Transcriptional Regulation of NF-E2 p45-related Factor (NRF2) Expression by the Aryl Hydrocarbon Receptor-Xenobiotic Response Element Signaling Pathway

DIRECT CROSS-TALK BETWEEN PHASE I AND II DRUG-METABOLIZING ENZYMES*

Weimin Miao, Lianggao Hu, P. James Scrivens, and Gerald Batist†

From the Montreal Center for Experimental Therapeutics in Cancer, Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis-Jewish General Hospital and Department of Oncology, McGill University, Montreal, Quebec H3T 1E2, Canada

The aryl hydrocarbon receptor (AHR) and NF-E2 p45-related factor (NRF2) are two distinct transcription factors involved in the regulation of drug-metabolizing enzymes. Increasing evidence from several studies implies that AHR and NRF2 have direct links, but the molecular mechanism remains unknown. In this work we demonstrate for the first time that Nrf2 gene transcription is directly modulated by AHR activation. DNA sequence analyses of the mouse Nrf2 promoter revealed one xenobiotic response element (XRE)-like element (XREL1) located at −712 and two additional XRE-like elements located at +755 (XREL2) and +850 (XREL3). Functional analysis using luciferase assay showed that XREL1, XREL2, and XREL3 are all inducible by 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment, with XREL2 being the most potent. The functionality of these XRE-like elements was further confirmed by mutagenesis and gel shift experiments. Finally, we used chromatin immunoprecipitation assay to show a direct binding of AHR to the Nrf2 promoter. Cells with silenced AHR expression using siRNA also lost Nrf2 mRNA induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin. These new data position Nrf2-antioxidant response element downstream in the AHR-XRE pathway. Moreover, direct regulation of Nrf2 by AHR contributes to couple phase I and II enzymes into an integrated system facilitating more effective xenobiotic and carcinogen detoxification.

Environmental exposure to carcinogens is an important risk factor for common cancers (1). Because carcinogen exposure is important in cancer risk, enhancing the body’s carcinogen-detoxifying capability is a promising approach for cancer prevention (2, 3). Like antibody-mediated immunity, nature has evolved a flexible, comprehensive enzyme system to detoxify a range of environmental toxicants, mutagens, and potential carcino gens. The cellular detoxifying system is divided into phase I and II drug-metabolizing enzymes (DMEs).1 The phase I enzymes consist of a gene superfamily of cytochrome P450s (CYPs); phase II enzymes include detoxifying and antioxidant enzymes such as glutathione S-transferases, γ-glutamylcysteine synthetase, NADPH:quinol oxidoreductase 1 (NQO1), and UDP-glucuronosyl transferases (4). Critical DNA sequences are frequently found single or multiply in the promoters of these genes, including antioxidant response element (ARE) and xenobiotic response element (XRE). The ARE is present in many phase II genes, whereas the XRE is found in both phase I and II genes (5).

XRE- and ARE-driven regulation of DME genes has two pathways that are generally thought to function independently. The XRE motif is the ultimate target of a protein complex that includes a ligand-activated aryl hydrocarbon receptor (AHR) translocating to the nucleus after binding to a chaperone nuclear transporter ARNT. The AHR-ARNT complex binds to the XRE motif and activates a battery of genes (4, 5). As well, a recent paper reported an alternate cis-acting element, called XRE II, which binds an as yet unknown protein factor with an AHR-ARNT heterodimer as a coactivator (6). AHR is a ligand-activated transcription factor that mediates toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as well as induction of three cytochrome P450 enzymes and a number of phase II enzymes including several glutathione S-transferase isoforms. AHR is a basic helix-loop-helix transcription factor that binds both synthetic chemicals such as TCDD and naturally occurred phytochemicals, sterols, and heme breakdown products. Whereas persistent activation of the AHR signaling pathway is responsible for the spectrum of adverse effects produced by metabolically stable AHR ligands such as TCDD, activation of AHR signaling by TCDD at nontoxic concentrations or by less persistent AHR agonists has also been shown to have some beneficial antitumorigenic/antiestrogenic activities (7, 8).

Binding of the ARE promoter by a number of proteins has been shown; however, studies in a mouse knock-out model have placed particular emphasis on the basic leucine zipper transcription factor NRF2 (NF-E2 p45-related factor 2) (9, 10). Electrophilic compounds stimulate the cytosolic protein KEAP1, activating and releasing the transcription factor NRF2 from the KEAP1-NRF2 complex. In the nucleus the activated

1 The abbreviations used are: DME, drug-metabolizing enzymes; CYP, cytochrome P450; GST, glutathione S-transferase; NQO1, NADPH:quinol oxidoreductase 1; ARE, antioxidant response element; XRE, xenobiotic response element; AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; NRF2, NF-E2 p45-related factor 2; EQ, ethoxyquin; siRNA, small interfering RNA; RT, reverse transcription.

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† To whom correspondence should be addressed: The Montreal Center for Experimental Therapeutics in Cancer, Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis-Jewish General Hospital, McGill University, 3755 Cote Sainte Catherine Rd., Montreal, Quebec H3T 1E2, Canada. Tel.: 514-735-1420; Fax: 514-735-7211; E-mail: gbatist@onc.jgh.mcgill.ca.

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NRF2 forms a complex with small Mafs (11, 12), and this protein complex binds to the ARE motif, activating the ARE gene battery. Exposure to a variety of inducers with specific KEAP1 sites releases NRF2 for nuclear localization in the ARE. Recent data show that NRF2 is degraded by the ubiquitin-dependent pathway (13–15) and that NRF2 phosphorylation leads to its transactivation activity (16–19). A range of enzymes involved in cellular detoxification are thus induced, including for example the rate-limiting enzyme in GSH synthesis γ-glutamylcysteine synthetase (20).

It is generally considered that the ARE and XRE pathways are distinct. The conventional explanation of how bi-functional agents activate both phase I and II DMEs is a sequential mechanism whereby XRE-driven enzymes metabolize the inducer to an intermediary capable of ARE activation. However, Jaiswal and Radjendirane (21) showed that TCDD, a metabolically stable AHR inducer, can induce the human NQO1 gene expression through ARE activation. Moreover, using our cell-based system for XRE/ARE-activating drugs, we showed that some phase II-activating agents (ARE inducers) require the presence of AHR, suggesting a more direct cross-talk between the ARE and XRE pathways (22). A more recent paper showed that induction of NQO1 by TCDD requires NRF2, implicating a link between AHR and NRF2 (23). In this work, we provide the first evidence that NRF2, the master transcriptional factor in the ARE pathway, can be directly modulated by the AHR-XRE activation, representing a novel molecular pathway in modulating drug-metabolizing enzymes.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—TCDD was from Wellington laboratories Inc. (ON, Canada). Anti-NRF2 antibody (h-300) was bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-AHR antibody (RPT9) was from Abeam Inc. (Cambridge, MA).

**Cell Lines and Tissue Culture**—AHR-deficient cell line tao, CYP1A1-deficient cell line c37, and their parent cell line mouse hepatoma 1c1c7 were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium.

**Western Blot**—Mouse hepatoma cells 1c1c7, tao, and c37 were treated with TCDD for indicated times before harvesting. 30 μg of cell lysate per each sample was loaded and run through a 7.5% SDS-PAGE gel, followed by being transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked with 5% fat-free milk solution and then sequentially incubated with primary antibody and enzyme-conjugated secondary antibody. The results are documented on x-ray film with ECL detection and autophotography.

**Quantitative RT-PCR**—Mouse hepatoma cells were treated with test compounds for an indicated time, and total RNA was prepared with TRIzol (Invitrogen). RT-PCR was performed in two steps. First, the mRNA was reverse-transcribed into cDNA using Expand reverse transcriptase (Roche Applied Science). Then the cDNA was used as the template for quantitative PCR detection using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science). The real-time PCR conditions were optimized as 95 °C for 7 min and 45 cycles of 95 °C for 10 s, 61 °C for 5 s, and 72 °C for 20 s followed by routine melting and cool conditions. The primers for amplifying mouse NRF2 were 5'-tgctggagacacctgct-3' and 5'-gctgcacagctatggtggt-3'. The **Experimental Procedures**

**FIG. 1.** NRF2 protein level regulated by TCDD treatment. A, 1c1c7 cells were treated with Me2SO vehicle and 1 and 10 nm TCDD for 2, 6, 16, and 24 h. NRF2 protein expression was monitored by Western blot detection. B, density analyses using Scion Image indicated that induction is 2–3 fold, starting from 16 h. Values represent the mean ± S.E. of three independent measurements. Statistical analysis (Student’s t test) was performed by comparison of treated and untreated cells (*, p < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**FIG. 2.** NRF2 mRNA level regulated by TCDD treatment. A, the quantitative RT-PCR was performed as described under “Materials and Methods.” A, the results showed that NRF2 mRNA is significantly induced by TCDD treatment up to 3.5 fold, and the induction started at 6 h and was sustained at 24 h. B, 1c1c7, tao, and c37 cell lines were treated with Me2SO vehicle and 10 nm TCDD for 16 h. The results indicated that NRF2 mRNA was significantly induced in 1c1c7 and c37 but not in tao. C, 1c1c cells were transfected with 100 pm siRNA or scramble control RNA. After 24 h the cells were treated with or without 10 nm TCDD for another 24 h. The left panel showed that AHR protein level was knocked down 85% by siRNA. The right panel showed that in this transient AHR-silenced cell, the induction of NRF2 mRNA was largely abolished. Values represent the mean ± S.E. of three independent measurements. Statistical analysis (Student’s t test) was performed by comparison of treated and untreated cells (*, p < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
primers for amplifying mouse glyceraldehyde-3-phosphate dehydrogenase were 5'-H11032-atgcagggatgatgttctgg-3' and 5'-H11032-tcaacgaccccttcattgac-3'.

RNA Interference—The coding sequence of mouse AHR was targeted with the following AHR siRNA: 5'-H11032-GCA GAA UCC CAC AUC CGC AUG AUU A-3' (Stealth™ RNA, Invitrogen). Briefly, 1c1c7 cells were seeded in a 6-well plate and transfected in the presence of a 100 pM concentration of either siRNA or scramble control RNA in a 250-µl volume with Lipofectamine™ 2000. At 48 h the efficiency of AHR silencing was analyzed by Western blot detection of AHR protein level.

Molecular Cloning and Vector Construction—Mouse Nrf2 promoter segments were generated by PCR amplification. Briefly, genomic DNA prepared from 1c1c7 cells was used as template, the upstream promoter segment (from −748 to +1000bp) was amplified separately and then cloned into the KpnI and MluI sites of pGL3-basic vector (Promega) containing a firefly luciferase reporter gene. A series of deletion mutants of the Nrf2 promoter was also generated by PCR and cloned into pGL3-basic vector. The sense and antisense oligonucleotides containing XREL1, XREL2, and XREL3 were synthesized by Invitrogen. The annealed oligos were ligated to the KpnI and MluI sites of pGL3-promoter vector (Promega) containing a heterologous SV40 promoter and a firefly luciferase report gene. All

FIG. 3. XRE-like elements in mouse Nrf2 promoter region. The genomic sequences (NT 039207) containing mouse Nrf2 promoter were retrieved from the NCBI mouse genome database. The 2-kilobase region around the mRNA starting site was searched for possible AHR response XRE elements. The results showed that in the 5'-flanking region of the Nrf2 promoter, in addition to two ARE-like sequences (located at −317 and −579, respectively) reported previously, there is one XRE-like sequence (XREL1) located at −712. In the downstream untranslated area, two additional XRE-like sequences were found located at +755 (XREL2) and at +870 (XREL3).
recombinant plasmids were sequenced to confirm the accuracy of cloning.

**Site-directed Mutagenesis**—Site-directed mutagenesis experiments were performed using a QuickChange site-directed mutagenesis kit (Stratagene). Briefly, the primers containing mutated nucleotides of AREL1, AREL2, and XREL1 were synthesized by Invitrogen. Mutation of AREL2, AREL1, and XREL1 were sequentially performed with the pGL3 recombinant plasmid −749 to +100. Three pGL3 plasmids containing AREL2, AREL1, plus XREL1 mutations were successfully generated (Fig. 6A). All constructs were sequenced to confirm the accuracy of mutagenesis. The AREL1 mutagenesis is gactcatccatctccctgg (the bold underlined letters represent the core sequences of the ARE, and the underlined letters represent mutated nucleotides).

**Transient Transfection and Luciferase Assay**—Cells were seeded at 9 × 10^4 per well using 24-well plates and grown overnight in normal media. The following day cells were transiently transfected using Lipofectamine (Invitrogen) with pGL3 luciferase reporter constructs. The plasmid pRL, containing a Renilla reniformis luciferase reporter gene, was co-transfected as internal control. Briefly, cells were incubated with DNA-Lipofectamine complexes for 5 h, after which they were washed gently and cultured in fresh serum-supplemented media. Then cells were treated with test compounds for 24 h before harvesting for luciferase assay. Luciferase activities were analysed in 20 μl cell extracts with the dual luciferase assay kit (Promega) on a Lumat LB 9500 luminometer (Berthold Technologies, Bad Wildbad, Germany). The relative luciferase activities reported are expressed as a ratio of the pGL3 reporter activity to that of the control plasmid pRL. −Fold induction is expressed as the ratio of induction from treated cells versus untreated. Values represent the mean ± S.E. of three independent measurements. Statistical analysis (Student’s t test) was performed by comparison of treated and untreated cells (*, p < 0.05).

**Electrophoretic Mobility Shift Assay**—Gel shift assays were performed as described previously (40). Briefly, cells were treated with TCDD for 2 h before preparation of nuclear extracts. For DNA–protein binding reactions, 5 μg of nuclear protein was mixed with 1 μg of poly(dI-dC) in a 24-μl buffer containing 30 mM HEPES-KOH, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 14% glycerol. The reaction mixture was preincubated for 10 min at room temperature, after which the probe DNA was added. Incubation was continued for another 20 min at room temperature. Competition reactions were carried out under the same conditions with the addition of 200-fold unlabeled oligos or 1 μg of related antibody. Protein-DNA complexes were resolved through a 5% polyacrylamide gel using 0.25× Tris borate/EDTA buffer. The gel was then dried and subjected to autoradiography with an intensifying screen at −80 °C overnight.

**Chromatin Immunoprecipitation Assay**—chromatin immunoprecipitation assay was performed using a kit from Upstate Biotechnology Co. Briefly, hepatoma 1c1c7 cells were exposed to TCDD for 1.5 h, and then cells were treated with formaldehyde for protein-DNA cross-linking. The soluble chromatin of these cells was prepared according to the kit protocol. Ten percent of the diluted chromatin solution was reserved as protocol. The chromatin solution was incubated with either an anti-AHR antibody or pre-immune IgG. Immunoprecipitation was performed overnight at 4 °C with rotation. After washing and elution, DNA was purified with phenol-chloroform extraction and ethanol precipitation and then re-suspended in 20 μl of water. 2 μl of DNA solution was used as template for 35 cycles of PCR amplification with designated primers. Specific primer pairs were used to amplify the regions of CYP1A1 (Fig. 10A). The primer sequences for CYP1A1 were 5′-ctctttcaacccacccacaa-3′ and 5′-ctctttcaaggtgacgaggg-3′. The primer sequences for Nrf2/XREL1 were 5′-ggagtctttggctgttggtc-3′ and 5′-tcaggtctttgcatcttctc-3′. The primers for Nrf2/XREL2 were 5′-accctctctctctctctct-3′ and 5′-ggtcttctctctctctct-3′.

**RESULTS**

**NRF2 Expression Is Directly Regulated by AHR Activation**—Mouse hepatoma 1c1c7 cells were treated with TCDD for 2, 6, 16, and 24 h. In our Western blot experiments, NRF2 protein was identified as a doublet band at 100 kDa by comparing with a recombinant NRF2 protein standard (Fig. 2A). The quantitative RT-PCR experiments revealed that the Nrf2 mRNA level was remarkably elevated up to 3.5-fold by TCDD treatments; up-regulation started at 6 h and was sustained at 24 h (Fig. 2A). These data imply that modulation happens at the gene transcription level. To further clarify if AHR is necessary for this regulation, the AHR-deficient cell line tao and CYP1A1-deficient cell line c37 were each treated with 10 nM TCDD for 16 h, and their Nrf2 mRNA expressions were compared. The quantitative RT-PCR data showed that Nrf2 mRNA was substantially induced in 1c1c7 and c37 cell lines, but the induction was largely abolished in tao cell line (Fig. 2B), suggesting an AHR-dependent mechanism. To exclude the possibility that the AHR-deficient cell line tao have other differences that account for the phenotype, we created a transient AHR-silenced cell (80–90% AHR knock-down at 48 h) by transfection of AHR-targeted siRNA (Fig. 2C). Induction studies were performed in 1c1c7 cells, transfected with either 100 pm AHR-targeted siRNA or the scramble control RNA. After 24 h the cells were treated with or without 10 nM TCDD for another 24 h before RNAs were harvested. The quantitative RT-PCR data showed that the induction of Nrf2 mRNA by TCDD seen in controls cells is abolished in the AHR-silenced cell (Fig. 2C). Collectively, these data indicate that NRF2 expression may be regulated through AHR activation at the gene transcription level.
XRE-like Elements Found in mouse Nrf2 Promoter—To study transcriptional regulation of Nrf2, sequences (NT 039207) containing mouse Nrf2 promoter were retrieved from the NCBI mouse genome data base. Computer-aided analyses showed that in the 5'-flanking region of the Nrf2 promoter, in addition to the two ARE-like elements (located at −317 and −579, respectively) reported previously, there is also one XRE-like sequence (XREL1) located at −712. The downstream untranslated region, which is often important for transcriptional regulation, was also analyzed. Two additional XRE-like elements were found located at +755 (XREL2) and at +870 (XREL3) within the first intron of the gene (Fig. 3).

Molecular Cloning of Mouse Nrf2 Promoter—Using mouse genomic DNA as template, PCR was used to amplify the upstream promoter segment (−749 to +100), the downstream promoter segment (−300 to +904), and the whole promoter segment (−749 + 904) separately. The PCR products were then cloned into pGL3-basic vectors, which were used in subsequent functional analyses. The luciferase assays showed that in 1c1c7 cells, the upstream promoter was induced 2.6-fold, the downstream promoter was up-regulated 3.8-fold, and the whole promoter was induced 5.2-fold in response to treatment of 10 nM TCDD (Fig. 4A), but all inductions were diminished or absent in the tao cell line (Fig. 4B).

Series Deletion Mutant Analyses—To assess functionality of XRE-like elements in the promoter, a series of deletion mutants were created for both upstream and downstream promoter fragments (Fig. 5A). The luciferase data showed that the −749 + 100 fragment, which contains both XREL1 and ARE elements is potent, induced 2.9-fold at 10 nM TCDD; however, the −698 + 100 fragment containing only the ARE elements but no XREL1 is less active and induced 1.8-fold at 10 nM TCDD. The −300 + 904 fragment containing neither XREL1 nor AREs had only a marginal response (Fig. 5B). In experiments with the downstream promoter segment, the −300 + 859 fragment containing both XREL2 and XREL3 is most potent and induced 4.4-fold at 10 nM TCDD treatment; the −300 + 859 fragment containing only XREL2 is also active and induced 3.2-fold at 10 nM TCDD treatment, whereas the −300 + 740 fragment containing neither XREL2 nor XREL3 has no significant response (Fig. 5C).

Site-directed Mutagenesis—To further evaluate functions of the XRE and ARE elements in the upstream promoter, site-directed mutagenesis was performed (Fig. 6A). The luciferase assay showed that wild type promoter (−749 + 100) responded robustly to both TCDD or EQ (ethoxyquin). Mutation of two ARE elements only slightly reduced induction by TCDD but completely abolished the response to EQ. However muta-
transcription at the XRE1 site substantially diminished the response to TCDD but did not affect the response to EQ. Simultaneous mutation of XRE1, ARE1, and ARE2 sequences completely abolished the response to both TCDD and EQ (Fig. 6B). These data indicate that ARE elements are responsible for EQ activation; however, both XRE and ARE elements respond to TCDD treatments.

**Molecular Cloning of XRE-like Elements of Mouse Nrf2 Promoter**—To compare the potency of three XRE elements, oligos containing mutated (m) ARE1, ARE2, or XRE2, or XRE1 was created by the protocol described “Experimental Procedures.” B, 1c1c cells were transfected with these wild type (Wt) and mutated promoter constructs and then exposed to 10 nM TCDD or 10 μM EQ for 24 h before luciferase activity was assayed. The relative luciferase activities reported were expressed as a ratio of the plasmid reporter activity to that of the control plasmid pRL. Fold induction was recorded as relative luciferase activity of treated versus untreated cells. Values represent the mean ± S.E. of three independent measurements. Statistical analysis (Student’s t test) was performed by comparison of treated and untreated cells (*, p < 0.05). DMSO, Me_SO.

**Electrophoretic Mobility Shift Assay**—Gel shift experiments were also performed to evaluate XRE elements found in the Nrf2 promoter. The results show that XRE1 (Fig. 8A) and XRE3 (Fig. 8B) form a retarded band (indicated by an arrow), which can be induced by TCDD. Competition assay showed that the band was competed by excess unlabeled XRE3 oligos or AHR antibody but not by unrelated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Fig. 8, A and B). In the case of XRE2 (Fig. 9), we observed multiple retarded bands, but only one of which (indicated by the arrow) was significantly induced by TCDD. Competition assays showed that nearly all bands can be competed by unlabeled XRE2 oligos, but only the indicated band can be competed by DRE3 or AHR antibody (Fig. 9). The result suggests that the indicated band is specific to the XRE core sequence and AHR binding, and we hypothesize that other bands may be related to the flanking sequences in the XRE1.

**Chromatin Immunoprecipitation Assay**—1c1c7 cells were treated with 10 nM TCDD for 1.5 h, and soluble chromatin preparations were used for immunoprecipitation with AHR antibody. The results show that the sequences specific to the XREs found in the Nrf2 promoter as well as from the CYP1a1 promoter can be recovered from AHR immunoprecipitates but not from pre-immune Ig precipitates (Fig. 10B). Moreover, substantially more Nrf2 promoter sequences were recovered from AHR immunoprecipitates after treatment with 1 or 10 nM TCDD compared with vehicle Me_SO treatment samples (Fig. 10C).

**DISCUSSION**

A large body of evidence, based on preclinical and clinical research, indicates that modulation of the body’s DME could provide an effective approach for cancer prevention (2, 24). Understanding the molecular mechanism of DME modulation is critically important in designing rational cancer preventive agents. Although many naturally occurring or synthetic compounds have shown robust effects on DME modulation, the underlying molecular mechanisms are not fully understood. It has long been recognized that nearly all bi-functional inducers can activate XRE and ARE pathways concomitantly. The most common explanation is an indirect link. Briefly, bi-functional compounds first activate the AHR-XRE pathway and induce phase I enzymes including CYP1A1, which in turn metabolize these compounds. The resulting electrophilic intermediary metabolites further activate the Nrf2-ARE pathway. A series of studies conducted by Shertzer and co-workers (25, 26) showed...
that TCDD can induce reactive oxygen species and that the induction is dependent on the AHR, which implicates another indirect approach of activating phase II enzymes through reactive oxygen species.

Using gel shift experiments, Nebert and co-workers (27) reported that AHR may bind to both XRE and ARE consensus sequences, suggesting that one transcription factor may activate two distinct cis-acting regulatory elements (27). This suggested direct interaction between the two pathways unrelated to the metabolic products of either, but it required further proof. In addition, Jaiswal and Radjendirane (21) reported that TCDD, a metabolically stable AHR inducer, can induce NQO1 via an ARE element (21). Using Nrf2-null cells, Ma et al. (23) showed that NRF2 is involved in the activation of NQO1 by TCDD, but the underlying molecular mechanism was not known. We have also shown that some bi-functional compounds including TCDD can only activate the ARE element in the presence of AHR (22). Based on the experimental evidence from different laboratories, we hypothesized that NRF2, the master transcription factor for the ARE pathway, could be downstream target of regulation by AHR activation.

We did find that NRF2 expression is induced by TCDD at the transcriptional level. Furthermore, experiments with AHR-deficient cells showed that AHR is an indispensable factor involved in the induction of NRF2 by TCDD. These preliminary findings led us to examine the Nrf2 promoter sequences. The mouse Nrf2 promoter was cloned in 1996 (28), with further analysis in 2002 showing two ARE-like elements in the 5' flanking region, at least one of which is activated by Nrf2 binding (29). Because the Nrf2 promoter has not been studied in detail, we retrieved the mouse promoter sequence of Nrf2 from the NCBI genome data base. Using computer-aided analyses, we found that one XRE-like element is located at -712, which is close to the functional ARE-L2 (-579). It has been reported that functional XRE and ARE elements exist in the proximity in promoters of several important detoxifying genes including GST (30) and NQR (31). Using series deletion and site-directed mutagenesis, we showed that both XRE and ARE elements are responsible for the induction induced by TCDD.
In recent years mounting evidence showed that the promoter downstream region, especially the untranslated sequences within introns, are very important in transcriptional regulation (32–35). In our previous work we found an XRE element within the first intron of rGSTA5 that is active in regulating transcription (36). Therefore, we also checked the sequences downstream of the mRNA initiation site of Nrf2. We found no ARE-like elements but did find two XRE-like elements at \( /H11001 \) and \( /H11001 \), respectively.

The existence of multiple copies of XREs in DME gene promoters has been extensively studied before (37, 38). Our data also showed that tandem arrangement of these two XREs is responsible for Nrf2 promoter response to a greater extent than the single upstream XRE sequence. Finally, a chromatin immunoprecipitation assay showed that AHR specifically binds the Nrf2-XRE sequence.

We compared the DNA sequence of mouse Nrf2 promoter with those of the rat and human Nrf2 promoters. The comparisons showed that mouse and rat have a high degree of homology in promoter sequence, and both have three copies of XRE-like elements in 2-kilobase region of the promoter. Although the human promoter sequence has a very low percentage of homology with those of rodents, it does possess 5 copies of XRE-like elements in the 2-kilobase region of the promoter, although the location and sequence of these human XRE-like elements are distinct from those of rodent species. The conservation of multi-copy XREs in Nrf2 promoters from all three species suggests an important role in regulating Nrf2 gene expression.

These novel observations demonstrate that Nrf2 gene expression is at least partly regulated by AHR inducers by activating multiple XRE elements in its promoter. This molecular
event establishes a direct relation between AHR and NRF2 and places the NRF2-ARE pathway downstream of AHR-XRE activation in certain scenarios. This newly found pathway adds important knowledge in exploring the molecular mechanism of DME regulation (Fig. 11). It also clarifies previous data.

The DME system is composed of phase I and II enzymes, which have generally been thought to operate distinctly and at times in series. Among investigators working on modifiers of drug-metabolizing enzymes for chemoprevention, it is also considered that phase II enzymes are exclusively involved in detoxifying xenobiotics and carcinogens, and this has led researchers to targeting at NRF2 and monofunctional agents preferentially. Meanwhile, phase I enzymes such as CYP1As are considered to have two-sided effects. On the one hand they do detoxify some xenobiotics and carcinogens, and on the other, they can also metabolically activate some pro-carcinogens into toxifying xenobiotics and carcinogens, and this has led researchers to targeting at NRF2 and monofunctional agents preferentially. Meanwhile, phase I enzymes such as CYP1As are at least as important, if not more so, in detoxification as in metabolic activation. One conclusion, supported further by our work reported here, is that targeting the AHR/XRE pathway in chemoprevention is legitimate and may be very fruitful. Although NRF2 targeting agents for chemoprevention generally aim at releasing NRF2 from KEAP1, a plausible additional approach to increase NRF2 expression adds another dimension.

In this work, our finding of direct modulation of NRF2 expression by AHR activation demonstrates a molecular mechanism whereby phase I and II enzymes are closely integrated, and their regulations are linked at the promoter transcription level. In view of these findings, it is clear that the induction of phase II enzymes happens virtually concurrently in both enzyme groups. In this way, phases I and II work together to make a coordinated and highly efficient system of detoxification.

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Weimin Miao, Lianggao Hu, P. James Scrivens and Gerald Batist

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