

Regulation of NAD(P)H:Quinone Oxidoreductase 1 Gene Expression by CYP1A1 Activity

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ABSTRACT

The dioxin 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) induces phase I and II xenobiotic metabolizing enzymes (XME) which act sequentially to eliminate different classes of xenobiotics. The transcriptional effects of TCDD are generally mediated by the arylhydrocarbon receptor (AhR). We hypothesized that TCDD could also act indirectly, by increasing the activity of cytochrome P450 1A1 (CYP1A1), a phase I gene, which could then mediate the induction of other XME genes, such as the NAD(P)H:quinone oxidoreductase 1 (NQO1). To test this hypothesis, NQO1 gene expression was monitored after either overexpression of CYP1A1 or siRNA-mediated knock-down of CYP1A1 activity in the hepatoma cell line HepG2. Overexpression of CYP1A1 in the absence of TCDD was carried out using either adenoviral infection or the "Tet-off" system. Recombinant adenoviruses were produced encoding no protein, CYP1A1 (Ad1A1), or a mutated inactive CYP1A1 (Ad1A1mut).

In the HepG2 Tet-off cell line, CYP1A1 expression was induced by the removal of doxycycline (dox) from the cell medium. Ad1A1 infection or dox removal induced CYP1A1 activity and H₂O₂ production similarly to TCDD treatment. Moreover, in both systems, the amount of NQO1 mRNA increased to the same level as after TCDD treatment (approximately 2-fold). The UDP-glucuronosyl transferase 1A6 (UGT1A6) gene is also similarly regulated. NQO1 gene expression was not induced when mutant, inactive CYP1A1 was overexpressed or when the antioxidant *N*-acetyl cysteine (NAC) was added to Ad1A1. Finally, either NAC or siRNA directed against CYP1A1 mRNA decreased the induction of NQO1 gene expression by TCDD. We conclude that, after exposure to TCDD, the NQO1 gene expression can be controlled by CYP1A1 activity through an oxidative stress mediated pathway.

Humans are exposed to a large variety of xenobiotics such as environmental pollutants and food contaminants, which tend to accumulate and perturb several cellular functions. One of these xenobiotics is the dioxin 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD), which is released by the combustion of organic compounds in the presence of chlorine. In rodents, TCDD, as well as polycyclic aromatic hydrocarbons (PAH), induces a variety of toxic effects, including immunotoxicity, reproductive disorders, hepatotoxicity, and cancers (Huff et al., 1994; Pohjanvirta and Tuomisto, 1994). However, TCDD toxicity is highly dependent on the species (Hengstler et al., 1999). In humans, chloracne was unequivocally found (Needham et al., 1997) but high TCDD exposure

(100–1000 times higher than those experienced by the general population) results in an excess of all cancer combined without any marked specificity (Steenland et al., 1999).

Cells respond to the continuous exposure to potentially toxic xenobiotics by the induction of xenobiotic metabolizing enzymes (XME). The most common phase I metabolic enzymes are cytochrome P450 monooxygenases (P450s), which function by inserting one atom of oxygen into the substrate. Other XMEs, such as glutathione-*S*-transferases, UDP-glucuronosyltransferases, or sulfotransferases, add a highly hydrophilic group to the oxygenated substrate to facilitate its elimination. NAD(P)H:quinone oxidoreductase 1 (NQO1) can also decrease the toxicity of the toxic metabolites by modifying their redox status. Finally, transport proteins excrete the modified compound out of the cell. A stringent control of the metabolic pathway enzymes is critical for an efficient detox-

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ABBREVIATIONS: TCDD, 2,3,7,8 tetrachloro-dibenzo-*para*-dioxin; PAH, polycyclic aromatic hydrocarbon; XME, xenobiotic-metabolizing enzyme; BaP, benzo[a]pyrene; AhR, aryl hydrocarbon receptor; XRE, xenobiotic responsive element; NQO1, NADPH quinone oxidoreductase 1; ROS, reactive oxygen species; H₂DCF-DA, dihydro 2',7'-dichlorofluorescein diacetate; DCPIP, 2,6-dichlorophenolindophenol; CMV, cytomegalovirus; PCR, polymerase chain reaction; FBS, fetal bovine serum; tTA, tetracycline-controlled transactivator; TRE, Tet responsive element; Adnull, adenovirus encoding no protein; Ad1A1, adenovirus encoding CYP1A1; Ad1A1mut, adenovirus encoding an inactive mutated CYP1A1; dox, doxycycline; UGT1A6, UDP-glucuronosyl transferase 1A6.

ification of these xenobiotics. This is well illustrated by the metabolism of benzo[*a*]pyrene (BaP), a PAH that is found in tobacco smoke, diesel particles, and barbecued meat. BaP is a procarcinogen that undergoes several steps of metabolic activation by various enzymes, including CYP1A1 (Shimada et al., 1994), CYP1B1, and epoxide hydrolase (Shimada et al., 1999), to yield highly toxic intermediate metabolites, such as the *anti* or *trans* isomer of BaP-*r*-7,*t*-8 dihydrodiol-*t*-9,10-epoxide. Some intermediate metabolites can be conjugated by XMEs, such as glutathione-*S*-transferase and UDP-glucuronosyltransferase, and then eliminated (Jin et al., 1993; Srivastava et al., 1999). Other BaP metabolites containing a quinone function are detoxified by NQO1 (Joseph and Jaiswal, 1994). Studies in mice lacking a functional NQO1 gene demonstrated that NQO1 is also an endogenous protector against BaP-induced carcinogenicity (Long et al., 2000). Therefore, in the case of BaP exposure, an adequate balance between the activities of the various XMEs involved in its metabolism is critical to avoid accumulation of intermediate toxic metabolites.

TCDD and PAH induce the expression of a set of XMEs: *CYP1A1*, *CYP1A2*, *CYP1B1*, *NQO1*, *UGT1A6*, and *GSTYα*. These genes constitute the so-called "Ah gene battery" (Nebert et al., 1990, 2000). The best characterized pathway by which TCDD and PAH exert their effects is the arylhydrocarbon receptor (AhR) pathway: these compounds bind to the AhR, which in turn heterodimerizes with the AhR nuclear translocator Arnt. The AhR-AhR nuclear translocator complex binds to xenobiotic responsive elements (XRE) of target genes and activates these promoters (Okey et al., 1994). Large-scale gene expression experiments using DNA microarrays have shown that a large number of genes (more than 100) are induced by TCDD (Puga et al., 2000; Frueh et al., 2001). Interestingly, the induction of the majority of these genes is prevented by cycloheximide treatment, an inhibitor of protein synthesis, suggesting that induction by dioxin can also be a secondary response (Puga et al., 2000).

Regulation by TCDD and PAH differs among the members of the Ah gene battery. Indeed, although CYP1A1 is the most potently induced gene (up to 50-fold) (Whitlock, 1999), other XMEs of the Ah gene battery are moderately induced (Paulson et al., 1990; Jaiswal, 1991; Munzel et al., 1996). Furthermore, XME genes such as *NQO1* and *GSTYα* are induced by various oxidants and compounds involved in redox cycling (Nguyen et al., 1994; Jaiswal, 2000), and this regulation is mediated by the antioxidant-responsive element through the binding of several transcription factors such as Nrf2 (Venugopal and Jaiswal, 1998; Dhakshinamoorthy et al., 2000; Jaiswal, 2000). In the case of the human *NQO1* gene, an AhR-independent regulation of TCDD has been demonstrated. This gene does not contain active XREs and is induced by TCDD through the antioxidant-responsive element pathway (Radjendirane and Jaiswal, 1999). Furthermore, previous studies have shown that the P450 catalytic activity is accompanied by the release of reactive oxygen species (ROS) (Bondy and Naderi, 1994; Puntarulo and Cederbaum, 1998). ROS production was implicated in the autoregulatory loop repressing the *CYP1A1* gene promoter (Morel and Barrouki, 1998; Morel et al., 1999). Another study showed that the CYP2E1 promoter was similarly inhibited by ROS production by CYP1A1 (Morel et al., 2000). Although these studies were done mainly by cotransfection experiments, they

clearly suggested that increased CYP1A1 activity could alter gene expression by a ROS-mediated mechanism. These observations together with genetic evidence from several mutant mouse hepatoma cell lines (Raychaudhuri et al., 1990; Vasiliou et al., 2000) raised the possibility that P450 activity could play a modulatory function in the expression of other xenobiotic-metabolizing enzyme genes.

Our working hypothesis is that the activity of phase I enzymes, particularly CYP1A1, regulates the gene expression of other XME genes, such as the *NQO1* gene, to convey a better elimination of xenobiotics out of the cell without any adverse effects caused by the accumulation of toxic metabolites. To demonstrate the role of CYP1A1 in the regulation of *NQO1* expression, we overexpressed CYP1A1 using two independent methods which allow increase of CYP1A1 activity without activating the AhR pathway. We showed that overexpression of CYP1A1 using an adenoviral vector or the "Tet-off" inducible system leads to the induction of both the *NQO1* and the UDP-glucuronosyl transferase 1A6 (*UGT1A6*) endogenous genes in HepG2 cells. This induction is probably mediated by the enzymatic activity of CYP1A1 and the reactive oxygen species produced by CYP1A1.

Materials and Methods

Chemicals. TCDD was purchased from LCG Promochem (Molsheim, France). Ethoxy-resorufin, 2',7'-Dichlorofluorescein diacetate (H₂DCF-DA), 2,6-dichlorophenolindophenol (DCPIP), and all other chemicals were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Oligonucleotides were obtained from Genset (Paris, France).

Recombinant Adenovirus Production. Adenovirus production was performed by the vector core facility of the University Hospital of Nantes (supported by the Association Française contre les Myopathies). We used a plasmid shuttle (pShuttleCMV) in which we inserted either human CYP1A1 cDNA or human CYP1A1 cDNA with a single amino acid substitution (Cys457→Ser) between the CMV promoter and the polyadenylation site of the plasmid. The wild-type CYP1A1 cDNA was obtained by XbaI and KpnI digestion of pRSV-CYP1A1 vector, which contains 1560 base pairs of the human CYP1A1 cDNA (Morel et al., 1999). It was subcloned into XbaI-digested pShuttleCMV using a KpnI-XbaI linker (5'-CTAGGTAC-3'). Therefore, the recombinant CYP1A1 lacks the 3' nontranslated region of the endogenous gene and uses a vector polyadenylation site.

The next step was to allow the recombination of these plasmids with pAd-Easy, a plasmid that contains the adenovirus genome, except the sequences covering mainly the E1 gene, which is responsible for the virulence of the adenoviruses. Three types of viruses were produced: those encoding the CYP1A1 protein (Ad1A1), the mutated CYP1A1 protein (Ad1A1mut), or those encoding no protein (Adnull). They were all amplified in 293 cells and titered by plaque assay on 293 cells as described previously (He et al., 1998).

Generation of an Inactive CYP1A1. To obtain an inactive CYP1A1 that cannot bind the heme, we mutated the heme axial cysteine of CYP1A1, Cys457 (Kawajiri et al., 1986; Xia et al., 1993). This mutated CYP1A1 has no catalytic activity and is unable to produce oxidative stress. The mutation was generated by PCR using the pRSV-1A1 plasmid and four oligonucleotides: A, 5'-GTTCTA-GAGGAACCATGC-3' (XbaI site underlined, initiation codon in bold); B, 5'-CCGGTACCTTAGGCCTCAG-3' (KpnI site underlined, stop codon in bold); C, 5'-AGCGGAAGTCCATCGGTG-3' with the mutation (TGT→TCC) in CYP1A1 sequence that changes Cys-457 into Ser; D, 5'-TCACCGATGGACCTTCCGC-3' with the mutation (ACA→GGA) in CYP1A1 sequence.

The PCR-amplified products A-D and B-C were purified using the QIAquick PCR purification kit from QIAGEN (Les Ulis, France) and

another PCR reaction with fragments A-D and B-C using oligonucleotides A and B was carried. This amplification yielded the PCR product A-B, which was digested by XbaI and KpnI and subcloned into XbaI-digested pShuttleCMV by using the KpnI-XbaI linker.

Tet-Off Plasmid Construction. The pTet-off and pTRE2pur vectors were obtained from BD Biosciences Clontech (Montigny-le Bretonneux, France). The pTet-off vector encodes the tetracycline-controlled transactivator (tTA) that binds specifically to the Tet response element (TRE). tTA activates transcription of promoters containing TRE in the absence of tetracycline or doxycycline. The pTRE2pur vector consists of the TRE and the minimal CMV promoter upstream a multiple cloning site and includes an internal selection gene encoding puromycin resistance.

The EcoRV-KpnI fragment of pRSV-1A1 containing the human CYP1A1 cDNA was inserted into EcoRV-digested and dephosphorylated pTRE2pur in the presence of a phosphorylated KpnI-EcoRV linker: 5'-GTAC-3'. The resulting pTRE2pur-1A1 vector comprises the CYP1A1 cDNA downstream of the TRE- minimal CMV promoter.

Cell Culture and Adenoviral Infection. Human hepatocarcinoma HepG2 cells were cultured at 37°C in 50% Dulbecco's minimal essential medium and 50% Ham's F12 medium (Invitrogen, Cergy Pontoise, France) containing 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere in 5% CO₂. Infection with adenoviruses was carried as described previously (Burcin et al., 1999). Briefly, on day one, 5×10^5 cells per well were seeded into six-well plates (Falcon). On day 2, they were allowed to grow to 60 to 70% confluence and were infected with Adnull, Ad1A1, or Ad1A1mut at a multiplicity of infection of 160 (8×10^7 infectious particles per well). This multiplicity leads to 95 to 100% cell infection as determined using an Adβ-gal infection followed by X-Gal staining. The infection lasted for 4 h in a medium containing 2% FBS. Then, the medium was replaced by 10% FBS containing medium. 24 to 48 h later, cells were collected for EROD activity assay, oxidative stress measurement, or RNA preparation.

Establishment of HepG2 Subclones that Overexpress CYP1A1 under the Control of Tetracycline: HepG2 Tet-off Cells. Five million HepG2 cells were first cotransfected with 5 µg of pTet-off vector and with 1 µg of pSV2-Neo, a vector which provides neomycin or G418 resistance (BD Biosciences Clontech). Transfection was performed using a standard calcium phosphate coprecipitation technique described previously (Morel and Barouki, 1998). Clones selected with 800 µg/ml G418 for about 4 weeks were then isolated and tested for their transactivating activity by transient transfection with pTRE2-Luc, a vector that contains the luciferase gene downstream of the TRE- minimal CMV promoter (BD Biosciences Clontech). Luciferase assays were performed using a Promega kit (Promega, Madison, WI) on cells grown in the presence or absence of 1 µg/ml doxycycline (dox). Using this approach, we identified a clone displaying a 40-fold luciferase induction upon removal of doxycycline. This clone was then grown in HepG2 medium complemented with 2 µg/ml dox and 500 µg/ml G418 and 5 million cells were transfected with 4 µg of pTRE2pur-1A1. Transfected cells were selected using 2 µg/ml dox, 500 µg/ml G418, and 0.4 µg/ml puromycin, and the surviving clones were screened for the integration of CYP1A1 cDNA by measuring EROD activity after 8 days of culture with or without dox. One HepG2-Tet-off clone was selected for its high CYP1A1 activity after 8 days of culture without dox; EROD activity, oxidative stress measurement, and RNA expression were performed on this clone.

Ethoxyresorufin O-deethylase Activity Assay. EROD assays were performed as follows: 5×10^5 cells per well were plated on six-well plates. Four to 40 h after treatment or adenoviral infection, the medium was removed, and each well was washed with 2 ml of phosphate-buffered saline (Invitrogen) and then incubated with 5 µM ethoxyresorufin and 2 mM salicylamide in 1.5 ml of medium at 37°C. Kinetic reading with a Bio-Tek FL-600 fluorometer (Fisher, Elancourt, France) was performed over 40 min every 2 min with an

excitation wavelength of 530 nm and an emission of 590 nm. The slope of the resulting standard curve corresponding to the increased fluorescence over the time (milliunits of fluorescence per minute) reflects the CYP1A1 activity in the cells.

Measurement of Intracellular H₂O₂ Generation using H₂DCF-DA Probe. The oxidation-sensitive probe H₂DCF-DA diffuses into cells, where it is hydrolyzed by endogenous esterases into H₂DCF. Oxidation of H₂DCF yields compound 2',7'-dichlorofluorescein (DCF), which is fluorescent. Cells (5×10^5 per well) were cultured on six-well plates. Twenty-four hours after adenoviral infection, the medium was removed, and each well was incubated with 20 µM H₂DCF-DA in 2 ml of medium at 37°C for 40 min. The fluorescence of DCF was then measured with the FL-600 fluorometer (excitation at 485 nm; emission at 530 nm). In each well, 131 measures were made with a 3-mm diameter optic to cover the whole well surface. The null values obtained when no cells were over the optic were eliminated, and the result given was the sum of all the other values.

Isolation of RNA and Northern Blot Hybridization. Total RNA were isolated using the RNeasy midi kit from QIAGEN. Northern blot were performed as already described previously (Morel and Barouki, 1998) using 10 µg of ARN. The probe used to detect UGT1A6 mRNA consists of the first 700 base pairs of the coding sequence of UGT1A6 cDNA, it has been isolated by NcoI-BamHI digestion of pET11d-UGT1A6 (Harding, 1988). UGT1A6 probe covers the first exon of the gene. The probe used to detect NQO1 is a 795-base pair fragment (nucleotide 624-1419) of human NQO1 gene obtained by PCR from HepG2 genomic DNA using oligonucleotides 5'-TAGCATTGGGCACACTCCAGCAGACGCC-3' and 5'-AAGCTGACCAAGAGTGGTACTTGTGCTAGG-3'. The CYP1A1 probe consists of the entire coding sequence of the cDNA. A 3'-restricted probe specifically labels the endogenous CYP1A1 mRNA. A probe detecting the GRP94 mRNA was isolated by reverse transcription-PCR (nucleotide 633-1033 of human Grp94 cDNA) using oligonucleotides 5'-CAAAATGACTGAAGCACAGG-3' and 5'-GCTTCATCATCAGAT-TCTTC-3'. These probes were labeled using the Megaprime DNA labeling system (Amersham Biosciences, Orsay, France) and the hybridization was performed in the Rapid-hyb buffer (Amersham Biosciences) following the manufacturer's instructions. The membrane was washed for 45 min at 65°C with 2× SSC and 0.1% SDS and for 35 min at 65°C with 0.5× SSC and 0.1% SDS. Quantifications were performed with a PhosphorImager and the ImageQuant software (Amersham Biosciences).

Cellular Extracts. Twelve million HepG2 cells infected with Ad1A1 or Ad1A1mut were trypsinized. All subsequent steps were performed at 4°C. Cells were washed twice in 5 ml of PBS and centrifuged at 1500 rpm for 5 min. The pellets were then resuspended in an hypertonic buffer (0.25 mM sucrose, 10 mM Tris, and 1 mM EDTA, pH 7.4) containing an antiprotease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). Cells were sonicated 30 s with a Vibracell (Fisher Bioblok Scientific, Illkirch, France). Then a centrifugation of 20 min at 15,000 rpm was performed. The pellets were resuspended in 200 µl of buffer (100 mM NaPO₄, 10 mM MgCl₂, and 20% glycerol, pH 7.4). The protein concentration was measured according to the Bradford method (Bradford, 1976).

Western Blot. Each sample contained 50 µg of protein in 10% glycerol, 75 µM Tris, pH 6.8, 1% SDS, 0.5% pyronine, and 5% β-mercaptoethanol. Before loading, samples were boiled for 2 min and run at 8 mA for 16 h in a 9% polyacrylamide gel. Proteins were then transferred to nitrocellulose (Hybond C; Amersham Biosciences) by electroblotting (3 mA/cm², 1.5 h). The blot was incubated 3 h in PBS-0.5% Tween 20 with 0.1% polyvinyl pyrrolidone (Sigma-Aldrich) and then overnight at 4°C with a rabbit polyclonal CYP1A1 (De Waziers et al., 1990). After six washes of 5 min with PBS-Tween 20, the blot was incubated 30 min at 37°C with an anti-rabbit or an anti-goat IgG coupled to horseradish peroxidase (DakoCytomation Denmark A/S, Glostrup, Denmark). After six additional washes, the

peroxidase activity was measured with the enhanced chemiluminescence kit (Amersham Biosciences).

NQO1 Activity Assay. HepG2 cells were either treated by TCDD or infected by adenovirus for 30 h. Cells were washed 2 times with Hanks' balanced salt solution 1×, then scrapped in 3 ml of buffer (25 mM Tris, 250 mM sucrose, and 5 μ M FAD⁺) and sonicated. After 10 min of centrifugation at 15,000g, the supernatant was collected and stored at -80°C until assayed for NQO1 activity. The assay was performed as described by Ernster (1967) using DCPIP as a substrate in presence or absence of 20 μ M dicumarol. The reaction mix contained 25 mM Tris, pH 7.4, 0.07% bovine serum albumin (w/v), 200 μ M NADH, and 80 μ M DCPIP. The assay consisted in the monitoring of the decrease in absorbance at 600 nm because of the reduction of DCPIP. NQO1 activity was deduced by subtracting the residual activity obtained in the presence of dicumarol.

Design of CYP1A1-Specific Short Interfering RNA. CYP1A1-specific siRNA duplex sequence (CYP1A1-siRNA) was designed according to QIAGEN parameters (<http://www.qiagen.com/siRNA>). We verified that the selected region (+349 to +369 relative to the translation start site of human CYP1A1 cDNA (5'-AATGGTCAGAGCAT-GTCCTTC-3')) did not match any other human gene using the NCBI standard nucleotide-nucleotide BLAST program. CYP1A1-siRNA as well as control-siRNA (a nonsilencing siRNA designed by QIAGEN) were synthesized by Xeragon oligoribonucleotides, a QIAGEN company. The lyophilized oligoribonucleotides were resuspended in sterile annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, and 2 mM magnesium acetate, pH 7.4) to obtain a 20 μ M solution. They were heated at 90°C for 1 min followed by 1 h at 37°C to allow duplex formation and disrupt higher aggregates and then stored at -20°C .

siRNA Transfection. Twenty-four hours before transfection, HepG2 cells were trypsinized and transferred to six-well plates (5×10^5 cells/well with 2 ml of HepG2 medium). Cells were transfected with siRNA duplexes using the Transmessenger transfection reagent (TMTR; QIAGEN) as described by the manufacturer for adherent cell lines. For each well, 2 μ g of siRNA were diluted with 4 μ l of Enhancer R (ratio 1:2) and buffer EC-R (final volume, 100 μ l) and mixed by vortexing. After 5 min of incubation at room temperature, 10 μ l of TMTR was added to the mixture (ratio of micrograms of siRNA/microliters of TMTR; 1:5). After 10 min of incubation during which the cells were washed with sterile PBS, the siRNA-transfection mixture was diluted in 2 ml of HepG2 medium without serum and antibiotics and added to the cells. Three to 4 h later, the medium was changed and HepG2 medium with FBS and antibiotics was added. Cells were also cotreated or not with 25 nM TCDD. Twenty-four hours later, RNA were prepared from these cells and analyzed by Northern Blotting.

Statistics. Student's two-tailed *t* tests were performed using the Statview software (Abacus Concepts, Inc., Berkeley, CA).

Results

Overexpression of CYP1A1 by Adenoviral Infection of HepG2 Cells. Optimal conditions for adenoviral infection were established using an adenovirus expressing the β -galactosidase gene. Infected cells were revealed by X-Gal staining and the lowest viral load that elicited more than 95% X-Gal-stained cells was selected for the following experiments (a multiplicity of infection of 160 was chosen). HepG2 cells were infected with Adnull, Ad1A1, or Ad1A1mut or treated with TCDD and the resulting CYP1A1 activity was measured 24 h later by EROD assay (Fig. 1A). Cells infected with Adnull or Ad1A1mut (which encodes an enzymatically inactive CYP1A1), as well as control cells (without treatment or adenoviral infection), lacked CYP1A1 activity. In contrast, Ad1A1 infection elicited a potent CYP1A1 activity (200,000

mU of fluorescence/min). HepG2 cells treated with 25 nM TCDD displayed a CYP1A1 activity similar to the CYP1A1 activity obtained by Ad1A1 infection.

Time Course of CYP1A1 Activity. A time course experiment of CYP1A1 activity was performed 4 to 40 h after Ad1A1 infection or TCDD treatment (Fig. 1B). Values of CYP1A1 activities were close and reached, after 24 h,

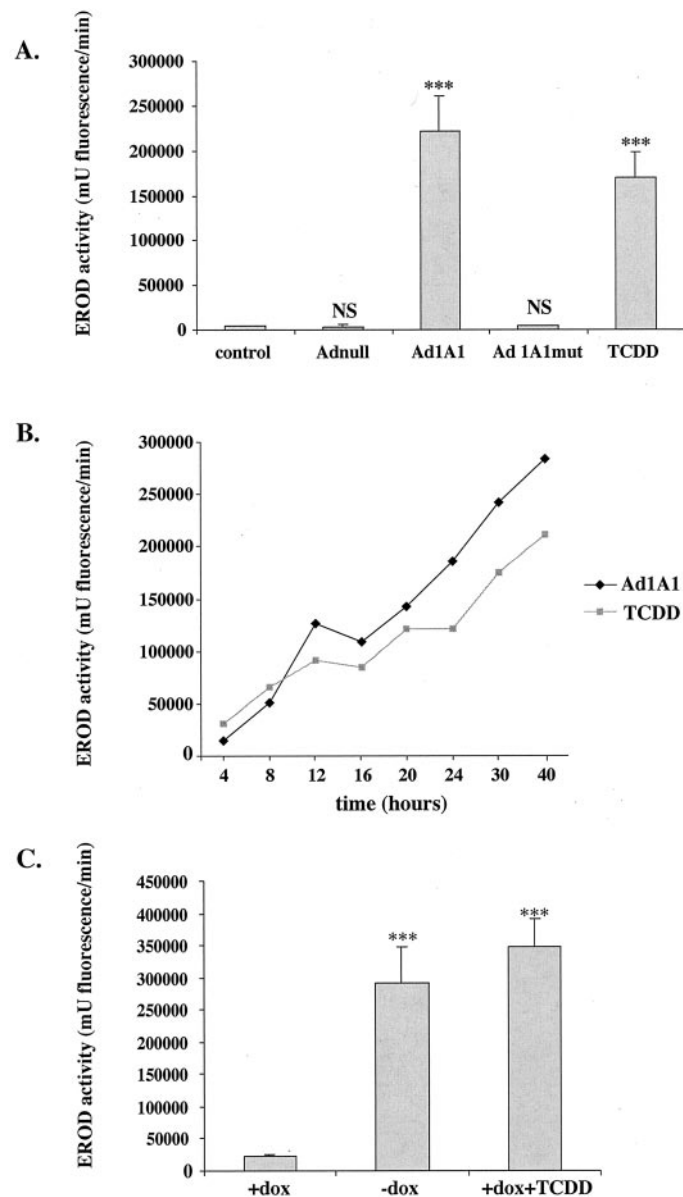


Fig. 1. CYP1A1 activity measured by EROD assay in HepG2 cells. A, adenoviral infection. Cells were either untreated (control), treated for 24 h with 25 nM TCDD, or infected with one of three different adenoviral preparations, Adnull, Ad1A1, or Ad1A1mut, at a multiplicity of infection of 160. Data shown are the means \pm S.D. (bars) of six independent determinations. The differences between values of control cells or of cells treated by Ad1A1 or TCDD are significant (***, $P < 0.001$). NS, nonsignificant difference. B, time course of CYP1A1 activity in HepG2 cells treated with 25 nM TCDD or infected with Ad1A1. C, CYP1A1 activity in HepG2 Tet-off cells. EROD activity was measured after 8 days of culture with dox (+dox, uninduced Tet-off *CYP1A1* gene), without dox (-dox, induced Tet-off *CYP1A1* gene), or with dox and 25 nM TCDD for 24 h (+dox+TCDD, induced endogenous *CYP1A1* gene). Data shown are the means \pm S.D. (bars) of six independent determinations. Induction of CYP1A1 in the absence of doxycycline in the medium is significant (***, $P < 0.001$).

150,000 mU of fluorescence/min for TCDD and 200,000 mU of fluorescence/min for Ad1A1 infection. The CYP1A1 activities continued to increase similarly up to 40 h after infection or addition of TCDD.

CYP1A1 Activity in HepG2 Tet-off Cells. HepG2 Tet-off cells were obtained after two stable transfection steps [see *Materials and Methods* (Freundlieb et al., 1997)]. After the first step, a HepG2 clone stably expressing tTA was isolated. The plasmid pTRE2pur-1A1 was stably introduced into these cells and several clones were selected and cultured. These clones were screened for their CYP1A1 activity after withdrawal of doxycycline from the cell medium. We focused on one of the clones that displayed a high CYP1A1 activity upon dox removal. A time-course study was carried out to determine the optimal time point at which the CYP1A1 activity elicited by dox removal was the most similar to the one induced by 24-h TCDD treatment. A dox concentration that does not interfere with gene expression and that yields the lowest background level of CYP1A1 was selected (2 μ g/ml) (data not shown). As shown in Fig. 1C, removal of dox for 8 days from Tet-off HepG2 cells yielded a CYP1A1 activity that was similar to the one induced by 24-h TCDD treatment. It should be noted that the increase in CYP1A1 activity started more than 6 days after dox removal. However, the CYP1A1 background activity level in the presence of dox remained higher than in untreated HepG2 cells (approximately 4-fold).

Effect of Overexpression of CYP1A1. NQO1 mRNA levels were assayed by Northern blot in untreated HepG2 cells, cells treated with 25 nM TCDD, and cells infected with Adnull or Ad1A1 for 24 h (Fig. 2A). TCDD treatment induced NQO1 approximately 2-fold. Interestingly, Ad1A1-infected cells that overexpress CYP1A1 and that were not treated by TCDD displayed a 2-fold NQO1 induction compared with Adnull-infected cells. A similar regulation was observed for the *UGT1A6* gene. As expected, endogenous CYP1A1 was induced by TCDD but not modified by Ad1A1 infection. Quantification of the mRNA levels from five independent experiments, showed that the fold induction of NQO1 and *UGT1A6* mRNAs by TCDD (TCDD-treated cells versus untreated cells) and by CYP1A1 overexpression (Ad1A1 versus Adnull infected cells) were very similar (approximately 2-fold) although the basal mRNA level was lower in adenoviral infected cells compared with control cells (Fig. 2B).

Using the Tet-off system overexpressing CYP1A1, Northern Blots were carried out to evaluate NQO1 and *UGT1A6* mRNA levels in untreated or TCDD-treated cells, with or without dox. Both NQO1 and *UGT1A6* mRNA levels increased after dox removal or addition of TCDD (Fig. 3A). Endogenous CYP1A1 and the transgene Tet-off CYP1A1 could be detected using a CYP1A1 probe common for both of them. In the presence of dox, despite the residual EROD activity, no CYP1A1 mRNA could be detected; the residual CYP1A1 mRNA was probably below the detection limit of the Northern blot assay. In contrast, dox removal induced the CYP1A1 transgene, whereas TCDD induced the endogenous *CYP1A1* gene. The mRNA level of the Tet-off CYP1A1 in the absence of dox and that of the endogenous CYP1A1 in the presence of both dox and TCDD cells were of the same magnitude. Quantification of NQO1 and *UGT1A6* mRNA levels (Fig. 3B.) showed that the fold-induction by dox removal in the Tet-off system and by Ad1A1 infection were similar. It should be noted that the basal *UGT1A6* mRNA levels were

very low in HepG2 cells. We therefore carried the additional characterization of the effect of CYP1A1 on the NQO1 gene expression.

Oxidative Stress Produced by CYP1A1 Activity. H_2O_2 production in HepG2 Tet-off cells and in cells infected with recombinant adenovirus was measured by the oxidation of H_2DCF probe into fluorescent DCF (see *Materials and Methods*). Compared with cells cultured with dox (uninduced CYP1A1 Tet-off gene, control cells), cells cultured without dox (induced Tet-off CYP1A1 gene) displayed an increased fluorescence of more than 50% (Fig. 4A). The increase in H_2O_2 production was similar for cells treated with TCDD in the presence of dox. With the adenoviral system (see Fig. 4B), the increased fluorescence compared with control cells in Ad1A1 infected cells was about 50% and in TCDD treated cells of 30%. Interestingly, Adnull-infected cells and Ad1A1mut-infected cells displayed no increase in oxidized DCF suggesting that Adnull or Ad1A1mut infection did not elicit an oxidative stress in the cell.

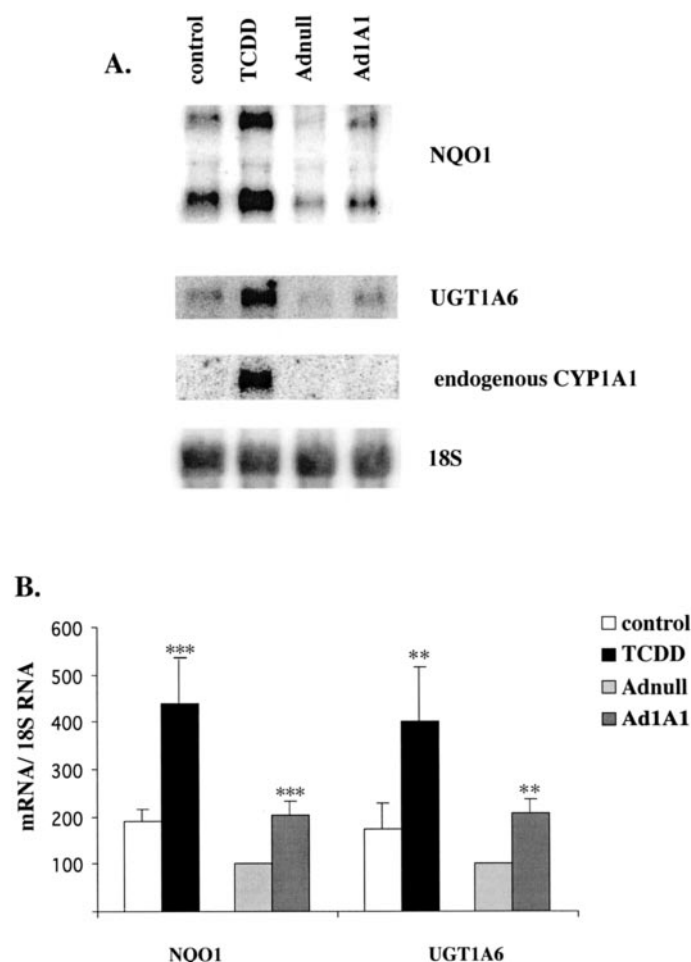


Fig. 2. Induction of *NQO1* and *UGT1A6* genes expression by adenoviral CYP1A1. HepG2 cells were treated with 25 nM TCDD or infected with Adnull or Ad1A1 for 24 h. A, Northern blots were hybridized with NQO1, *UGT1A6*, endogenous specific CYP1A1 probes, and then with an 18S RNA probe. B, semiquantification of NQO1 and *UGT1A6* mRNA levels normalized to 18S RNA levels. 100% corresponds to the mRNA ratio in cells infected with Adnull. Data shown are the means \pm S.D. (bars) of five independent experiments. Significant differences in the amount of NQO1 and *UGT1A6* mRNAs are found between control and TCDD-treated cells as well as between Adnull and Ad1A1-infected cells, respectively (***, $P < 0.001$; **, $P < 0.01$).

Induction of *NQO1* Gene by CYP1A1 Depends on CYP1A1 Activity and ROS. As shown in Fig. 2, *NQO1* mRNA was induced by TCDD and by Ad1A1 infection. To test whether *NQO1* induction is caused by the increase of the CYP1A1 protein content or to the CYP1A1 activity, we infected cells with Ad1A1mut, an adenovirus that expresses approximately the same amount of CYP1A1 protein as shown by Western blot (Fig. 5A) but in an inactive form (Fig. 1A). Ad1A1mut did not induce the *NQO1* gene after infection of the HepG2 cells. Quantification of *NQO1* mRNA levels from three experiments (Fig. 5B) confirmed that the overexpression of CYP1A1 by Ad1A1 infection led to an 80% increase in *NQO1* mRNA levels, whereas infection with Ad1A1mut did not lead to any significant difference with the control. The increase of *NQO1* mRNA by TCDD and Ad1A1 was accompanied by a similar yet lower increase in *NQO1* enzyme

activity. Indeed, 30 h of TCDD treatment elicited a 1.75 ± 0.22 fold increase of the enzyme ($n = 3$), whereas Ad1A1 elicited a 1.48 ± 0.31 increase compared with Ad1A1mut ($n = 3$). Longer time points could not be tested because of the relative toxicity of adenoviral infection. Addition of the antioxidant *N*-acetyl cysteine (NAC) inhibited the induction of *NQO1* in the Ad1A1-infected cells. Thus, the oxidative stress produced by CYP1A1 activity could mediate the induction of *NQO1* by CYP1A1 overexpression. The previous experiments suggest that CYP1A1 activity induces *NQO1* gene expression through an oxidative stress-mediated pathway. To determine the role of ROS in the induction of *NQO1* by TCDD, we treated HepG2 cells by TCDD in the presence or absence of the antioxidant NAC. As shown in Fig. 5C, the increase in *NQO1* mRNA by dioxin was partially prevented by NAC, suggesting a contribution of ROS to this regulation.

Inhibition of *NQO1* Gene Expression during TCDD Treatment Using CYP1A1-siRNA. We next assessed the actual contribution of increased CYP1A1 activity to the induction of *NQO1* by TCDD. We used a specific siRNA directed against CYP1A1 mRNA to knock down *CYP1A1* gene

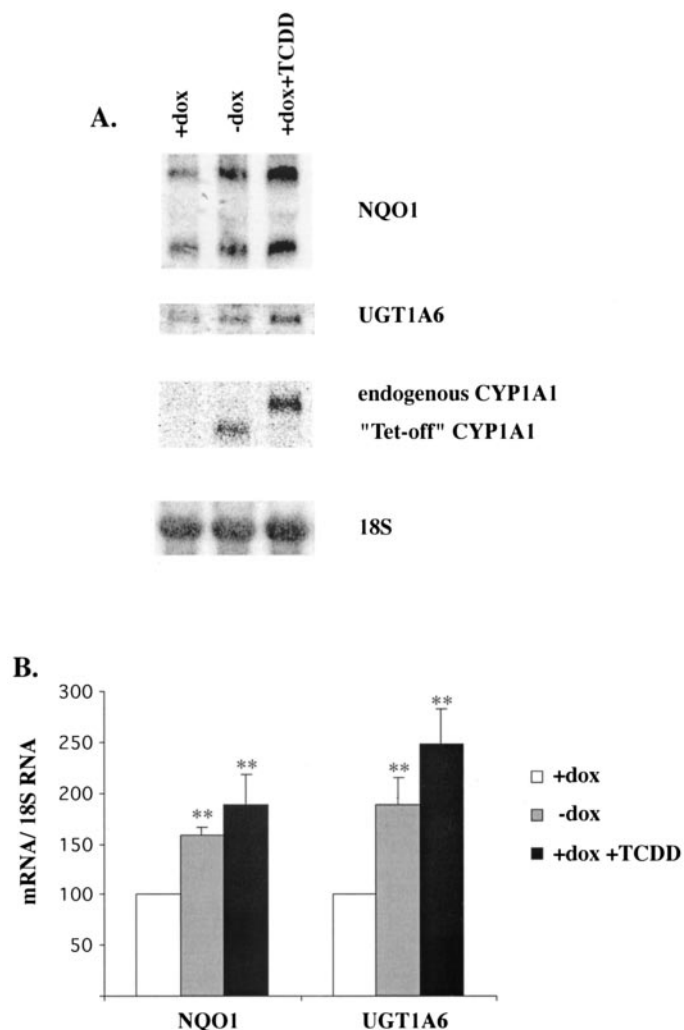


Fig. 3. Induction of *NQO1* and *UGT1A6* genes expression by Tet-off CYP1A1. HepG2 Tet-off cells were cultured for 8 days with 2 μ g/ml dox (+dox, uninduced Tet-off CYP1A1) or without dox (-dox, induced Tet-off CYP1A1) or treated with 25 nM TCDD for 24 h before RNA isolation. A, Northern blots were hybridized with *NQO1*, *UGT1A6*, and CYP1A1 probes and then with an 18S RNA probe. B, semiquantification of *NQO1* and *UGT1A6* mRNA levels normalized to 18S RNA levels. 100% corresponds to the mRNA ratio in HepG2 Tet-off+dox cells. Data shown are the means \pm S.D. (bars) of three independent experiments. Significant increases in *NQO1* and *UGT1A6* mRNA levels are observed between control cells (+dox) and CYP1A1-induced cells (-dox) and between control and TCDD-treated cells (+dox+TCDD) (**, $P < 0.01$).

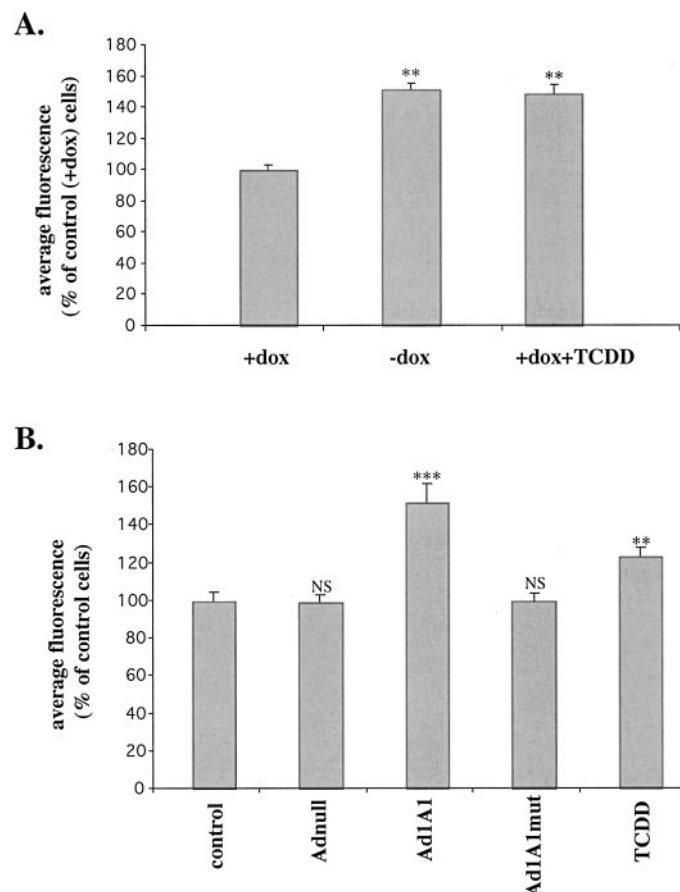


Fig. 4. Oxidative stress produced by CYP1A1 induction. A, HepG2 Tet-off cells. H_2O_2 levels within HepG2 Tet-off cells were assayed after 8 days of culture with or without 2 μ g/ml dox or 25 nM TCDD for 24 h. Results of four independent experiments were expressed as means \pm S.D. normalized to 100% for HepG2 Tet-off+dox cells. Significant differences from control values are indicated (**, $P < 0.01$). B, after adenoviral infection. H_2O_2 levels were assayed after 24 h of infection with Adnull, Ad1A1, and Ad1A1mut or treatment with 25 nM TCDD. Results of five independent experiments were expressed as means \pm S.D. normalized to 100% for control cells. Significant differences from control values are indicated (***, $P < 0.001$; **, $P < 0.01$; NS, nonsignificant).

expression. As shown in Fig. 6, CYP1A1 siRNA led to a partial decrease in CYP1A1 mRNA level (approximately 30%); more importantly, however, it also specifically prevented NQO1 induction by TCDD (approximately 50%). Control-siRNA did not significantly modify CYP1A1 or NQO1 mRNA levels. The inhibition was specific because CYP1A1-siRNA did not modify the expression of another gene, the *GRP94* gene. These data clearly show that the increase in CYP1A1 expression, after TCDD addition, contributes significantly to the induction of NQO1.

Discussion

In the present study, we have attempted to determine the contribution of increased cytochrome P450 activity to the effects of xenobiotics on gene expression. More specifically, we have focused on the role of increased CYP1A1 in the fine-tuning of the xenobiotic metabolizing enzyme *NQO1*

gene expression. The aim of such fine-tuning would be to avoid the accumulation of intermediate toxic metabolites. The *CYP1A1* and several other XME genes, such as *NQO1*, *GSTY α* , and *UGT1A6*, are inducible by dioxin and several polyaromatic hydrocarbons (Nebert et al., 1993). Several studies have previously shown that the latter genes are also regulated by other signaling pathways, in particular those involving oxidative stress (Dhakshinamoorthy et al., 2000) and that dioxin induction of the human *NQO1* gene is mediated by antioxidant-responsive element (Radjendirane and Jaiswal, 1999; Ma et al., 2003). Genetic studies using mutant mouse hepatoma cell lines provided indirect evidence for an interaction between the AhR pathway and the other pathways regulating these genes and predicted that increased CYP1A1 could affect the gene expression of other XME genes (Nebert et al., 2000). Additional studies supporting such predictions or not were still needed. The data presented here provide direct evidence for the induction of the *NQO1* and *UGT1A6* genes by CYP1A1 and suggest that the reactive

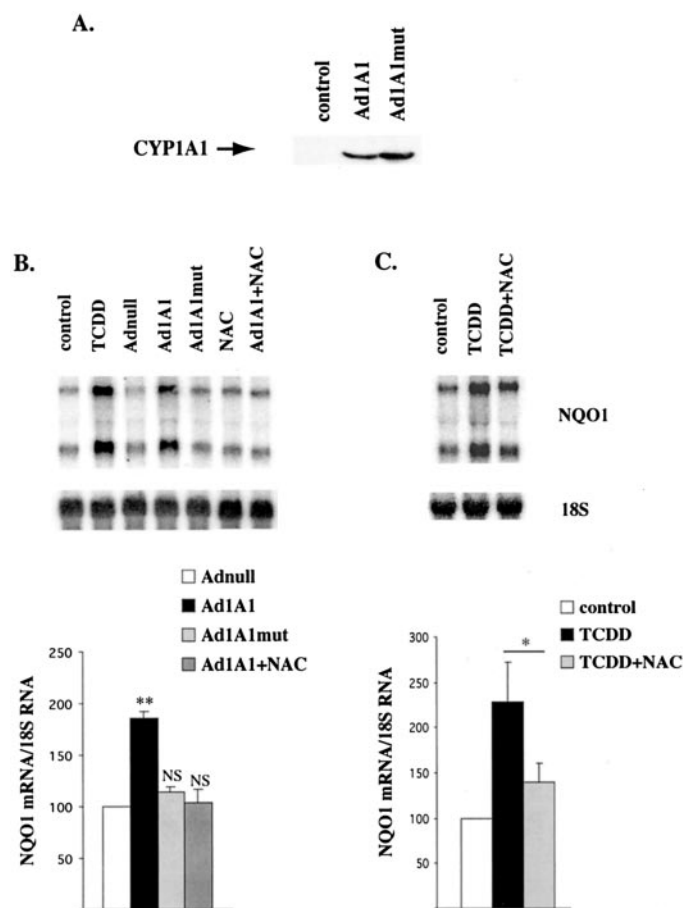


Fig. 5. Induction of NQO1 by CYP1A1 depends on CYP1A1 activity. A, Western blot analysis of proteins from HepG2 cells that were untreated (control) or infected with Ad1A1 or Ad1A1mut. B and C, Northern blot analysis. HepG2 cells were infected with Adnull, Ad1A1, or Ad1A1mut for 24 h and cotreated or not with NAC (B). HepG2 cells were treated with 25 nM TCDD and/or 10 mM NAC for 24 h (C). Northern blots were hybridized with NQO1 and then with an 18S RNA probe. Semiquantification of NQO1 mRNA levels were normalized to 18S RNA levels. 100% corresponds to the mRNA ratio in cells infected with Adnull (B) or to the ratio in untreated cells (C). Data shown are the means \pm S.D. (bars) of three independent experiments. Significant differences of NQO1 mRNA levels are found between Adnull and Ad1A1-infected cells (B) and between TCDD+NAC- and TCDD-treated cells (C). No significant difference was found between Adnull and Ad1A1mut or Ad1A1+NAC cells. (**, $P < 0.01$; NS, nonsignificant).

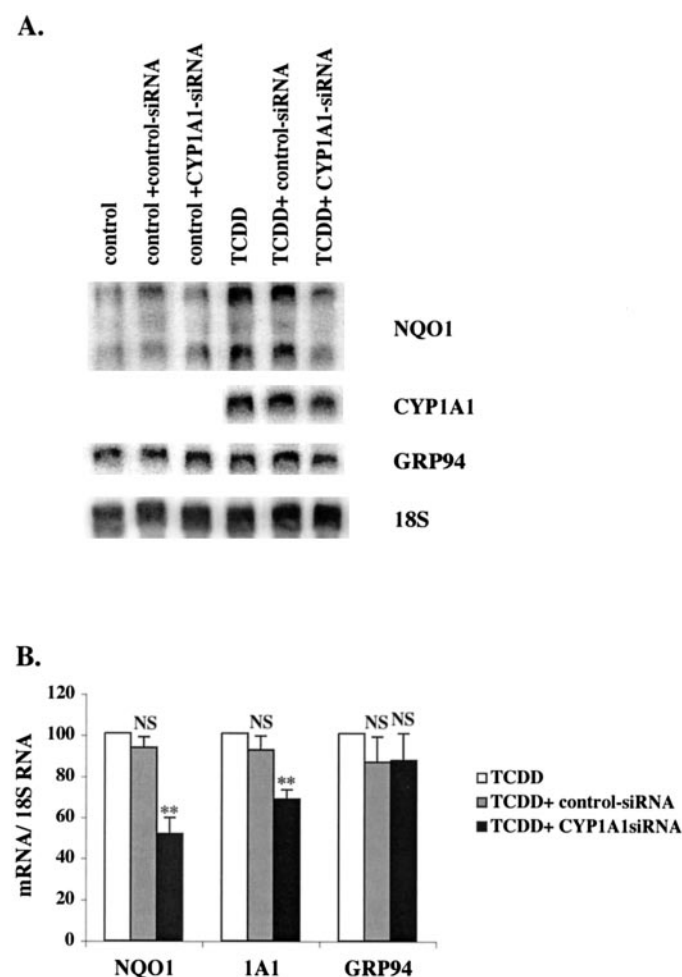


Fig. 6. Decrease of NQO1 mRNA expression by CYP1A1 knockdown using CYP1A1-siRNA. Northern blot analysis. HepG2 cells were transfected for 24 h with 2 μ g of siRNA (control- or CYP1A1-siRNA) and cotreated or not with 25 nM TCDD before RNA isolation. A, Northern blots were hybridized with NQO1, CYP1A1, GRP94 probes and then with an 18S RNA probe. B, semiquantification of NQO1 mRNA levels normalized to 18S RNA levels. 100% corresponds to the mRNA ratio of TCDD-treated cells. Data shown are the means \pm S.D. (bars) of three independent experiments. Significant differences from TCDD values are indicated (**, $P < 0.01$; NS, nonsignificant).

oxygen species released by this enzyme are required for such a regulation.

Increased CYP1A1 activity was obtained using two independent procedures, adenovirus infection and the Tet-off system. These procedures were selected because they provided an AhR-independent increase in CYP1A1 activity and because the increase in CYP1A1 activity was obtained in up to 100% of the cells, which allows study of the regulation of the endogenous genes. Furthermore, in contrast with the generation of a cell line constitutively overexpressing the *CYP1A1* gene using a strong viral promoter, the procedures used here provide a short-term infection method or an inducible overexpression system mimicking the treatment with an AhR agonist. In addition, CYP1A1 activity can be adjusted in both systems by varying either the viral load or the dox concentration to attain an activity similar to that elicited by TCDD treatment. Nevertheless, each of the experimental settings presents specific advantages and limitations. Although adenovirus infection is a very efficient procedure, it can modify the basal expression of several genes and could lead to cell toxicity with time. However, the use of control empty vectors as well as vectors overexpressing a mutant CYP1A1 gene validated our observations. The Tet-off system is, a priori, closer to the TCDD-inducible situation, although the time course is much longer (8 days versus 24 h). This is most probably caused by the actual half-life of dox. Indeed, although no significant modification in CYP1A1 activity could be detected in HepG2 Tet-off cells at day 7 after dox removal, an increase similar to the one elicited by a 24-h TCDD treatment could be observed at day 8. Thus, in this case too, the actual increase in CYP1A1 activity occurs within a 24-h period after the derepression of the promoter. The leakage of the Tet repressor leads to a small basal P450 activity that is not present in nontransfected cells. Because both experimental procedures demonstrate an induction of the *NQO1* gene, it is likely that this induction is caused by the increase in CYP1A1 activity. The CYP1A1 knockdown experiments using CYP1A1-specific siRNA show that the increase in CYP1A1 expression directly contributes to the induction of the *NQO1* gene by TCDD. Thus, both overexpression and knockdown studies support our hypothesis.

Several experiments described in this study proved that the induction of the *NQO1* gene by CYP1A1 overexpression is related to the increased activity of this enzyme. First, the CYP1A1 mutant, which is devoid of any enzymatic activity, did not induce the *NQO1* gene expression although it was overexpressed to the same level as the wild-type enzyme. These results indicate that the activity of the CYP1A1 proteins is critical, not the increase in CYP1A1 proteins. Second, the wild-type CYP1A1, but not the mutant enzyme, triggered an increase in reactive oxygen species within the cells. Furthermore, the anti-oxidant NAC inhibited the effect of CYP1A1. This suggests that the induction of *NQO1* gene expression is mediated by the increase of ROS elicited by CYP1A1 activity.

Previous genetic and molecular studies have suggested a possible regulation of XME genes expression by CYP1A1 activity. Two pathways have been suggested to account for such a control: the first pathway predicts the presence of a putative endogenous ligand of the AhR that could be metabolized by CYP1A1, whereas the second pathway highlights the role of the reactive oxidized species released by CYP1A1.

Our previous studies, showing a down-regulation of *CYP1A1* gene expression by CYP1A1 activity and ROS release, were in support of the latter pathway (Morel et al., 1999, 2000). The present demonstration of the induction of the *NQO1* and *UGT1A6* gene expression by CYP1A1 activity and ROS release is in good agreement with the regulation of phase II genes by oxidative stress and also supports this model. The former pathway is supported by previous studies done in mutant mouse hepatoma cell lines (RayChaudhuri et al., 1990; Vasiliou et al., 1992; Nebert et al., 2000). In those studies, a cell line with an inactive *CYP1A1* gene displayed high basal *CYP1A1* and *NQO1* gene expression. In a cloned cell derived from this cell line and constitutively overexpressing an active *CYP1A1* gene, the expression of *CYP1A1* and *NQO1* genes was repressed because of the degradation of an endogenous AhR ligand by CYP1A1 (Nebert et al., 2000; Vasiliou et al., 2000). Those results are compatible with ours in the case of *CYP1A1* gene expression but differ from ours in the case of *NQO1*. There are several important differences between those experiments and ours. The first one is that we have used inducible systems for overexpression and have not derived clones with constitutively overexpressed genes. In the latter case, some of the observations could be related to long-term adaptation of the cell. Second, the species that have been studied are different. This point may be critical because the structure of the *NQO1* promoter differs between humans and rodents. Although both the human and rodent promoters contain antioxidant responsive elements, only the rodent promoter contains an obvious XRE (Favreau and Pickett, 1991). Thus, the human gene, upon which our study is focused, may be predominantly controlled by ROS and CYP1A1 activity during TCDD treatment, whereas the rodent gene could also be directly regulated by the AhR and its putative endogenous ligands. The possibility that the oxidative stress could activate the AhR-XRE pathway was assessed by transfection of a plasmid containing the luciferase gene driven by a promoter consisting of three XREs. This promoter was potently induced by TCDD (300-fold) but only marginally by hydrogen peroxide (2- to 3-fold) (data not shown).

The data presented in this article are particularly relevant for the understanding of the fine-tuning of xenobiotic-metabolizing enzyme gene expression. Indeed, we have shown that CYP1A1, a phase I enzyme, directly signals the induction of another XME gene expression: *NQO1*. Our data suggest that the *UGT1A6* gene, despite its weak expression in HepG2 cells, is also regulated by CYP1A1. In previous studies, we and others have shown that CYP1A1 activity represses its own gene expression as part of an autoregulatory loop (RayChaudhuri et al., 1990; Jorgensen and Autrup, 1996; Morel et al., 1999). Thus, upon the induction of the XME genes by a xenobiotic receptor such as the AhR, increased CYP1A1 activity constitutes a signal to further induce other XME genes and to down-regulate its own gene. This should prevent the uncontrolled elevation of CYP1A1 activity and maintain an adequate ratio between XME. It is likely that the main metabolic consequence of such fine-tuning is to avoid the accumulation of highly reactive intermediates. Indeed, increased *NQO1* and phase II enzymes activities ensures either the reduction of these oxidized intermediates or the addition of hydrophilic groups and their elimination.

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