* (3-fold).

Wild-Type Treated and Wild-Type Control. The altered transcriptional profile in this comparison group is the result of treatment with sulforaphane. Thirty-three genes and 17 ESTs were induced by sulforaphane. The most responsive genes in this class of comparison were *GSTs* (2.5–6-fold), catalytic subunit of *GCS* (4-fold), *fibroblast growth factor related protein* (3-fold), *carboxyl esterase* (4-fold), and *NQO1* (2.5-fold).

***Nrf2*-deficient Treated and *Nrf2*-deficient Control.** This profile also includes genes induced by sulforaphane; however, Nrf2 does not regulate them. There were 59 genes and 62 ESTs, of which the most responsive genes were histone genes (*H2B* and *H2A*; 5-fold) and mouse immunoglobulin-active λ-1-chain V-region (*V-J*) gene (2.5-fold).

Nrf2-dependent Genes. To identify the gene targets of Nrf2 based on the transcriptional pattern of gene expression, we adopted a specific strategy of analysis that is depicted in Fig. 1. Our analysis revealed 77 up-regulated genes whose expression was influenced by Nrf2 (Table 1). They can be classified into three different categories based on their dependence on Nrf2. For basal expression, the expressions of this class of genes were elevated in wild-type control mice compared with *nrf2*-deficient mice and were not effected by sulforaphane in either genotype, suggesting that these genes require Nrf2 for their basal but not inducible expression. For inducible expression, this subset of genes was elevated in the sulforaphane-treated, wild-type group relative to vehicle-treated, wild-type and *nrf2*-deficient mice and were not induced in sulforaphane-treated, *nrf2*-deficient mice, demonstrating their dependence on Nrf2 for induction. For basal and inducible expression, this class of genes was elevated in vehicle-treated, wild-type mice when compared with the *nrf2*-deficient mice and selectively induced in response to sulforaphane in the wild-type mice only. Thus, these genes are dependent on Nrf2 for basal as well as inducible expression. All genes belonging to these three groups are presented in Table 1, together with fold change and mode of dependence on Nrf2. Genes elevated in the *nrf2*-deficient, treated and *nrf2*-deficient, control comparison are considered to be Nrf2-independent genes and are included as supplementary data at the web site.

We have further categorized the Nrf2 up-regulated genes based on their functions (Table 1). The majority of the up-regulated genes at the inducible and/or basal level are associated with various metabolic reactions (hydrolysis, reduction, oxidation, and conjugation with endogenous ligands: glutathione and glucuronide) involved in detoxication of electrophiles and free radicals. Antioxidative genes such as *glutathione peroxidase*, *glutathione reductase*, *ferritin*, and *haptaglobin* and the genes encoding NADPH-generating enzymes, *i.e., glucose 6-phosphate dehydrogenase*, *malic enzyme*, and *6-phosphogluconate dehydrogenase* were also dependent on Nrf2 for expression.

Validation of Microarray Data by Northern Blot. Four well-characterized Nrf2 regulated genes, *NQO1, GST Ya, γ-GCS(h),* and *UGT 1A6* and two new genes identified by this screening, *malic enzyme* and *glutathione reductase*, were selected for verification of the transcriptional changes using Northern hybridization. The fold changes [NQO1, 2.5-fold; GST Ya, 2 fold; γ-GCS(h), 3-fold; UGT 1A6, 1.4-fold; malic enzyme, 1.8-fold; and glutathione reductase, 2-fold] in sulforaphane-treated, wild-type mice compared with vehicle-treated, wild-type mice are in close agreement with the oligonucleotide array data (Fig. 2*A*). Furthermore, lower expression of these genes in *nrf2*-deficient mice compared with wild-type mice is consistent with the transcriptional changes observed by microarray.

Enzyme Assays of Selected Genes. Six genes were selected for enzymatic assays (Figs. 2*B* and 3). NQO1 and GST enzyme activities were induced 1.6- and 1.3-fold, respectively, by treatment of wild-type mice with sulforaphane. Moreover, their basal activities were significantly lower in the untreated *nrf2*-deficient mice when compared with the untreated wild-type mice. Malic enzyme and glucose 6-phosphate dehydrogenase activities were induced to 1.8- and 10.3-fold, respectively, and there were significant differences in basal activities between the wild-type and knock-out mice. There was 1.3-fold difference in the basal activity of UDP-glucose dehydrogenase between wild-type and *nrf2*-deficient mice, and significant induction was seen with sulforaphane treatment in wild-type mice. Carboxylesterase activity showed a modest induction of 1.3-fold in response to sulforaphane only in wild-type mice (Fig. 3). In general, the fold increases in enzyme activities were lower than those observed for the increases in transcript levels.

DISCUSSION

Previous studies using biochemical and Northern blot analyses in wild-type and *nrf2*-deficient mice have demonstrated that Nrf2 controls the constitutive expression of antioxidative enzymes such as the light regulatory subunit of γ-glutamylcysteine synthetase, heme oxygenase 1, and peroxiredoxin MSP23 (29) and also mediates induction of hepatic and intestinal NQO1 and GST enzymes by butylated hydroxytoluene, oltipraz, and sulforaphane (8, 11). In the present investigation, we have compared the transcriptional profile of small intestine of *nrf2* wild-type and *nrf2*-deficient mice with and without sulforaphane treatment, a potent chemopreventive agent that activates the Nrf2 pathway (30), and identified several new target genes that are positively regulated at the basal and/or inducible level by Nrf2. Interestingly, most of the proteins that are positively regulated through

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Table 1 *Nrf2 up-regulated genes obtained from the screening*

Group/Class and accession no.	Gene	Nrf2 regulated expression[a]	Fold change ± SE
Hydrolysis			
Y12887	*Carboxyl esterase*	B & I	15.2 ± 3.7 and 4 ± 0.3
U89491	*Epoxide hydrolase*[b]	B & I	11.8 ± 1.6 and 1.6 ± 0.1
M29961	*γ-Glutamyl peptidase*	I	1.7 ± 0.15
Reduction			
U31966	*Carbonyl reductase*	B	1.9 ± 0.1
AI840094	*Aflatoxin aldehyde reductase*[c]	B	1.62 ± 0.1
U12961	*NQO1*[b]	B & I	3.7 ± 0.2 and 2.4 ± 0.2
U04204	*Aldose reductase (fibroblast growth factor regulated protein)*	B & I	2.9 ± 0.2 and 3.1 ± 0.2
AB027125	*Aldo-keto reductase*	I	1.5 ± 0.1
Oxidation			
U96401	*Aldehyde dehydrogenase*[d]	B	2.4 ± 0.2
U07235	*Aldehyde dehydrogenase*	B	1.7 ± 0.1
AI848045	*Monoamine oxidase*[c]	B	1.7 ± 0.1
AI197481	*Amino oxidase*[c]	B	1.6 ± 0.1
AA596710	*NADP-dependent leukotreine B4 12-hydroxydehydrogenase*[c]	B & I	1.7 ± 0.1 and 2.5 ± 0.1
Glucuronidation pathway			
XO6358	*UDP-glucuronosyl-transferase 2 family*[b]	B	8.0 ± 0.7
AF061017	*UDP-glucose dehydrogenase*	B	1.5 ± 0.1
Glutathione transferases			
AW124337	*Rat glutathione S-transferase*[b,c]	B	1.9 ± 0.1
J04696	*GST class mu (GST5-5)*[b]	B	1.9 ± 0.1
AI326397	*GST M2* (muscle)[b,c]	B	1.8 ± 0.1
AI843448	*Microsomal GST 3*[b,c]	B	1.7 ± 0.1
J03952	*GST GT8.7*[b]	B & I	2.7 ± 0.2 and 4.6 ± 0.2
J03953	*GST GT9.3*[b]	B & I	6.1 ± 0.4 and 2.4 ± 0.1
AI841270	*GST mu 1*	B & I	4.7 ± 0.3 and 2.8 ± 0.2
X65021	*GST α3*[b]	I	4.9 ± 0.6
JO3958	*GST α2* (Yc2)[b]	I	1.6 ± 0.5
L06047	*GST α1* (Ya)[b]	I	1.8 ± 0.1
AA919832	*Microsomal GST 2*[b,c]	I	1.8 ± 0.1
Glutathione synthesis			
U95053	*GCS, regulatory*[b]	I	2.4 ± 0.2
U85414	*GCS, catalytic*[b]	I	4.1 ± 0.4
Antioxidants			
X61399	*Glutathione peroxidase*[c]	B	1.7 ± 0.1
AV097950	*Ferritin*[c,d]	B	1.6 ± 0.1
AI841295	*Haptaglobin*[c]	B	1.6 ± 0.1
AI851983	*GR*[c]	I	1.7 ± 0.1
Protective proteins			
AA833514	*Multidrug resistance protein*[c]	B	1.6 ± 0.1
AW120711	*HSP 40 (DnaJ)*[c]	I	1.8 ± 0.1
NADPH regenerating enzymes			
Z11911	*G6PDH*	B	1.7 ± 0.1
J02652	*Malic enzyme*	B	3.4 ± 0.2
AW120625	*6PGDH*[c]	I	1.6 ± 0.1
Metabolic enzymes			
AI790931	*Fructose bisphosphatase*[c]	B	1.6 ± 0.1
U67611	*Transaldolase*	I	1.5 ± 0.1
U05809	*Transketolase*	I	1.5 ± 0.1
Inflammatory suppressive gene			
AW046181	*Glucocorticoid-regulated kinase*[c]	I	1.7 ± 0.1
Miscellaneous			
AI841464	*Tryptophan hydrolase*[c]	B	1.5 ± 0.1
X64837	*Ornithine aminotransferase*	B	1.5 ± 0.1
AI849587	*Calcium channel, voltage dependent*[c]	B	1.6 ± 0.1
AI842432	*Phosphoglucomutase*[c]	B	1.7 ± 0.1
AF071068	*Aromatic amino acid decarboxylase*	I	1.9 ± 0.1
AI845584	*Dual-specificity protein tyrosine phosphatase*[c]	I	1.7 ± 0.1
AJ238636	*Nucleoside diphosphatase (ER-UDPase gene)*	I	1.9 ± 0.1
AF042491	*Membrane-associated progesterone receptor component*	I	1.6 ± 0.1
EST (unannotated)			
AW123697		B	1.6 ± 0.1
AW125453		B	1.9 ± 0.2
AI788959		B	1.6 ± 0.1

[a] B, basal gene; I, inducible gene; B & I, basal and inducible gene.
[b] Genes containing ARE, which are known to be regulated by Nrf2.
[c] Annotated ESTs.
[d] Genes with ARE.

Nrf2 are implicated directly or indirectly in counteracting the cellular stress induced by a wide spectrum of electrophiles and free radicals (Table 1).

Fig. 1 depicts the strategy and the outcomes of the comparisons of transcriptional profiles obtained from the different treatment groups. The strategy we used of comparative analysis of transcriptional profile resulted in both up-regulated and down-regulated genes in each category of comparison. Our focus in the present investigation has been only on genes that are positively regulated by Nrf2, because most of the detoxifying phase 2 proteins depend on this transcription factor for their constitutive and or inducible synthesis. The down-regulated genes in sulforaphane treated/vehicle control comparison using *nrf2*-deficient mice cannot be attributed to Nrf2; however, the role of Nrf2 in the repression of these genes that are present exclusively in "wild

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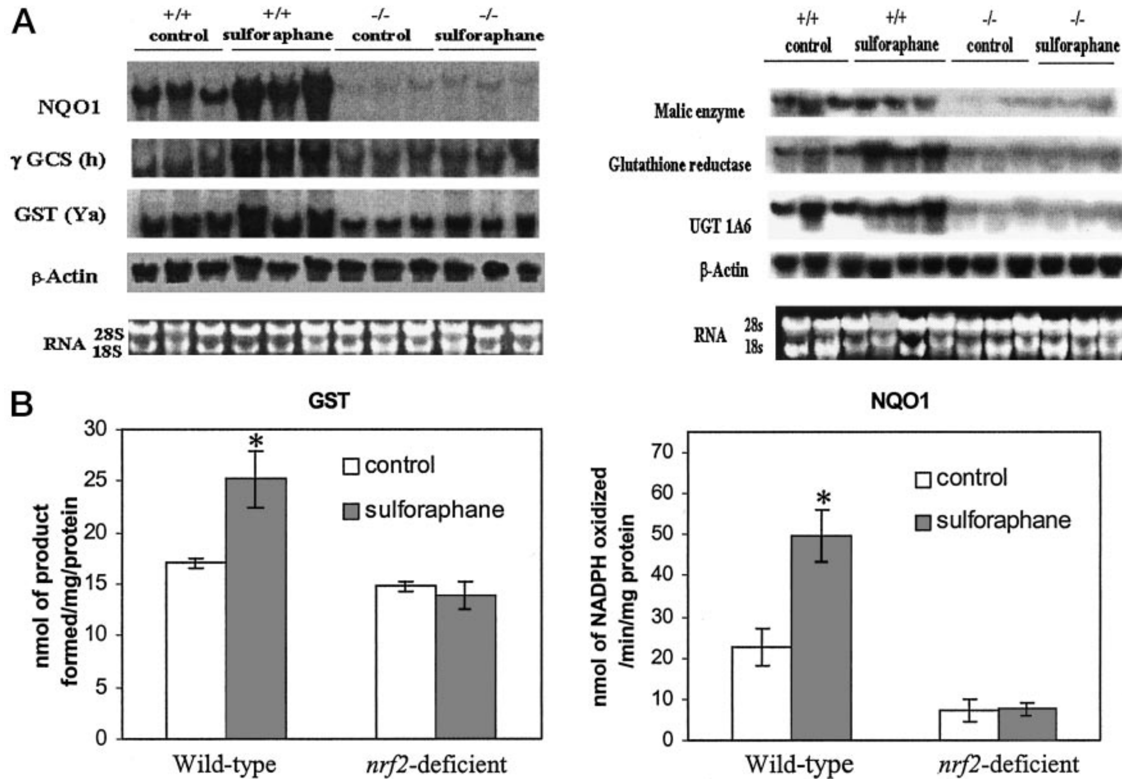

Fig. 2. *A*, Northern blot analysis of mRNA levels of NQO1, GST (Ya), GCS (h), UGT, malic enzyme, and glutathione reductase in small intestine of wild-type [*nrf2* (+/+)] and *nrf2*-deficient [*nrf2* (−/−)] mice treated with either vehicle (corn oil) or sulforaphane to show the basal and inducible expression. *B*, GST and NQO1 activities in the cytosolic fractions of small intestine of wild-type [*nrf2* (+/+)] and *nrf2*-deficient [*nrf2* (−/−)] mice treated with vehicle (corn oil) or sulforaphane. The activities of both enzymes are elevated only in sulforaphane-treated *nrf2*+/+ mice. Values represent means ($n = 3$); *bars,* SE. *, significantly greater from control of same genotypes, $P < 0.05$ (analyzed by Student's *t* test).

type treatment/wild type control" comparisons cannot go unnoticed. It is reported that the balance in expression of Nrf2 and its small maf binding proteins can affect the positive or negative regulation of some genes (31).

Two major cellular pathways of detoxication, *i.e.,* glucuronidation and glutathione conjugation, appeared to be even more dependent on Nrf2-regulated genes than thought previously. The glucuronidation pathway enhances the elimination of many lipophilic xenobiotics and

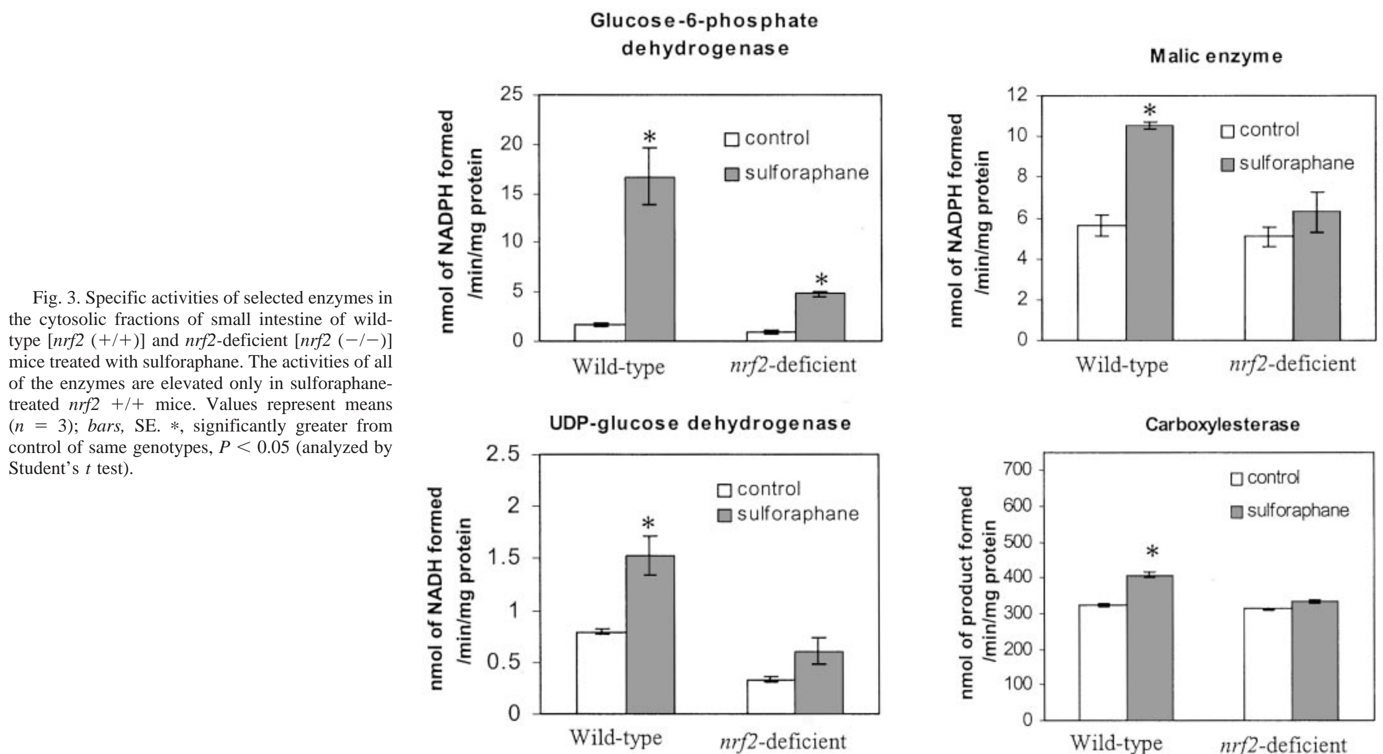

Fig. 3. Specific activities of selected enzymes in the cytosolic fractions of small intestine of wild-type [*nrf2* (+/+)] and *nrf2*-deficient [*nrf2* (−/−)] mice treated with sulforaphane. The activities of all of the enzymes are elevated only in sulforaphane-treated *nrf2* +/+ mice. Values represent means ($n = 3$); *bars,* SE. *, significantly greater from control of same genotypes, $P < 0.05$ (analyzed by Student's *t* test).

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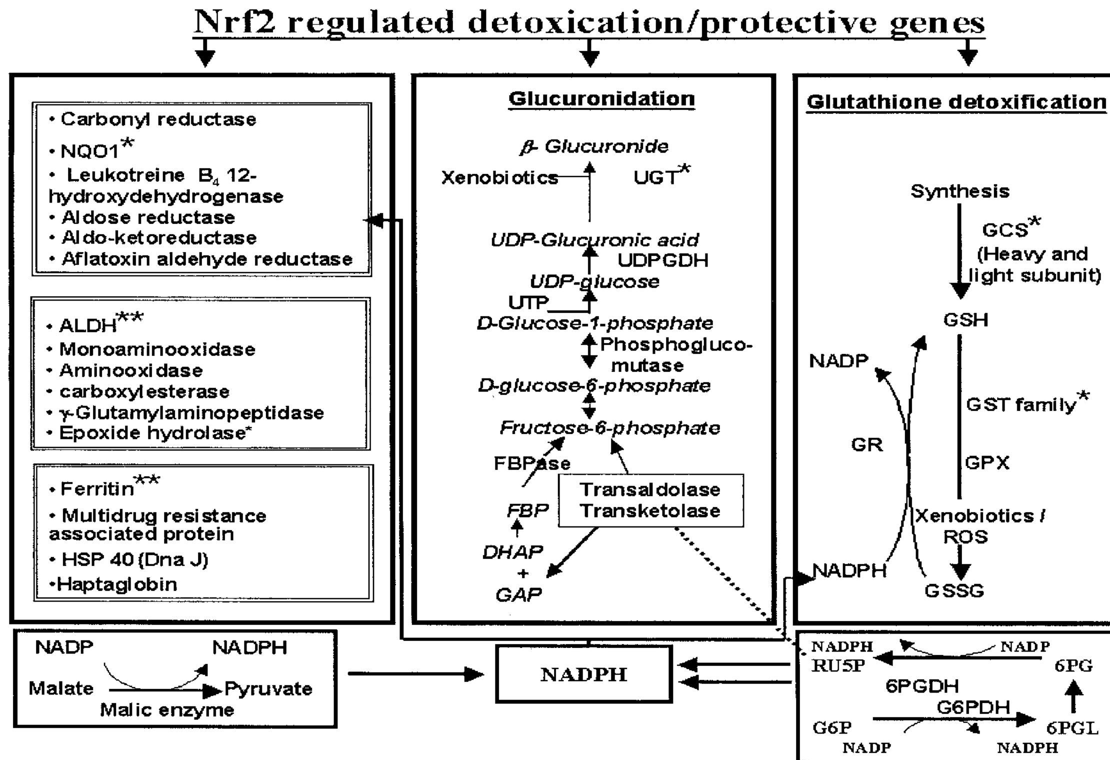

Fig. 4. Probable network of Nrf2-regulated genes involved in the detoxication process. *, genes containing ARE that are known to be regulated by Nrf2. **, genes with ARE.

endobiotics by conversion to more water-soluble compounds. Glucuronidation requires the cofactor UDP-glucuronic acid, and the reaction is catalyzed by a family of UGTs, some members of which are well-characterized Nrf2-regulated genes (32). In the present investigation, we found several new Nrf2-dependent genes (*transketolase, transaldolase, fructose bisphosphatase*, *phosphoglucomutase,* and *UDP-glucose dehydrogenase*), which are associated with metabolic pathways that may directly or indirectly aid the glucuronidation process (Fig. 4). Transketolase and transaldolase catalyzes the formation of fructose-6-phosphate from the products derived from ribulose-5-phosphate. Furthermore, fructose 1,6-bisphosphatase also catalyzes the conversion of fructose 1,6-bisphosphate to fructose-6-phosphate. Both these reactions may increase the influx of fructose-6-phosphate, which after conversion to glucose-6-phosphate forms glucose-1-phosphate by the action of phosphoglucomutase (Fig. 4). The UDP-glucose that provides the glucuronic acid for UGT-mediated conjugation with xenobiotics is formed from glucose-1-phosphate by UDP-glucose dehydrogenase.

GSTs constitute a family of enzymes that detoxify xenobiotics by conjugating glutathione to a range of electrophilic substrates. The cytosolic GSTs are currently divided into at least eight classes on the basis of their physical and chemical properties (33). In our study, we have found several Nrf2-dependent genes coding for isozymes of GST, including *GST mu* (*GST5-5*), *GST mu 1, GST GT8.7, GST GT9.3, GST α3, GST α2* (*Yc2*), *GST α1* (*Ya*). In addition, a few ESTs that were homologous to human GST [GST M2 (muscle), microsomal GST 3, and microsomal GST 2] and rat GST required Nrf2 for either basal or inducible expression.

Glutathione using genes such as *GR* and *GPX* [other than the well-known Nrf2 targets, *GST* (33) and *γ-GCS* (34)] were up-regulated in wild-type mice. *GR,* which is involved in the production of reduced glutathione by using NADPH, is up-regulated only in wild-type sulforaphane-treated mice, suggesting the role of Nrf2 in its induction, whereas *GPX,* which is involved in detoxifying various free radicals and peroxides by consuming glutathione (35), was found to be elevated only in the wild-type control mice, indicating the dependence of Nrf2 for basal expression. Lack of or low expression of all these glutathione-associated genes in *nrf2*-deficient mice undoubtedly makes them more susceptible to xenobiotic toxicities (12, 14).

Other than NQO1 (36) and epoxide hydrolase (3), which are known Nrf2 targets, additional targets of xenobiotic detoxication genes regulated by Nrf2 obtained from the screening can be grouped as oxido-reductase, hydrolytic, and oxidative detoxication enzymes. Carbonyl reductase, aldose reductase (fibroblast growth factor regulated protein), aldo-keto reductase, and aflatoxin aldehyde reductase belong to a class of NADPH-dependent oxido-reductases catalyzing the reduction of aldehyde and keto groups of several endogenous and exogenous compounds. The broad range of substrates includes acrolein, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, prostaglandins, steroids, pterins, and biogenic amines (37–42). Carboxylesterase and peptidases belong to the hydrolytic class of detoxication enzymes. Carboxylesterase hydrolyzes xenobiotics containing functional groups such as carboxylic esters (procaine), amides (procainamide), and thioesters (spironolactone; Ref. 43). Several oxidative enzymes, such as aldehyde dehydrogenase, monoamine oxidase, and amino oxidase, are also regulated by Nrf2. ALDH enzymes are involved in the oxidation of xenobiotic aldehydes (44) and also possess esterase activity. *ALDH2,* one of the genes identified in our investigation, is a mitochondrial enzyme that appears to be regulated by Nrf2. It is primarily responsible for oxidizing simple aldehydes such as acetaldehydes (45). Interestingly, the presence of AREs in the 5′ upstream region of ALDH was shown recently (46). This observation is consistent with our findings and suggests the positive regulation by Nrf2 on these classes of genes. Monoamine oxidase, an integral protein of the mitochondria outer membrane is a flavoenzyme that is dependent on Nrf2 for basal expression. It catalyzes the oxidation of structurally diverse amines and xenobiotics such as the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (47). Leukotriene B_4 hydroxydehy-

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drogenase is another basal and inducible oxidoreductase regulated by Nrf2 that has been found to be effective in catalyzing the hydrogenation and detoxication of wide variety of cytotoxic and mutagenic α,β-unsaturated aldehydes and ketones that are major toxic environmental pollutants and products of lipid peroxidation (48–50).

The genes coding for NADPH generating enzymes, such as *G6PDH* and *malic enzyme,* were elevated only in the wild-type control mice, suggesting the dependence of these genes on Nrf2 for basal expression. Recently, *malic enzyme* has been reported to contain an ARE in its promoter region, which corroborates well with our observation (51). However, another NADPH-generating enzyme, 6PGDH, was up-regulated in wild-type mice in response to sulforaphane, indicating that its induction is mediated by Nrf2 (52, 53). Increased formation of NADPH may prove to be beneficial because it is involved in the microsomal monooxygenation of xenobiotics, reductive biosynthesis, maintenance of redox state, and also acts as a potent antioxidant (direct and indirect; Ref. 54). The hexose shunt enzymes, G6PDH and 6PGDH, are also responsible for generating the ribose-5-phosphate necessary for nucleic acid biosynthesis and repair. The coordinated expression of all of these genes involved in detoxication, antioxidant status, and repair suggests an important role for Nrf2 in regulating the cellular defenses against carcinogenic challenges by increasing the reductive capacity of the cell. The microarray data were verified by the observed increases in enzyme activity of G6PDH and malic enzyme in the intestine of wild- type mice treated with sulforaphane (Fig. 3). Although the impact of Nrf2 genotype on the transcript levels of the genes coding G6PDH and malic enzyme appeared to be on basal expression, the activities of these enzymes were elevated in wild-type mice by sulforaphane treatment (nearly 10-fold and 2-fold higher when compared with vehicle, respectively). Also, there was significant increase in G6PDH activity in *nrf2*-deficient mice after treatment with sulforaphane, suggesting that the induction of this enzyme is partly controlled by other transcription factors.

Apart from enzymes, several cytoprotective proteins involved in lessening electrophile toxicity and oxidative stress appear to be regulated through Nrf2. Ferritin (light chain), an antioxidant, known to possess an ARE (55), requires Nrf2 for basal expression. HSP 40, a cofactor for HSP 70, the expression of which is up-regulated by a variety of cellular stresses (56), requires Nrf2 for induction. Another antioxidant protein, haptaglobin, an acute phase protein capable of binding to hemoglobin, thus preventing iron loss and renal damage, is also dependent on Nrf2 for basal expression (57). Multidrug resistance protein is elevated only in wild-type control, suggesting that Nrf2 contributes to its basal expression. Multidrug resistance protein is a ubiquitously expressed protein with several physiological functions, such as protection against heavy metal oxyanions, modulation of the activity of ion channels, and transport of leukotriene C_4 and other glutathione conjugates and glucuronides (58).

We have identified a number of Nrf2-dependent genes (for either basal or inducible expression) that are involved in different biological functions such as *ornithine aminotransferase* [protects against ammonia intoxication (59)], *tryptophan hydrolase*, *aromatic amino acid decarboxylase*, *nucleoside diphosphatase*, *putative membrane-associated progesterone receptor*, and *glucocorticoid-regulated kinase*. The screening also enabled us to identify genes that are induced in response to sulforaphane but not regulated by Nrf2 (genes up-regulated in *nrf2*-deficient treated/*nrf2*-deficient control comparison).

The time of sampling point is very critical to monitor the transcriptional activation of any gene. Probably in the present investigation at the selected sampling point (24 h after the last dose), there may be certain genes whose transcriptional activity might have returned to basal expression levels; such differential outcome between transcript and protein levels has been observed with hepatic GST Ya in oltipraz-treated rats (60). Similarly, with G6PDH and malic enzyme, enzyme activities were high at the selected time point after sulforaphane treatment, but the transcript levels were basal as determined by our microarray data analysis. In addition to the dynamic influence of time of sampling, potency and efficacy of different Nrf2 activators will vary with chemical class and target tissue. Thus, studies of the transcriptional profiles with different Nrf2 activators in various tissues at a range of sampling points may result in identification of additional sets of Nrf2-dependent genes that our study is unable to reveal.

This study expands the scope of the positive, coordinated regulation of a wide variety of cellular defense proteins by Nrf2 and underscores the potential of Nrf2 activation as a strategy for achieving cancer chemoprevention. The genes regulated by Nrf2 include detoxication enzymes as well as antioxidative and cytoprotective proteins that can collectively alleviate the toxicities mediated by a broad range of electrophiles and reactive oxygen species. Future studies aimed at searching for AREs in the promoter of these target genes of Nrf2 will help in deciphering the direct and indirect role of this transcription factor in these actions. In conclusion, this study expands the molecular basis by which the cancer preventive agents such as sulforaphane exert protective efficacy against a broad spectrum of exogenous and endogenous toxicants. Furthermore, understanding of the downstream molecular targets of these anticarcinogens will facilitate their development and use in clinical interventions (61).

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