Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction

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TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dixoin) induces phase II drug-metabolizing enzyme NQO1 [NAD(P)H:quinone oxidoreductase; EC 1.6.99.2; DT-diaphorase] in a wide range of mammalian tissues and cells. Here, we analysed the molecular pathway mediating NQO1 induction by TCDD in mouse hepatoma cells. Inhibition of protein synthesis with CHX (cycloheximide) completely blocks induction of NQO1 by TCDD as well as the basal expression and induction by phenolic antioxidant tBHQ (2-t-butylbenzene-1,4-diol), implicating a labile factor in NQO1 mRNA expression. The inhibition is both time- and concentration-dependent, requires inhibition of protein synthesis, and occurs at a transcriptional level. Inhibition of NQO1 transcription by CHX correlates with a rapid reduction of the CNC bZip (cap 'n' collar basic leucine zipper) transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) through the 26 S proteasome pathway. Moreover, blocking Nrf2 degradation with proteasome inhibitor MG132 increases the amount of Nrf2 and superinduces NQO1 in the presence of TCDD or tBHQ. Finally, genetic experiments using AhR (aryl hydrocarbon receptor)-, Arnt (aryl hydrocarbon receptor nuclear translocator)- or Nrf2-deficient cells reveal that, while induction of NQO1 by TCDD depends on the presence of AhR and Arnt, the basal and inducible expression of NQO1 by either TCDD or tBHQ requires functional Nrf2. The findings demonstrate a novel role of Nrf2 in the induction of NQO1 by TCDD and provide new insights into the mechanism by which Nrf2 regulates the induction of phase II enzymes by both phenolic antioxidants and AhR ligands.

Key words: NQO1 [NAD(P)H:quinone oxidoreductase 1], Nrf2 (nuclear factor erythroid 2-related factor 2), AhR (aryl hydrocarbon receptor), ARE (antioxidant response element), DRE (dioxin response element), TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin).

INTRODUCTION

The environmental contaminant TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is a potent agonist of the AhR (aryl hydrocarbon receptor). TCDD induces a broad spectrum of genes that may mediate TCDD's effects on xenobiotic metabolism, cell growth, apoptosis, immune function, endocrine homoeostasis and embryonic development [1–4]. Induction of gene transcription by TCDD is best studied for induction of CYP1A1. These analyses have revealed an 'AhR/DRE (dioxin response element) paradigm' for the induction, in which AhR is activated by a ligand, binds to DRE sequences located in the enhancer of the gene with partner protein Arnt (aryl hydrocarbon receptor nuclear translocator), and thereby mediates transcription [1,5]. While induction of many genes by TCDD is found to require AhR and DRE, recent studies revealed variations in the signalling pathways for induction of several target genes of TCDD under certain physiological/pathophysiological conditions. These include synergies between TCDD and hypoxia in the induction of erythropoietin, between TCDD and lipopolysaccharide in the inhibition of AhR and nuclear factor κB-mediated gene transcription, and between AhR and retinoblastoma protein which enhances gene induction by AhR [6–8].

NQO1 [NAD(P)H:quinone oxidoreductase; EC 1.6.99.2; DT-diaphorase] catalyses the obligatory two-electron reduction of a wide range of endogenous and environmental quinones and quinoid compounds such as benz[*a*]pyrene-3,6-quinone, vitamin K, vitamin E α-tocopherol, benzene quinones and anthraquinone-based anti-tumour drugs such as mitomycin C [9–13]. Epidemiological and genetic studies reveal that a loss or reduction of NQO1 activity is associated with increased risks for a number of pathological lesions, including benzene-induced haematotoxicity [14], acute leukaemia in adults [15] and children [16], secondary leukaemia after chemotherapy in cancer patients [17], increased myelogenous hyperplasia [18] and decreased therapeutic effect of chemotherapy in patients with disseminated peritoneal cancer [19]. On the other hand, increased activities of NQO1 are found to contribute to chemoprotection against cancer and chemical toxicity by natural or synthetic compounds [20,21].

NQO1 is broadly expressed in mammalian tissues and cell types (i.e. constitutive expression). Moreover, NQO1 is highly inducible by AhR agonists such as TCDD and polycyclic aromatic

Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; Nrf2, nuclear factor erythroid 2-related factor 2; CNC bZip, cap 'n' collar basic leucine zipper; Arnt, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; ARE, antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase; GST, glutathione S-transferase; HO-1, haem oxygenase-1; Aldh3a1, aldehyde dehydrogenase 3a1; tBHQ, 2-t-butylbenzene-1,4-diol; CHX, cycloheximide; BNF, β-naphthoflavone; ANF, α-naphthoflavone; MEF cell, mouse embryonic fibroblast cell; DIG, digoxigenin; ROS, reactive oxygen species.

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hydrocarbon or by phenolic antioxidants such as tBHQ (2-t-butylbenzene-1,4-diol) [9]. TCDD and polycyclic aromatic hydrocarbon are also termed bifunctional inducers, which induce both phase I and phase II genes, whereas tBHQ and similar inducers are designated as monofunctional inducers, which induce phase II genes [22]. Early analyses of the enhancer sequences of rat NQO1 by Favreau and Pickett [23,24] identified DNA response elements necessary for NQO1 induction by polycyclic aromatic hydrocarbon or tBHQ, which were designated as DRE and ARE (antioxidant response element), respectively. Recent genetic and biochemical studies on phase II enzyme induction implicate Nrf2 (nuclear factor erythroid 2-related factor 2), a redox-sensitive member of the CNC bZip (cap 'n' collar basic leucine zipper) family of transcription factors [25], as a principal mediator of NQO1 induction by phenolic antioxidants [26–28]. Nrf2 is localized in the cytoplasm in a complex with Keap1 [29]. In the presence of tBHQ, Nrf2 dissociates from Keap1 and translocates into the nucleus, followed by dimerization with a Maf (musculoaponeurotic fibrosarcoma) protein, binding to ARE, and transcription of the gene. The mechanism by which AhR ligands induce NQO1 is not well understood at present. Induction of rat NQO1 by TCDD and BNF (β-naphthoflavone) was found to require a DRE (also termed XRE) upstream of the promoter of the gene [23], whereas induction of human NQO1 by TCDD was suggested to be mediated through ARE independently of AhR [30]. We have previously reported that induction of NQO1 mRNA by TCDD is susceptible to inhibition by the protein synthesis inhibitor CHX (cycloheximide), but induction of CYP1A1 is not [31], suggesting different signalling pathways for induction of NQO1 and CYP1A1 by AhR agonists. Like NQO1, other phase II enzymes such as GST (glutathione S-transferase) and UDP-glucuronosyltransferase are induced by both AhR ligands and phenolic antioxidants; therefore, analyses of NQO1 induction can provide insights into transcriptional regulation of phase II enzymes by xenobiotics. In particular, these studies can facilitate identifying protein factors mediating the induction of phase II genes by AhR ligands and potential cross-reactions between the DRE- and ARE-dependent signal transduction in the inductions.

In this study, we utilized biochemical and genetic approaches to examine the signalling pathway of NQO1 induction by TCDD. The findings reveal that inhibition of protein synthesis by CHX blocks the transcription of NQO1 for its basal and inducible expression by either TCDD or tBHQ. Inhibition of NQO1 transcription by CHX correlates with a rapid reduction in the protein level of Nrf2, due to rapid turnover through the ubiquitin-26 S proteasome pathway. Genetic evidence reveals that induction of NQO1 by TCDD requires AhR, Arnt and Nrf2. Together these results demonstrate that Nrf2 is required for both basal and inducible transcription of NQO1 and suggest that the AhR-mediated signal transduction cross-reacts with Nrf2 functions in the induction of phase II enzymes.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other general molecular biology reagents were purchased from New England Biolabs (Beverly, MA, U.S.A.), Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.), Invitrogen (Carlsbad, CA, U.S.A.) and Promega (Madison, WI, U.S.A.). DMSO, tBHQ, CHX, BNF, ANF (α-naphthoflavone) and puromycin were purchased from Sigma (St. Louis, MO, U.S.A.). MG-132 and lactacystin were from Bio-Mol (Plymouth Meeting, PA, U.S.A.). TCDD was from AccuStandard (New Haven, CT, U.S.A.). Cell culture materials were from Invitrogen. Reagents for Northern and immunoblotting are described below.

Cell culture and treatment

Mouse hepa1c1c7 and its AhR-defective or Arnt-defective variant cells were provided by Dr J. P. Whitlock, Jr (Stanford University, Stanford, CA, U.S.A.). The cells were grown as monolayer in α-minimal essential medium containing 10 % fetal bovine serum and 5 % CO_2, as described previously [32]. Cells were treated with chemicals as described in the Figure legends. DMSO was used as a solvent control for TCDD, tBHQ, BNF and ANF. CHX and puromycin were dissolved in water.

Derivation of MEF cells (mouse embryonic fibroblast cells)

Nrf2-null mice, in which the Nrf2 gene is disrupted and non-functional by targeted gene knockout, are described in [25]. The mice have the genetic backgrounds of 129SVJ and C57BL/6 mice, and were re-derived in Jackson Laboratory to ensure that they were free of pathogens before entering the animal quarters at NIOSH (National Institute for Occupational Safety and Health, Morgantown, WV, U.S.A.). The $Nrf2^{-/-}$ and wild-type control mice were maintained in the animal quarters according to the guidelines for animal care and use at NIOSH. Male and female $Nrf2^{-/-}$ or $Nrf2^{+/+}$ mice were paired and pregnancy was monitored. Embryos were obtained 18 days after pairing under aseptic conditions. Embryo heads were used for confirming Nrf2 genotypes by PCR. Embryo bodies were minced into small pieces and cultured in Dulbecco's modified Eagle's medium with 10 % fetal bovine serum at 37 °C and 5 % CO_2. MEF cells grew out of the embryo tissues; the cells were collected using standard procedures and were stored in liquid nitrogen for further use. MEF cells from three embryos of each genotype were used for experiments.

RNA preparation and Northern blotting

Total RNA was isolated from cells using a Qiagen total RNA isolation kit (Valencia, CA, U.S.A.). RNA samples of 5 μg each were fractionated in a 1 % agarose-formaldehyde gel and transferred to a Nytran membrane. The blot was probed with a DIG (digoxigenin)-labelled riboprobe prepared with the DIG-labelling kit (Roche Molecular Biochemicals) for mouse CYP1A1, NQO1, HO-1 (haem oxygenase-1) and Aldh3a1 (aldehyde dehydrogenase 3a1), according to established procedures [33]. Signals were visualized by chemiluminescence using a DIG RNA detection kit with CDP Star as a substrate (Roche Molecular Biochemicals). Parallel blots of the same samples were probed with a DIG-labelled actin probe to ensure equal loading of the samples. Results shown were repeated two to three times in separate experiments with consistent observations.

Immunoblotting

Cells were collected and lysed in a cell lysis buffer (Promega). Total cell extracts were prepared by centrifugation at 13000 ***g***. The cell extracts (5 μg each) were fractionated in 10 % SDS/polyacrylamide gels, transferred to nitrocellulose membranes and blotted with antibodies according to established procedures. For immunoblotting of Nrf2, an affinity-purified rabbit polyclonal antibody against mouse Nrf2 was used (kindly provided by Dr C. B. Pickett, Schering-Plough Research Institute, Kenilworth, NJ, U.S.A.) [34]. Horseradish peroxidase-conjugated anti-rabbit

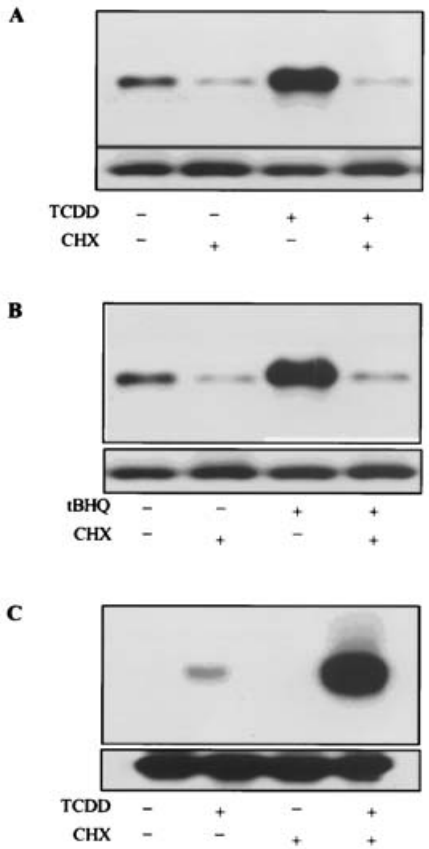

Figure 1 Inhibition of NQO1 induction by CHX

Hepa1c1c7 cells were treated with DMSO, CHX (10 μg/ml), TCDD (1 nM), TCDD plus CHX, tBHQ (100 μM) or tBHQ plus CHX for 5 h. Total RNA of 5 μg each was analysed by Northern blotting for NQO1 (**A** and **B**) or CYP1A1 (**C**). The same RNA samples were blotted for actin mRNA expression to ensure equal loading (bottom panels).

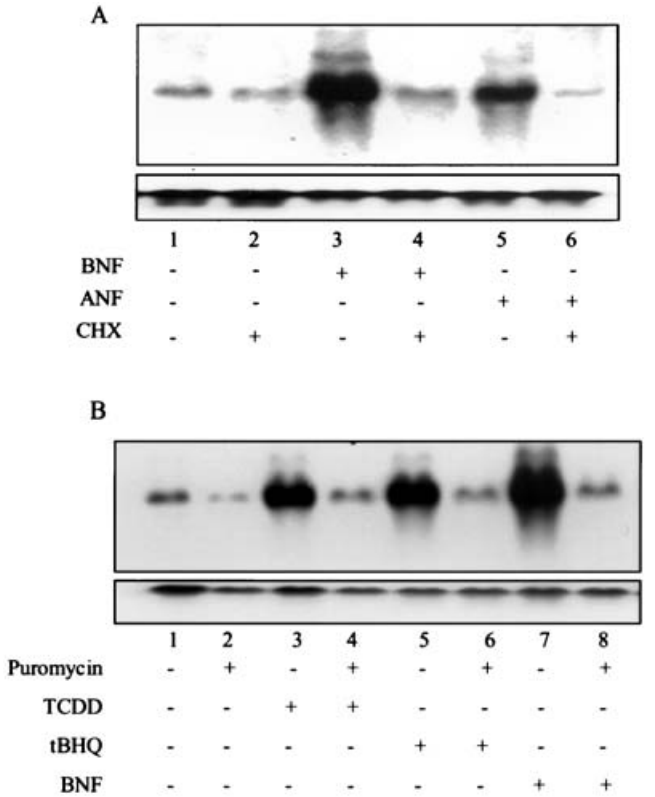

Figure 2 Effect of inhibition of protein synthesis on NQO1 induction by different inducers

Hepa1c1c7 cells were treated as indicated and total RNA was analysed by Northern blotting for NQO1 (upper panels) and actin (lower panels). (**A**) Cells were treated with BNF (10 μM) or ANF (10 μM) in the absence or presence of CHX (10 μg/ml) for 5 h. (**B**) Cells were treated with TCDD (1 nM), tBHQ (100 μM) or BNF (10 μM) in the absence or presence of puromycin (10 μg/ml) for 5 h. Northern blotting was carried out as described in the Materials and methods section.

IgG antibodies (Promega) were used as the secondary antibody. The blots were visualized by chemiluminescence using an ECL® kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). The same blots were reprobed with a monoclonal goat anti-actin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), followed by incubation with alkaline phosphatase-conjugated anti-goat IgG (Promega) and colour visualization. The amount of actin detected in the blots was used as an internal control to ensure equal loading of the samples.

RESULTS

Basal and inducible expressions of mouse NQO1 require a label factor

We have previously observed that induction of mouse NQO1 by TCDD is inhibited by CHX, an inhibitor of protein synthesis, implying that a labile or inducible protein factor(s) is required for the induction [31]. To elucidate the molecular steps of mouse NQO1 induction, we analysed the mechanism by which inhibition of protein synthesis blocks NQO1 induction in hepa1c1c7 cells, a highly responsive murine cell line to AhR agonists. As shown in Figures 1(A) and 1(B), NQO1 mRNA is constitutively expressed (lanes 1, numbering from left) and is induced by TCDD (1 nM, 5 h) or tBHQ (100 μM, 5 h) for >5-fold increases (lanes 3) by Northern analysis. CHX (10 μg/ml) alone inhibited the basal expression to less than 20 % of control (Figures 1A and 1B, compare lanes 2 and 1); co-treatment with CHX completely blocks NQO1 induction by TCDD or tBHQ to the level of CHX alone (Figures 1A and 1B, compare lanes 4 with 2 and 3). Thus, protein synthesis is required for the basal, TCDD-inducible and tBHQ-inducible expression of mouse NQO1. Inhibition of NQO1 expression by CHX is not due to cell toxicity or inhibition of gene transcription in general by CHX, because CHX does not repress, but enhances the induction of CYP1A1 mRNA by TCDD under similar conditions (e.g. 'superinduction'; Figure 1C, compare lanes 2 and 4) [33]. This differential effect of CHX on the induction of NQO1 and CYP1A1 mRNAs by TCDD indicates that induction of the two genes by AhR ligands involves different signalling pathways.

Benzoflavones BNF and ANF induce both DRE- and ARE-dependent transcription of NQO1 [22,23]. In Figure 2(A), we tested if CHX inhibits BNF- or ANF-induced expression of NQO1. BNF or ANF at 10 μM strongly induces NQO1, whereas co-treatment with CHX totally blocks the induction, suggesting that the CHX-sensitive factor is required for NQO1 induction by a broad range of inducers. Next, we examined whether inhibition of protein synthesis is sufficient to inhibit NQO1 induction. Puromycin inhibits protein synthesis with a similar potency to CHX by substituting amino acid-tRNA for protein synthesis. Puromycin inhibits the basal, TCDD-, tBHQ- or BNF-inducible

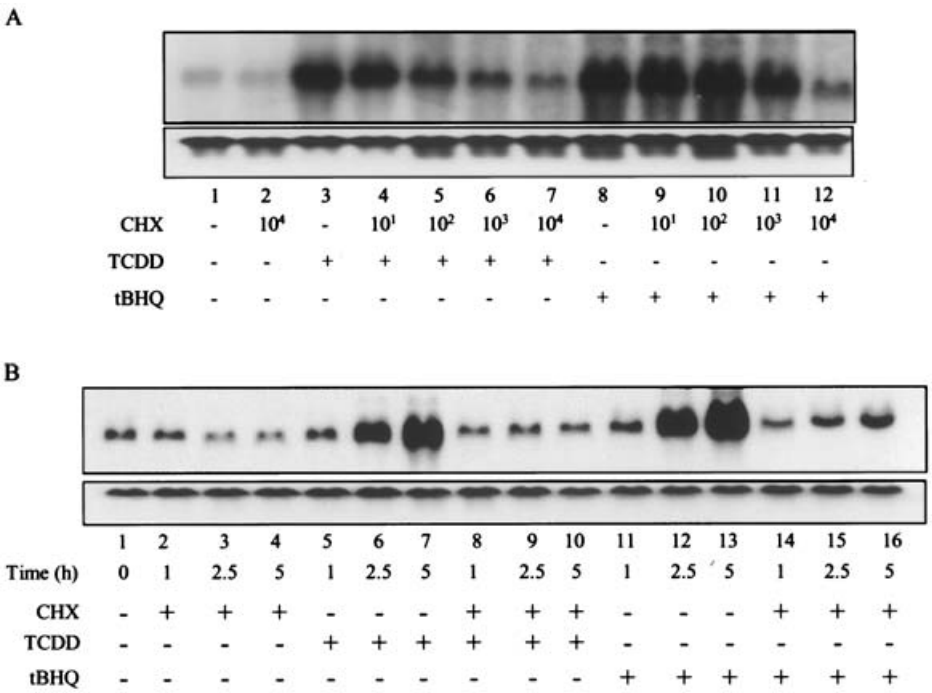

Figure 3 Concentration- and time-dependence of CHX inhibition of NQO1 induction

(**A**) Cells were treated with increasing amounts of CHX (ng/ml) in the presence of TCDD (1 nM) or tBHQ (100 μM) for 5 h as shown. (**B**) Cells were treated with CHX (10 μg/ml) in the presence of TCDD (1 nM) or tBHQ (100 μM) for the indicated times. Total RNA was analysed by Northern blotting for NQO1 and actin as described for Figure 1.

expression of NQO1 similarly to CHX at a concentration of 10 μg/ml (Figure 2B). Therefore, inhibition of protein synthesis is both necessary and sufficient for blocking the induction of NQO1 by protein synthesis inhibitors. Together, these findings indicate that a labile or newly induced protein factor functions as a common regulator for both constitutive and inducible expression of NQO1 by all inducers tested in the study.

Inhibition of NQO1 induction by CHX is transcriptional

To further characterize the inhibition of NQO1 induction by CHX, we examined the concentration- and time-dependence of the process. CHX blocks NQO1 induction by TCDD or tBHQ in a concentration-dependent manner (Figure 3A). CHX completely inhibits induction at a concentration of 10^4 ng/ml, at which over 90% of protein synthesis is inhibited in the cells [35]. Thus inhibition of NQO1 by CHX requires substantial inhibition of cellular protein synthesis. In Figure 3(B), TCDD (lanes 5–7) or tBHQ (lanes 11–13) induces NQO1 time-dependently during a 5 h induction course. Co-treatment with CHX blocks the induction throughout the time course (Figure 3B, lanes 8–10 and 14–16). The complete inhibition of NQO1 induction in both early and late phases of induction suggests that the function of the CHX-sensitive factor in NQO1 induction is dependent upon new protein synthesis to a large extent.

Inhibition of NQO1 mRNA expression can be transcriptional, in which CHX inhibits the synthesis of RNA, or due to a decrease in the stability of NQO1 mRNA, in which CHX increases the turnover of NQO1 mRNA. To distinguish these possibilities, the cells were treated either with TCDD alone for 2.5 or 5 h, or with TCDD for 2.5 h followed by TCDD plus CHX for an additional 2.5 h (Figure 4A). The result reveals that induction of

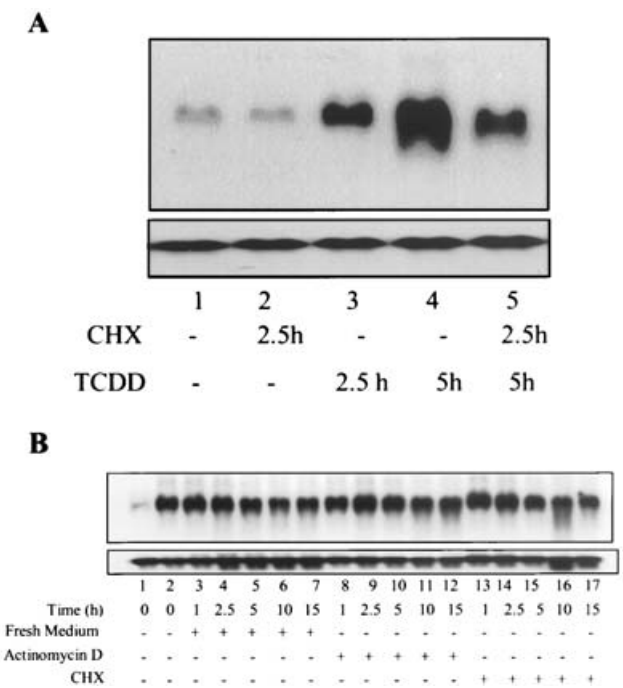

Figure 4 Effect of CHX on NQO1 mRNA stability

(**A**) Cells were treated with DMSO, CHX (10 μg/ml) for 2.5 h, TCDD (1 nM) for 2.5 h, TCDD for 5 h or TCDD for 2.5 h followed by TCDD + CHX for 2.5 h. (**B**) Cells were treated with DMSO (lane 1) or TCDD (1 nM, 5 h, lanes 2–17). The cells were washed with fresh medium three times and then treated as indicated. Actinomycin D was used at 2 μg/ml and CHX at 10 μg/ml. Total RNA was analysed for NQO1 and actin as described for Figure 1.

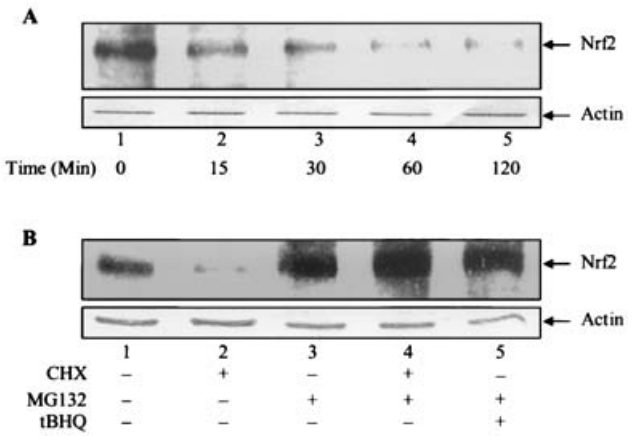

Figure 5 Degradation of Nrf2 by the 26 S proteasomes

(**A**) Hepa1c1c7 cells were grown in six-well plates and were treated with CHX (10 μg/ml) for increasing period of time. Total cell lysate was prepared and analysed by PAGE for Nrf2 as described in the Materials and methods section. The same samples were measured for actin protein to ensure equal loading. (**B**) Cells were treated with CHX (10 μg/ml), MG132 (25 μM), tBHQ (100 μM), or in combinations as shown, for 5 h. Total cell lysate was immunoblotted for Nrf2 and actin proteins as described for (**A**).

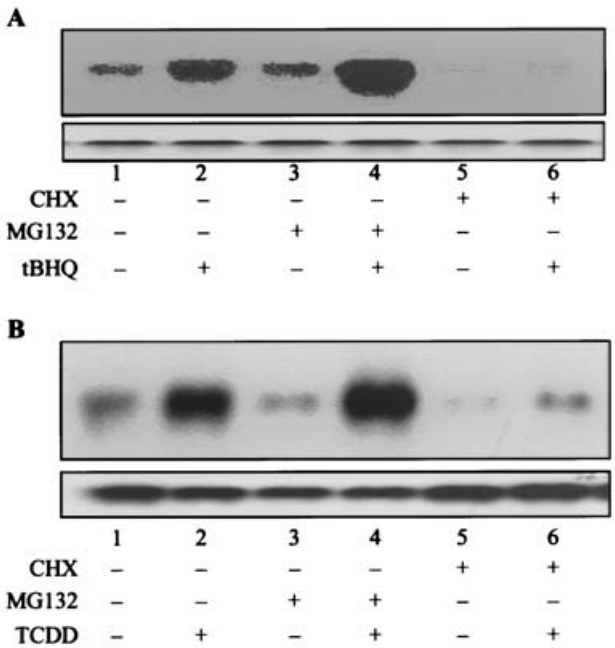

Figure 6 Effect of MG132 on NQO1 induction

(**A**) Cells were treated with tBHQ (100 μM), MG132 (25 μM), CHX (10 μg/ml), or in combinations as shown, for 5 h. (**B**) Cells were treated with TCDD (1 nM), MG132, CHX, or in combinations as shown, for 5 h. Total RNA was prepared and analysed by Northern blotting for NQO1 and actin mRNA as described for Figure 1.

NQO1 in cells treated with TCDD for 5 h plus CHX for 2.5 h is nearly the same as that treated with TCDD alone for 2.5 h, suggesting that CHX does not affect the level of existing NQO1 mRNA, but blocks the increase of newly synthesized mRNA. To examine whether CHX affects the turnover of NQO1 mRNA, the stability of NQO1 mRNA was analysed. As shown in Figure 4(B), mouse NQO1 mRNA is stable with a $t_{1/2}$ of >15 h in the absence (lanes 2–7) or presence (lanes 8–12) of actinomycin D, an inhibitor of mRNA synthesis. Treatment with CHX does not change the stability of NQO1 mRNA (Figure 4B, lanes 12–17). Taken together, the results reveal that the CHX-sensitive factor controls the basal and inducible expression of NQO1 mRNA expression at the level of gene transcription.

Nrf2 is rapidly degraded through the ubiquitin-26 S proteasome pathway

Nrf2, which mediates induction of phase II enzymes by tBHQ, was recently found to be involved in the basal expression of GST in mouse liver [36]. This observation raises the possibility that Nrf2 serves as a target molecule of CHX and is responsible for the inhibition of NQO1 transcription by CHX. To test the notion, we examined the turnover of Nrf2. Immunoblot analyses reveal that Nrf2 is constitutively expressed in mouse hepatoma cells (Figure 5A, lane 1). Treatment with CHX rapidly reduces the protein level of Nrf2 in a time-dependent manner (Figure 5A, lanes 2–5). The $t_{1/2}$ value of the Nrf2 protein is <30 min; thus murine Nrf2 is a labile protein in the absence of an activator. Similar observations were made in other laboratories for human and mouse Nrf2 proteins [34,37,38]. In Figure 5(B, lane 2), treatment with CHX for 5 h reduces the Nrf2 protein to less than 15% of the control; however, treatment with MG132 (25 μM, 5 h), a potent inhibitor of the 26 S proteasomes, increases the amount of Nrf2 (Figure 5B, lanes 3 and 1). Furthermore, co-treatment with MG132 and CHX blocks the reduction of Nrf2 by CHX (Figure 5B, compare lane 4 with lane 2). Co-treatment with MG132 and tBHQ (Figure 5B, lane 5) increases the amount of Nrf2 similarly to MG132 alone. Together, these findings suggest that the turnover of Nrf2 in the absence of an activator is mediated through the 26 S proteasome pathway. Next, the functional impact of Nrf2 degradation on NQO1 expression was examined (Figure 6). Expression of NQO1 mRNA is increased by tBHQ (100 μM, 5 h), whereas co-treatment with MG132 (25 μM, 5 h) enhances the induction (i.e. superinduction; Figure 6A, compare lanes 4 and 2), which is in agreement with increased amount of Nrf2 protein under similar conditions (Figure 5B, lane 5). However, MG132 alone does not change the level of NQO1 expression in the absence of a NQO1 inducer (Figure 6A, compare lane 3 with 1), even though it increases the protein level of Nrf2. Similar results were observed when treated with TCDD instead of tBHQ (Figure 6B). Therefore, blocking Nrf2 degradation by MG132 alone is not sufficient for increasing NQO1 transcription, but additional activator-induced modifications of Nrf2 are required for the induction of the gene. MG132 superinduces CYP1A1 in the presence of TCDD under similar conditions [33]. Thus superinduction of gene transcription by MG132 is inducer-dependent. Taken together, these results reveal that the function of Nrf2 is susceptible to inhibition of protein synthesis and may serve as the CHX-sensitive transcription factor for NQO1 transcription.

Nrf2 controls the basal expression and induction of NQO1 by both AhR ligands and phenolic antioxidants

We took a genetic approach to further confirm the role of Nrf2 in NQO1 gene transcription. MEF cells were isolated from Nrf2-knockout and wild-type mice. The MEF cells were examined for NQO1 expression and induction by various inducers. As shown in Figure 7(A), NQO1 is constitutively expressed in wild-type MEF cells ($Nrf2^{+/+}$) and is induced by tBHQ similarly to

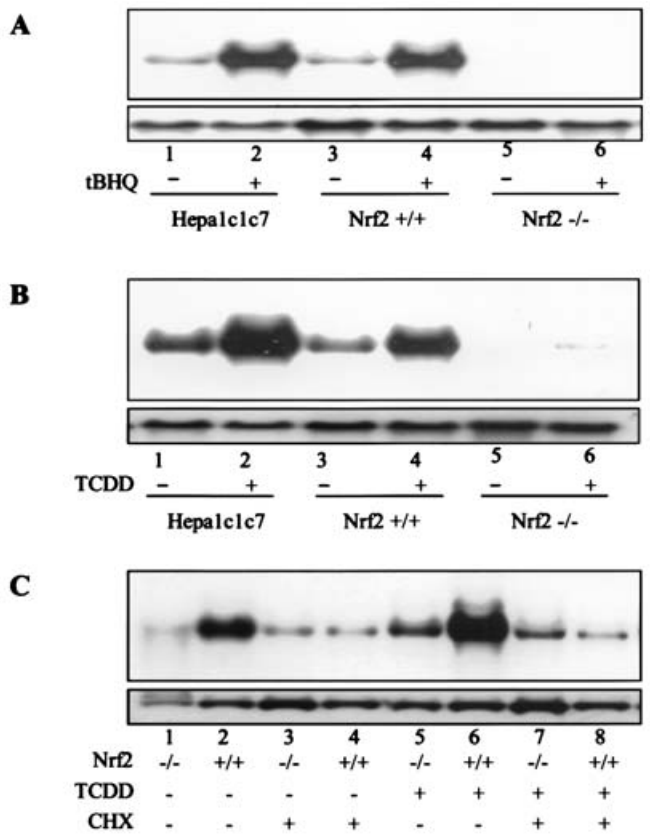

Figure 7 Nrf2-dependence of NQO1 induction

(**A**, **B**) Hepa1c1c7, MEF Nrf2$^{+/+}$ and MEF Nrf2$^{-/-}$ cells were treated with DMSO, tBHQ (100 μM; **A**) or TCDD (1 nM; **B**) for 5 h. (**C**) MEF Nrf2$^{+/+}$ or Nrf2$^{-/-}$ cells were treated with CHX, TCDD or both for 5 h. Total RNA was prepared and analysed by Northern blotting for NQO1 and actin as described for Figure 1.

induction in hepa1c1c7 cells (Figure 7A, compare lane 3 with 1 and lane 4 with 2). However, both the basal and tBHQ-inducible expressions are lost in Nrf2$^{-/-}$ MEF cells (Figure 7A, lanes 5 and 6). Similar experiments were carried out for induction by TCDD (Figure 7B). TCDD induces NQO1 in hepa1c1c7 and Nrf2$^{+/+}$ MEF cells, but not in Nrf2$^{-/-}$ MEF cells. In a separate experiment, the effect of CHX on NQO1 induction in the MEF cells was examined (Figure 7C). Treatment with CHX (10 μg/ml, 5 h) completely blocks the basal expression of NQO1 in Nrf2$^{+/+}$ cells (Figure 7C, compare lane 4 with 1, 2 and 3). Co-treatment with CHX and TCDD inhibits the induction of NQO1 by TCDD in Nrf2$^{+/+}$ cells (Figure 7C, compare lanes 8 and 6) and the slight induction in Nrf2$^{-/-}$ cells (Figure 7C, compare lanes 7 and 5). Together, these findings provide genetic evidence that Nrf2 is required for the basal expression and induction of murine NQO1 by both Nrf2 and AhR activators.

Because AhR and Arnt are required for induction of a number of TCDD-inducible genes by AhR ligands, we used AhR- or Arnt-defective variant hepatoma cells to examine the role of the AhR/Arnt pathway in the induction of NQO1. Induction of CYP1A1 by TCDD, which requires AhR and Arnt, was measured to ensure functional defect in AhR and Arnt (Figure 8, top panel). CYP1A1 mRNA is induced by TCDD and superinduced by TCDD plus CHX in wild-type cells (hepa1c1c7; Figure 8, top panel, lanes 5 and 6). The induction and superinduction of CYP1A1 are totally lost in Arnt-defective cells (Figure 8, top panel, lanes 7–12), but are detectable in AhR-defective cells (Figure 8, top panel, lanes 13–18), which express $\approx$5–10% of functional AhR compared with wild-type cells [32]. In wild-type cells, NQO1 is induced by tBHQ or TCDD and the induction is blocked by CHX as expected (Figure 8, middle panel, lanes 1–6). However, NQO1 is induced by tBHQ, but not TCDD, in Arnt-defective variants (Figure 8, middle panel, lanes 9 and 11). In AhR-defective cells, NQO1 is induced by tBHQ and, to a lesser extent, by TCDD (Figure 8, middle panel, lanes 15 and 17). Induction of NQO1 in Arnt- or AhR-defective cells is inhibited by CHX similarly to that in wild-type cells. Taken together, these results demonstrate that induction of NQO1 by TCDD requires AhR, Arnt and Nrf2, whereas induction by tBHQ requires Nrf2, but is independent of AhR and Arnt.

The observation that Nrf2 is labile suggests that other Nrf2-regulated genes are susceptible to inhibition by CHX. To test this possibility, we examined the effect of inhibition of protein synthesis on the induction of Aldh3a1, which encodes an aldehyde dehydrogenase, and HO-1, which is involved in haem catabolism. Aldh3a1 is inducible by tBHQ through ARE-dependent transcription [39]. Figure 9 shows that expression of Aldh3a1 is barely detectable in the absence of an inducer. Treatment with tBHQ induces the expression of Aldh3a1 (Figure 9, lane 3). Co-treatment with CHX reduces the induction by tBHQ to less than 15% (Figure 9, compare lanes 4 and 3).

HO-1 is highly inducible by a number of oxidative stress signals; biochemical evidence suggests induction is mediated through ARE-/Nrf2-dependent pathways [40]. As shown in

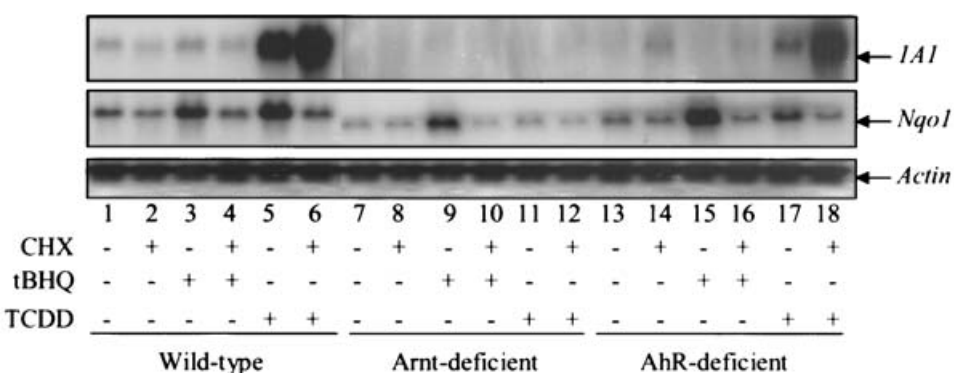

Figure 8 AhR- and Arnt-dependence of NQO1 induction

Wild-type (hepa1c1c7), AhR-defective and Arnt-defective cells were treated with DMSO, CHX, tBHQ, TCDD, or combinations as shown, for 5 h. Total RNA was prepared and analysed for expression of CYP1A1 (1A1; top panel), NQO1 (middle panel) and actin (bottom panel).

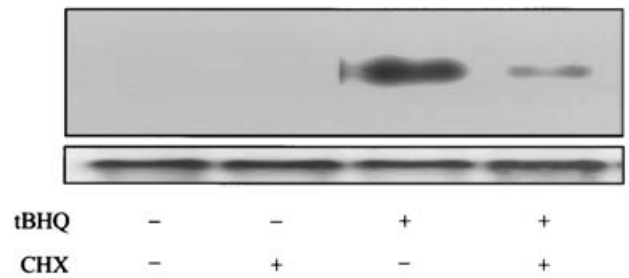

Figure 9 Effect of CHX on Aldh3a1 induction

Hepa1c1c7 cells were treated with tBHQ, CHX or both for 5 h. Total RNA was analysed by Northern blotting for induction of Aldh3a1 (upper panel) and actin expression (lower panel).

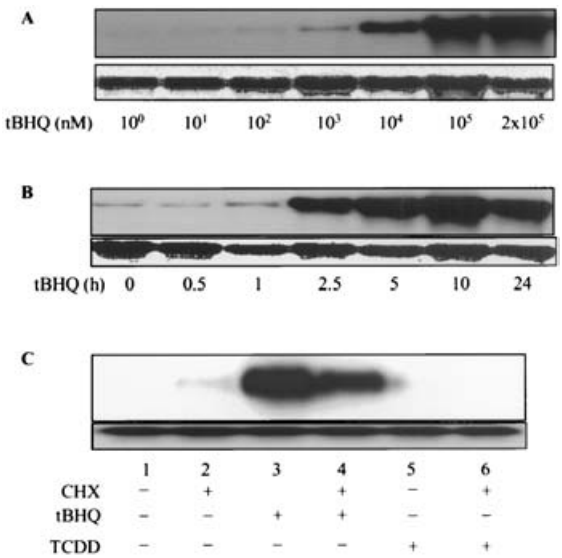

Figure 10 Effect of CHX and TCDD on HO-1 expression

(**A**) Dose-response study. Hepa1c1c7 cells were treated with increasing concentrations of tBHQ for 5 h. (**B**) Cells were treated with 100 μM tBHQ for indicated time periods. (**C**) Cells were treated with CHX, tBHQ, TCDD, or combinations as shown, for 5 h. Total RNA was prepared and analysed by Northern blotting for HO-1 (upper panels). Actin was measured to ensure equal loading (lower panels).

Figure 10(A) and 10(B), tBHQ induces HO-1 in hepatoma cells dose- and time-dependently; the EC_{50} and $t_{1/2}$ of maximal induction were at 10 nM and 1.5 h, which are similar to those of induction of NQO1 (results not shown). DMSO or CHX alone do not affect the expression of HO-1, but tBHQ induces HO-1 mRNA expression dramatically. Co-treatment with CHX inhibits the induction of HO-1 by tBHQ (Figure 10C). These findings are consistent with the notion that induction of HO-1 and Aldh3a1 by phenolic antioxidants involves Nrf2 or an Nrf2-like factor(s), which is labile and is inhibited by protein synthesis inhibitors. However, the low levels of basal expression of HO-1 and Aldh3a1 and incomplete inhibition of HO-1 induction by CHX in comparison with those of NQO1 suggest that gene transcription of NQO1, HO-1 and Aldh3a1 differs substantially.

It has been shown that TCDD treatment increases ROS (reactive oxygen species) production from mitochondria in mice; therefore, it is possible that TCDD induces ARE-dependent gene transcription by increasing ROS production, which leads to activation of Nrf2. To test the possibility, we examined whether TCDD induces HO-1. As shown in Figure 10(C), treatment with TCDD alone or TCDD plus CHX for 5 h has no observable effect on the expression of HO-1 as compared with tBHQ or tBHQ plus CHX. In similar experiments, tBHQ is shown to induce metallothionein 1, whereas TCDD fails to induce (results not shown; Y. Bi, R. D. Palmiter, K. M. Wood and Q. Ma, unpublished work). Thus TCDD does not affect expression of HO-1 and metallothionein 1, which are inducible by antioxidant/oxidative inducers.

DISCUSSION

Nrf2 is a master regulator of mouse NQO1 transcription

Phase II drug-metabolizing enzymes such as NQO1, GST and UDP-glucuronosyltransferase catalyse the metabolic reduction and conjugation reactions of endogenous and exogenous chemicals; substrates of phase II enzymes are involved in a broad range of biological functions, including drug therapy, hormonal homoeostasis, oxidative stress, chemical toxicity, cancer and certain diseases [9–11,14]. Phase II enzyme-catalysed host–chemical interactions are often considered as protective responses, because loss or reduction of the activities is often associated with increased risk of disease or chemical toxicity, whereas induction of the enzymes reduces such risks. As a group, phase II enzymes exhibit certain unique patterns of gene regulation: they are constitutively expressed in a broad range of animal tissues and cell lines, and are often highly inducible by both AhR ligands and phenolic antioxidants. Inducers of drug-metabolizing enzymes can be classified into two categories: bifunctional inducers (i.e. AhR ligands such as TCDD and benzo[*a*]pyrene) induce both phase I and phase II genes through DRE and AhR; whereas monofunctional inducers (i.e. diphenols, thiocarbamates, isothiocyanates and coumarins) induce phase II enzymes through ARE and Nrf2 [22,41]. Certain AhR agonists (for example BNF and ANF) can be metabolized to monofunctional inducers and thus can induce phase II genes through both DRE- and ARE-dependent pathways. Therefore, at least three different pathways exist to mediate the basal, TCDD-inducible and tBHQ-inducible expression of the enzymes. In this study, we analysed the molecular steps controlling the induction of mouse NQO1, which serves as a model for analysing mechanisms of gene regulation of phase II enzymes.

In an attempt to identify new proteins in the regulation of NQO1 transcription, we found that a CHX-sensitive transcription factor controls both basal and inducible expressions of NQO1 by AhR ligands and Nrf2 activators, because inhibition of protein synthesis by CHX or puromycin completely blocks the basal expression and induction of the gene. Two lines of evidence suggest that Nrf2 serves as the CHX-sensitive transcription factor controlling three types of NQO1 transcription: the basal, TCDD-inducible and tBHQ-inducible expression. First, analyses of Nrf2 turnover reveal that it is rapidly degraded through the ubiquitin-26 S proteasome-mediated proteolysis ($t_{1/2} < 30$ min). Blocking protein synthesis rapidly reduces the steady state level of Nrf2 protein (to less than 15 % of control in 5 h) in cells in the absence of an activator; reduction of Nrf2 protein by CHX correlates with inhibition of NQO1 induction. Secondly, genetic analyses using $Nrf2^{-/-}$ MEF cells demonstrate that the basal expression and induction by TCDD and tBHQ are lost in the absence of functional Nrf2 similarly to inhibition by CHX. While this paper was under review, Yamamoto and associates reported similar observations in which 3-methylcholanthrene (an AhR agonist and bifunctional inducer) fails to induce NQO1 in $Nrf2^{-/-}$ mouse liver [42]. Taken together, these findings support the notion that Nrf2 is required for the basal expression and induction of NQO1 by both mono- and

bifunctional inducers. The nature of the Nrf2 complex mediating NQO1 transcription is currently unclear. A number of 'small' and 'large' Maf proteins are capable of forming dimers with Nrf2 and related transcription factors [44]. The specificity and functional relevance of dimer formation between Nrf2 and the Maf proteins with respect to NQO1 gene regulation is not well understood at present. Further studies are needed to provide insights into the possible regulatory roles of the complex components other than Nrf2.

Cross-interaction between AhR and Nrf2 signal transduction in NQO1 transcription

Genetic analyses of NQO1 induction using AhR-, Arnt- or Nrf2-defective cells clearly established that induction of NQO1 by TCDD requires all three transcription factors. Furthermore, the findings suggest, for the first time, that a functional interaction between AhR- and Nrf2-mediated gene transcription is involved in NQO1 induction. The mechanism of such interaction is currently unclear. However, several possibilities can be proposed. Studies by Favreau and Pickett [23] identified the antioxidant-responsive and dioxin-responsive elements in rat NQO1. We have sequenced the enhancer region of mouse NQO1 and identified ARE and DRE that are similar in sequence and location to the rat elements. The putative ARE and DRE sequences are closely located to each other. The proximity of the two sites suggests a possible functional overlap between DRE and ARE. In this scenario, ARE and DRE function as a 'composite' response element to which both AhR and Nrf2 bind and mediate the induction of NQO1 by TCDD. Alternatively, AhR and Nrf2 may interact with each other directly or through an adaptor protein; such interactions are required for induction of NQO1 by TCDD. Interactions of AhR with other transcription factors have been found to be necessary for transcriptional regulation of a number of genes by TCDD [6–8]. Finally, treatment with TCDD may induce the activation of Nrf2, which mediates NQO1 induction. It is known that activation of Nrf2 by tBHQ involves modification of the phosphorylation status of the protein, because several kinase or phosphatase inhibitors can inhibit or potentiate Nrf2 functions [44]. We observed that increasing the protein level of Nrf2 by MG132 enhances induction of NQO1 in the presence of either TCDD or tBHQ, but not in the absence of an inducer, consistent with the notion that modification of the transcription factors occurs after activation by inducers. A recent study by Nebert and associates [45] suggests that TCDD induces the production of mitochondrial ROS, which is AhR-dependent. Thus it is possible that TCDD induces NQO1 by increasing ROS, which in turn activates the Nrf2/ARE pathway. However, the findings that induction of NQO1 by TCDD requires DRE and that TCDD fails to induce ROS-inducible genes HO-1 and metallothionein 1 in hepatoma cells but tBHQ does negate such a role of ROS in the induction of NQO1 by TCDD in our experimental system. Although we are unable to distinguish these possible mechanisms in the induction of NQO1 by TCDD at present, this model provides a testable scheme of NQO1 gene regulation, in which the basal, TCDD-inducible and tBHQ-inducible expression of the gene can be analysed and understood as an entity of gene transcription. Knowledge obtained from the model will provide new insights into gene regulation of phase II enzymes by endogenous and xenobiotic inducers.

We thank Dr C. B. Pickett (Schering-Plough Research Institute, Kenilworth, NJ, U.S.A.) for generously providing antibodies specific for Nrf2, Dr X. Hu and Dr S. Benkovic for NIOSH internal review, and I. Kent Reed for critical comments on the manuscript.

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Received 24 July 2003/17 September 2003; accepted 26 September 2003
Published as BJ Immediate Publication 26 September 2003, DOI 10.1042/BJ20031123