Chemico-Biological Interactions 180 (2009) 226–237

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

The role of aryl hydrocarbon receptor in regulation of enzymes involved in metabolic activation of polycyclic aromatic hydrocarbons in a model of rat liver progenitor cells

Jan Vondráček[a,b], Pavel Krčmář[b], Jiřina Procházková[a,b], Lenka Trilecová[b], Martina Gavelová[c], Lenka Skálová[c], Barbora Szotáková[c], Martin Bunček[d], Hana Radilová[d], Alois Kozubík[a], Miroslav Machala[b,*]

[a] Department of Cytokinetics, Institute of Biophysics ASCR, 612 65 Brno, Czech Republic
[b] Department of Chemistry and Toxicology, Veterinary Research Institute, 621 32 Brno, Czech Republic
[c] Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, 500 05 Hradec Králové, Czech Republic
[d] GENERI BIOTECH s.r.o., 500 11 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 9 December 2008
Received in revised form 10 March 2009
Accepted 16 March 2009
Available online 27 March 2009

Keywords:
Cytochromes P450
AhR
PAHs
Aldo–keto reductases
NQO1
NRF2

ABSTRACT

In contrast to hepatocytes, there is only limited information about the expression and activities of enzymes participating in metabolic activation of environmental mutagens, including polycyclic aromatic hydrocarbons (PAHs), in liver progenitor cells. In rat liver "stem-like" WB-F344 cell line, sharing many characteristics with rat liver progenitor cells, PAHs are efficiently activated to their ultimate genotoxic metabolites forming DNA adducts. The present study aimed to characterize expression/activities of enzymes of two major pathways involved in the metabolism of benzo[*a*]pyrene (BaP): cytochrome P450 (CYP) family 1 enzymes and cytosolic aldo–keto reductases (AKRs). We report here that, apart from induction of CYP1A1 and CYP1B1 expression and the corresponding enzymatic activity, both BaP and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced rat 3α-hydroxysteroid dehydrogenase (AKR1C9) expression and activity. In contrast, the aldehyde reductase AKR1A1 was not induced by either treatment. Thus, both CYP1 and AKR metabolic pathways were inducible in the model of liver progenitor cells. BaP and TCDD were efficient inducers of NAD(P)H:quinone oxidoreductase 1 (NQO1) expression and activity in WB-F344 cells, a principal enzyme of cellular antioxidant defense. Both compounds also induced expression of transcription factor NRF2, involved in control of enzymes protecting cells from oxidative stress. However, although BaP induced a significant formation of reactive oxygen species, it did not induce expression of heme oxygenase-1, suggesting that induction of oxidative stress by BaP was limited. Using shRNA against the aryl hydrocarbon receptor (AhR), we found that similar to CYP1A1 and CYP1B1, the AKR1C9 induction was AhR-dependent. Moreover, constitutive AKR1C9 levels in AhR-deficient rat BP8 hepatoma cells were significantly lower than in their AhR-positive 5L variant, thus supporting possible role of AhR in regulation of AKR1C9 expression. Taken together, both CYP1 and AKR1C9 appear to be AhR-regulated metabolic pathways, which may contribute to formation of pro-carcinogenic PAH metabolites in liver progenitor cells.

1. Introduction

The liver is the central organ of xenobiotic metabolism, which has enormous regenerative capacity being facilitated by different cell types [1]. When the regenerative capacity of mature hepatocytes is compromised by toxins or a disease, the liver may restore its original capacity through activation of unique progenitor cell populations, such as oval cells or small hepatocyte-like progenitor cells [1–5]. It is becoming increasingly evident that, apart from their role in liver regenerative response, these cells may participate in hepatocarcinogenesis [6,7]. Therefore, understanding the mechanisms contributing to their transformation is of major importance. However, there is currently only limited information about the expression and/or activity of xenobiotic-metabolizing enzymes in liver progenitor cells, including those participating in metabolic activation of promutagens, such as environmental polycyclic aromatic hydrocarbons (PAHs) and related compounds.

The metabolic activation of PAHs proceeds through activities of enzymes belonging to cytochrome P450 (CYP) 1 family being

* Corresponding author at: Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic. Tel.: +420 533331813; fax: +420 541211229.
E-mail address: machala@vri.cz (M. Machala).

0009-2797/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2009.03.011

under transcriptional control of the aryl hydrocarbon receptor (AhR), which is also a transcriptional regulator of a number of Phase II enzymes [8,9]. Although some early studies have suggested that bipotential liver progenitor cells have only limited expression/activities of CYP enzymes [10–12], there are contradictory findings regarding the expression of CYP1 enzymes in liver progenitor cells, which might be related to their different origin. It has been reported that there are no endogenous CYP1-dependent 7-ethoxyresorufin-*O*-deethylase (EROD) activities in freshly isolated or cultured oval cells, or in oval cell-derived cell lines [11,12]. On the other hand, it has been shown that in small hepatocyte-like progenitors, there is both inducible CYP1 expression and EROD activity, depending on their differentiation status [2,13]. The available data seem to suggest that expression and/or inducibility of CYP1A1/2 increase with differentiation of liver progenitors towards hepatocyte-like cells [2,10,14]. However, there is currently no information about the expression pattern of CYP1B1, a third member of mammalian CYP1 family, in liver progenitor cells.

Apart from peroxidases, yielding radical cations, and CYP1 enzymes, which, together with epoxide hydrolase, yield dihydrodiol epoxides, there is another important enzymatic pathway of PAH metabolism [15]. Members of the aldo–keto reductase (AKR) family may activate PAH *trans*-dihydrodiols by converting them to reactive and redox-active *o*-quinones, which further elicit production of reactive oxygen species (ROS) and DNA damage [16–19]. This pathway of PAH metabolism may contribute to PAH-induced carcinogenesis [19]. However, nothing is currently known about the regulation of AKR expression or activity in liver progenitor cells. Apart from eliciting oxidative stress via AKR activation [19], PAHs might also induce it indirectly through AhR activation followed by increased formation of ROS through the CYP1-catalyzed reactions [20]. Both oxidative stress and AhR have been also shown to activate cellular mechanisms aimed at reduction and detoxication of reactive quinones, such as the induction of NAD(P)H:quinone oxidoreductase 1 (NQO1), the enzyme playing a significant role in defense against reactive forms of oxygen [21]. Nothing is currently known about the regulation of NQO1 in liver progenitors in response to AhR ligands or oxidative stress inducers. Persistent AhR ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), may also increase the AhR-dependent expression of the basic leucine zipper transcription factor NRF2 (NF-E2 p45-related factor 2), which is known to provide a direct link between AhR- and ROS-dependent transcriptional regulation [22].

The rat epithelial WB-F344 cell line, isolated from the liver of male F344 rat, shares a number of properties with terminal biliary ducts or oval cells [23]. We have recently found that both CYP1B1 and CYP1A1 are inducible in WB-F344 cells by TCDD or benzo[*a*]pyrene (BaP) [24,25]. These cells provide an interesting model of partially differentiated liver progenitor cells, possibly resembling the expanding bile ductules, which progress towards hepatocyte-like cells in the partial hepatectomy/2-acetylaminofluorene model [10]. The inducible CYP1 enzymes in WB-F344 cells are sufficient to metabolize PAHs to their ultimate genotoxic metabolites, which can effectively form DNA adducts [24,26]. Moreover, we have also recently observed that in WB-F344 cells, expression of 3α-hydroxysteroid dehydrogenase mRNA, which is encoding AKR1C9 enzyme (EC 1.1.1.213) belonging to AKR family, is induced by PAHs or TCDD [24]. The WB-F344 are therefore a highly interesting cellular model for studies of both genotoxic and non-genotoxic effects of environmental contaminants, including both AhR activation and oxidative stress induction, as well as their interactions with endogenous signaling pathways [24,26–28].

The present study aimed to analyze the impact of a model PAH, BaP, on both expression and activities of enzymes involved in its metabolic activation, as well as on the proteins involved in oxidative stress defense, such as NQO1 and heme oxygenase-1 (HO-1). The inducibility of CYP1, AKR and NQO1 enzymes in WB-F344 cells was compared with AhR-positive and negative rat hepatoma cell lines. Finally, using the short hairpin (sh) RNA-mediated knock-down of AhR and hydrogen peroxide as a model oxidative stress inducer, respectively, we studied possible roles of AhR activation and ROS formation in AKR1C9 regulation in the model rat liver progenitor cell line.

2. Materials and methods

2.1. Chemicals

BaP (CAS No. 50-32-8, purity 99.9%) was provided by Ehrenstorfer (Augsburg, Germany). TCDD was from Cambridge Isotope Laboratories (Andover, MA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and kept in a dark. Rabbit polyclonal antibody against rat CYP1B1 and goat polyclonal antibody against rat CYP1A1 were obtained from BD Biosciences (San Jose, CA). Rabbit anti-AhR antibody was obtained from Biomol (Butler Pike, PA). Rabbit polyclonal antibody detecting rat AKR1C9 [29] was kindly provided by Trevor M. Penning (University of Pennsylvania School of Medicine, Philadelphia, PA). Mouse monoclonal antibody to β-actin was obtained from Sigma–Aldrich. Polyvinylidene difluoride (PVDF) membrane Hybond-P, and chemiluminescence detection reagents (ECLPlus) were purchased from GE Healthcare (Aylesbury, UK). All other chemicals were obtained from Sigma–Aldrich.

2.2. Cells

WB-F344 rat liver epithelial cells [23] were kindly provided by Dr. J.E. Trosko (Michigan State University, East Lansing, MI). The cells were cultured in D-MEM/F-12 Medium (Invitrogen, Carlsbad, CA), supplemented with 5% heat-inactivated fetal bovine serum as described previously [26]. Only the cells at passage levels 15–24 were used for the study. Rat hepatoma 5L cells and their AhR-deficient BP8 counterparts [30], kindly provided by Carsten Weiss (Research Center Karslruhe, Germany) were cultivated in D-MEM (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum. All cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were routinely maintained in 75 cm^2 flasks and subcultured twice a week. All other tissue culture reagents were obtained from Sigma–Aldrich.

2.3. Detection of enzymatic activities

Cells from 10 plates were homogenized in 0.1 M Na-phosphate buffer, pH 7.4. The microsomal and cytosolic fractions were obtained by fractional ultracentrifugation of the homogenate. Microsomes were finally resuspended in the homogenization buffer containing 20% glycerol (v/v) and both fractions were stored at −80 °C. The amount of protein was determined according to BCA method [31]. The EROD activity was determined using fluorimetric determination of resorufin [32] at 37 °C with the final concentrations of the substrate 2.5 μM and NADPH concentration 1 mM. The assays were conducted using the PerkinElmer luminescence spectrophotometer LS50B with the excitation and emission wavelengths of 530 nm and 585 nm, respectively. The EROD activity (corresponding to CYP1A1 and CYP1B1 activities in WB-F344 cells) was measured in the presence of 25 μM dicoumarol. The quinone reductase (DT-diaphorase, NQO1) activity was determined using fluorimetric measurement of resorufin consumption at 37 °C. The reaction mixture, with the final concentrations of resorufin 0.05 mM and NADPH 0.5 mM, contained 25 μl of cytosolic fraction,

0.1 M Tris–HCl buffer (pH 7.4) up to 1 ml. The assays were conducted using the PerkinElmer luminescence spectrophotometer LS 55 with the excitation and emission wavelengths of 530 nm and 585 nm, respectively. The activity was measured in the presence of 5 μM or 25 μM dicoumarol [33] as well. All activities were calculated using the standard amount addition technique [32,34]. The AKR activities were tested using acenaphthenol dissolved in DMSO as the substrate. The reaction mixture, with the final concentrations of acenaphthenol 0.1 M and NADPH 1 mM, contained 100 μl of cytosolic fraction, 0.1 M Tris–HCl buffer pH 8.0 up to 1 ml. Final concentration of DMSO was 1%. Spectrophotometric determination (detection wavelength 340 nm, 25 °C) of NADPH formation in the reaction mixture served for the assessment of AKR activity [16,35].

2.4. Real-time RT-PCR

Following the 24 h exposure of cells to test or control compounds, total RNA was isolated from cells using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). The levels of CYP1A1, CYP1A2, CYP1B1, NQO1 and AKR1C9 mRNAs were determined by quantitative real-time RT-PCR, using primer and probe sequences published previously [36]. The sequences of primers and probe for AKR1A1 (GeneBank accession number NM_031000) were: forward: 5′-CATCCCCAAAAGCATCACTCC-3′, reverse: 5′-GACTCTCTTCCCATCCACCGT-3′, probe: 5′-CCCGCATCCTTCAGAACA TTCAGGTA-3′. The sequences of primers and probe for NRF2 (GeneBank accession number NM_031789) were: forward: 5′-CATTTGTAGATGACCATGAGTCGC-3′, reverse: 5′-CCTGCTGTATGCTGCTTAAATCAG-3′, probe: 5′-CCCTGGATATTCCCAGCCACGTTGAGA-3′. The sequences of primers and probe for HO-1 (GeneBank accession number NM_012580) were: forward: 5′-CGCCCACAGCTCGACAGCA-3′; reverse: 5′-GGTACAAGGAGGCCATCACCAGC-3′; probe: 5′-AAAGCCTTCCCTGGACACCTGACCCTTCTGA-3′. All primer and probe sets were synthesized at Generi-Biotech (Hradec Králové, Czech Republic). The amplifications were run on the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using the conditions described previously [36]. All PCR reactions were performed in triplicates and changes in gene expression were calculated using the comparative threshold cycle method [37]. For absolute quantification of CYP1A2, in WB-F344 cells, where its levels were approaching detection limit, we used the following primers and FRET probes: forward: 5′-GAATGTCACCTCAGGGAATGC-3′; reverse: 5′-CCTGGATACTGTTCTTGTTGAAGTCT-3′; FL-probe: 5′-GACTTCTTTCCGGTCCTGCGC-3′-fluorescein; LC-probe: 5′-LC640-ACCTGCCCAACCCAGCCCT-3′. The primers and probes were designed and synthesized by TIB MolBiol (Berlin, Germany). The absolute quantification was performed using pCR4-ZeroBlunt-TOPO vector with inserted rCyp1a2 PCR product (Generi-Biotech) as a standard. The following reaction conditions were used: reverse transcription 50 °C, 20 min; denaturation 95 °C, 15 min; amplification 40 cycles – 95 °C, 15 s; 58 °C, 30 s; 72 °C, 30 s. The total RNA content was determined using Qubit™ fluorometer and Quant-iT™ RNA Assay kit (Invitrogen, Eugene, OR).

2.5. Western blotting

Whole cell lysates were prepared using SDS sample buffer (1% SDS, 10% glycerol, 100 mM Tris, pH 7.4) and protein concentration was estimated using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). The samples were separated by 10% SDS-PAGE, transferred to PVDF membrane and blocked with non-fat dry milk. The proteins were detected with appropriate primary antibodies, followed by anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies and ECLPlus reagent was used for visualization of selected proteins according to manufacturer's instructions.

2.6. Plasmid construction

The psiRNA vector system coding sequence specific siRNA cassette was cloned and the vector was constructed in the following manner: the blasticidine resistance gene (BSD) was cut-out (EcoRI, BglII) from pUB-BSD (Invitrogen) and cloned into (BamHI, EcoRI) pUC131 (DKFZ, Heidelberg, Germany). U6 promoter was PCR amplified from genomic DNA, verified by sequencing and cloned into pUC131-BSD, both digested with BglII and XbaI. The prepared psiRNA vector was used to clone respective siRNA cassette. This cassette was assembled by hybridization of two phosphorylated single stranded oligonucleotides. Oligonucleotides were designed so that they had cohesive ends for BamHI, HindIII restriction sites on both 5′- and 3′-termini, respectively:

- Rat_AhR_6+: 5′-GATCCAGTCCAATGCACGCTTGTTCAAGAGACAAG-CGTGCATTGGACTGGATTTTTTGGA.
- Rat_AhR_6−: 5′-AGCTTCCAAAAAATCCAGTCCAATGCACGCTTGTC-TCTTGAACAAGCGTGCATTGGACTG.

The siRNA cassette was then cloned into U6 promoter-based psiRNA vector digested with BamHI, HindIII. Plasmid was transformed and multiplied in *E. coli*. Individual clones were isolated and plasmid idenities were confirmed by restriction and sequencing analysis. As a negative control, luciferase specific shRNA (shRNA-luc; for non-specific control) was designed:

- shRNA_luc+: 5′-GATCCGTCGATGTACACGTTCGTCTTCAAGAGAGA-CGAACGT- GTACATCGACTTTTTTGGA-3′.
- shRNA_luc−: 5′-TCCAAAAAAGTCGATGTACACGTTCGTCTCTCTTG-AAGACGAACG TGTACATCGACGGATC-3′.

2.7. Transfections

WB-F344 cells were grown in D-MEM with 1× high glucose (L-glutamine 584 mg/l, sodium pyruvate 110 mg/l) medium supplemented with 5% heat-inactivated FCS (PAA Laboratories, Pasching, Austria). One day prior to transfection, the cells were seeded in 24-well plate at a density of 6–7 × 10^4 cells/well. Linearized vector (XmnI) of psiRNA_rat6 variant was transfected into WB-F344 cell line with effectene transfection reagent (Qiagen, Carslbad, CA) according to the manufacturer's recommendations. 30 h post-transfection medium was replaced with a blasticidine (Invitrogen) selective medium (5 μg/ml medium). Cells were grown at selective pressure for 2 weeks and individual clones were tested for AhR expression by the real-time RT-PCR.

2.8. Detection of ROS formation

Confluent WB-F344 cells were exposed to the test compounds for 24 h. Hydrogen peroxide (400 μM; exposure 5 min) was used as a positive control. After the exposure, the cells were twice washed with PBS, trypsinized, centrifuged, and resuspended with Hank's balanced salt solution (PANBioTech GmbH, Aidenbach, Germany) with 5% heat-inactivated fetal bovine serum. The cell suspension was incubated for 15 min with the fluorescent probe dichlorofluorescein diacetate (DCFH-DA) [38]; the final concentration was 20 μM. The cells were washed once again, centrifuged, and cooled on ice (except hydrogen peroxide-exposed cells). The fluorescence of dichlorofluorescein (DCF) was analyzed on FACSCalibur flow cytometer equipped with the CellQuest software (Becton Dickinson, San Jose, CA).

2.9. Statistical analysis

The results were expressed as means ± S.D. Multiple comparisons were made with one-way ANOVA and Tukey's post-hoc test, or with the two-way ANOVA, followed by Dunnett's post-hoc test vs. a respective control group. For two group comparisons, unpaired Student's *t*-test was used. Values of $p < 0.05$ and/or 0.01 (as indicated in legends to figures) were considered significant.

3. Results

3.1. BaP induces AKR1C9 and NQO1 expression, but it has no effect on AKR1A1 levels in WB-F344 cells

In the first step, we compared the effects of BaP and TCDD (as a model AhR ligand) on the expression of CYP1 enzymes with inducibility of NQO1 and two AKR family members, which have been previously shown to metabolize PAH *trans*-dihydrodiols [18,19] – AKR1C9 and AKR1A1. We found that BaP induced CYP1A1, CYP1B1, NQO1 and AKR1C9 mRNAs levels in WB-F344 cells in a dose-dependent manner after 24 h incubation, with already 100 nM concentration being effective (Fig. 1A). With exception of AKR1C9, where mRNA levels increased up to 10 μM concentration, the maximum induction of other enzymes was observed at 1 μM dose of BaP. Micromolar doses of BaP induced similar levels of CYP1A1, CYP1B1, NQO1 and AKR1C9 mRNAs as 1 nM TCDD, a model AhR ligand. Using relative quantification with TaqMan probes, we found that CYP1A2 was barely detectable in WB-F344 cells even after TCDD treatment. Therefore, using FRET probes and a plasmid-inserted PCR product as a standard, we performed absolute quantification of CYP1A2 in WB-F344 cells treated with DMSO (negative control) or 1 nM TCDD (positive control). We found that

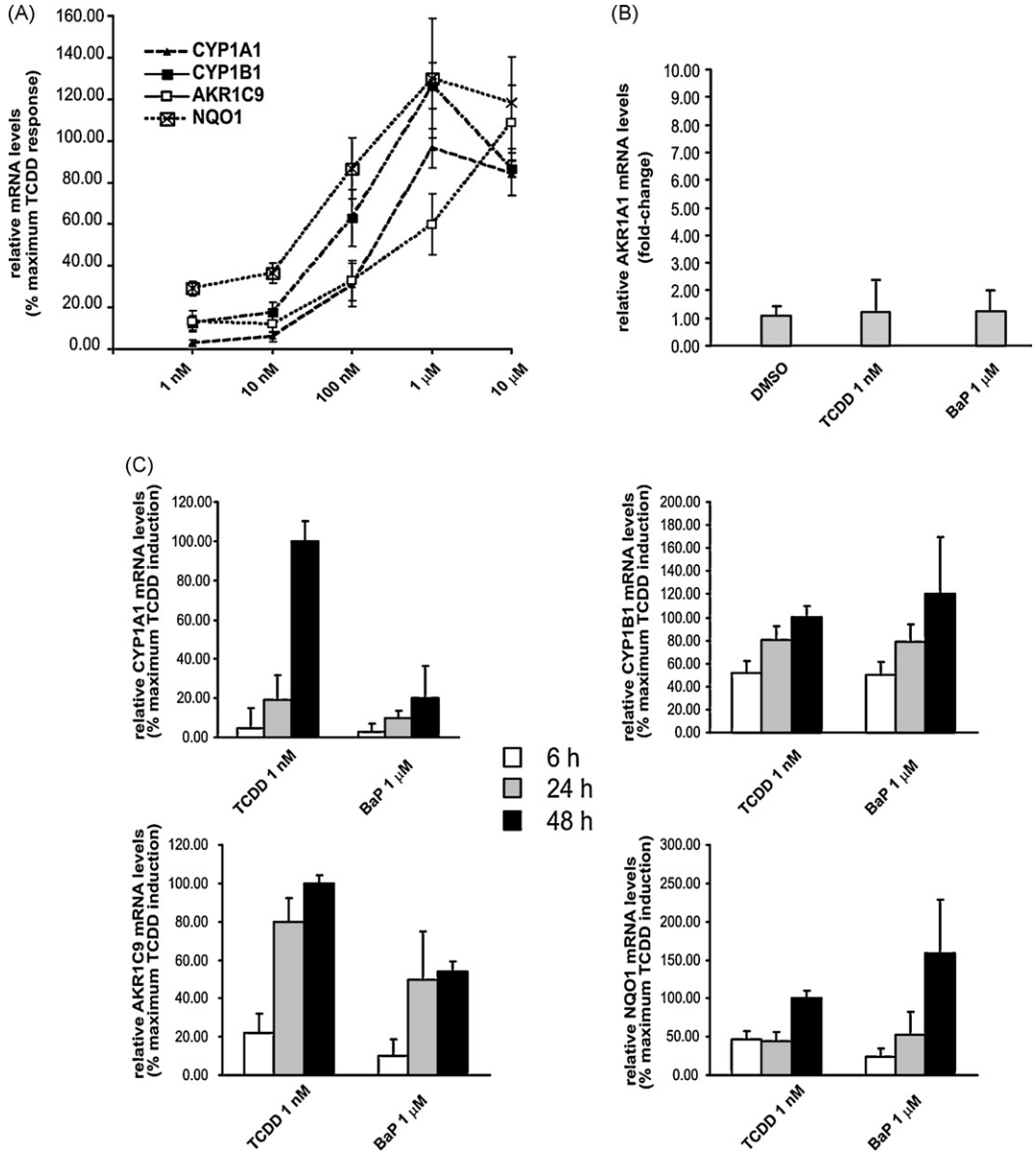


Fig. 1. Time and dose-dependent induction profiles of CYP1A1, CYP1B1, AKR1C9, and NQO1 mRNAs in rat liver epithelial WB-F344 cells treated with BaP, as compared to TCDD. (A) Cells were treated with DMSO (negative control), TCDD (1 nM), and a range of BaP concentrations (1 nM to 10 μM) for 24 h. (B) AKR1A1 mRNA levels after 24 h exposure to DMSO (solvent control), TCDD (1 nM) or BaP (1 μM). (C) Cells were treated with DMSO, TCDD, or BaP for 6, 24 and 48 h. Total RNA was isolated and quantitative real-time RT-PCR was performed as described in Section 2. The results in (A) and (C) were normalized to maximum 1 nM TCDD-induced mRNA level of respective enzyme. All results were expressed as mean ± S.D. of three independent experiments.

in DMSO-treated cells, CYP1A2 levels were below the detection limit of the assay (20 copies per reaction); in TCDD-treated samples, we detected only 137 ± 58 copies of the transcript per μg RNA. These results seem to imply that even induced CYP1A2 levels are very low in WB-F344, and it is consequently unlikely that CYP1A2 plays any role in metabolic activation of BaP or other PAHs in WB-F344 cells. The levels of AKR1A1 were not induced by either BaP or TCDD (Fig. 1B). We next compared time course profiles of CYP1A1, CYP1B1, NQO1 and AKR1C9 mRNAs induced by 1 nM TCDD and 1 μM BaP (Fig. 1C). For CYP1B1 and NQO1 induction, time course profiles were similar for both BaP and TCDD. In contrast, TCDD was a more potent inducer of CYP1A1 and AKR1C9, especially after 48 h incubation. This might be related to the metabolic clearance of BaP by induced enzymes. Therefore, although both compounds were potent inducers of all four enzymes, some target genes exhibited distinct profiles of expression in WB-F344 cells, when comparing persistent AhR ligand TCDD and BaP.

3.2. AhR ligands induce EROD, quinone reductase and aldo–keto reductase activities in WB-F344 cells

As all three types of enzymes were induced at the mRNA level, we next examined effects of BaP and TCDD on the respective enzymatic activities. As outlined in Fig. 2, TCDD was a more potent inducer of EROD activity in WB-F344 cells than BaP, despite having similar impact on CYP1A1 or CYP1B1 mRNA levels. This might be related to the known inhibition of EROD activity by BaP, which has been observed previously in rat hepatocytes [39]. Both compounds were efficient inducers of NQO1 activity and the specificity of this reaction was confirmed by using a specific inhibitor, dicoumarol [33], which significantly reduced both basal and induced NQO1 activities in a dose-dependent manner. As further shown in Fig. 2, both TCDD and BaP induced a significant increase of AKR activity in WB-F344 cells. As it has been shown that human AKR1C enzymes specifically catalyze the oxidation of 1-acenaphthenol [40], this seems to imply that the observed activity was related to increased AKR1C9 expression.

3.3. The shRNA-mediated AhR knock-down decreased CYP1A1, CYP1B1 and AKR1C9 mRNA induction

AhR is a critical regulator of CYP1 enzyme expression and its transactivation has been shown to play a role in NQO1 induction [reviewed in refs. [8,20]]. Nevertheless, the NQO1 has been also shown to be induced by a number of redox cycling agents or oxidative stress inducers through antioxidant response elements (ARE) being present in the enhancer region of the *Nqo1* gene [reviewed in refs. [21,41]]. Similarly, the human AKR1C enzymes have been suggested to be regulated via ARE, as they are up-regulated by ROS, PAHs and bifunctional enzyme inducers, and not by TCDD in human hepatoma or colon carcinoma cells [16]. Nevertheless, as shown above, both TCDD and BaP induced AKR1C9 expression in WB-F344 cells, suggesting that AhR might be involved in rat AKR1C9 regulation, at least in liver progenitor cells. Therefore, we next analyzed the possible role of the AhR in the regulation of AKR1C9 expression in WB-F344 cells stably transfected with a construct coding for shRNA targeted against AhR (Fig. 3A), using known AhR gene targets (CYP1A1 and CYP1B1) as a control for AhR-dependent expression.

As shown in Fig. 3B, induction of CYP1A1 was significantly reduced in the cells expressing the AhR shRNA, as compared to wild-type cells or cells transfected with a control vector coding for the shRNA targeting luciferase. Expression of anti-AhR shRNA also resulted in significant down-regulation of both CYP1B1 and AKR1C9 mRNA levels in cells treated with either BaP or TCDD. Nev-

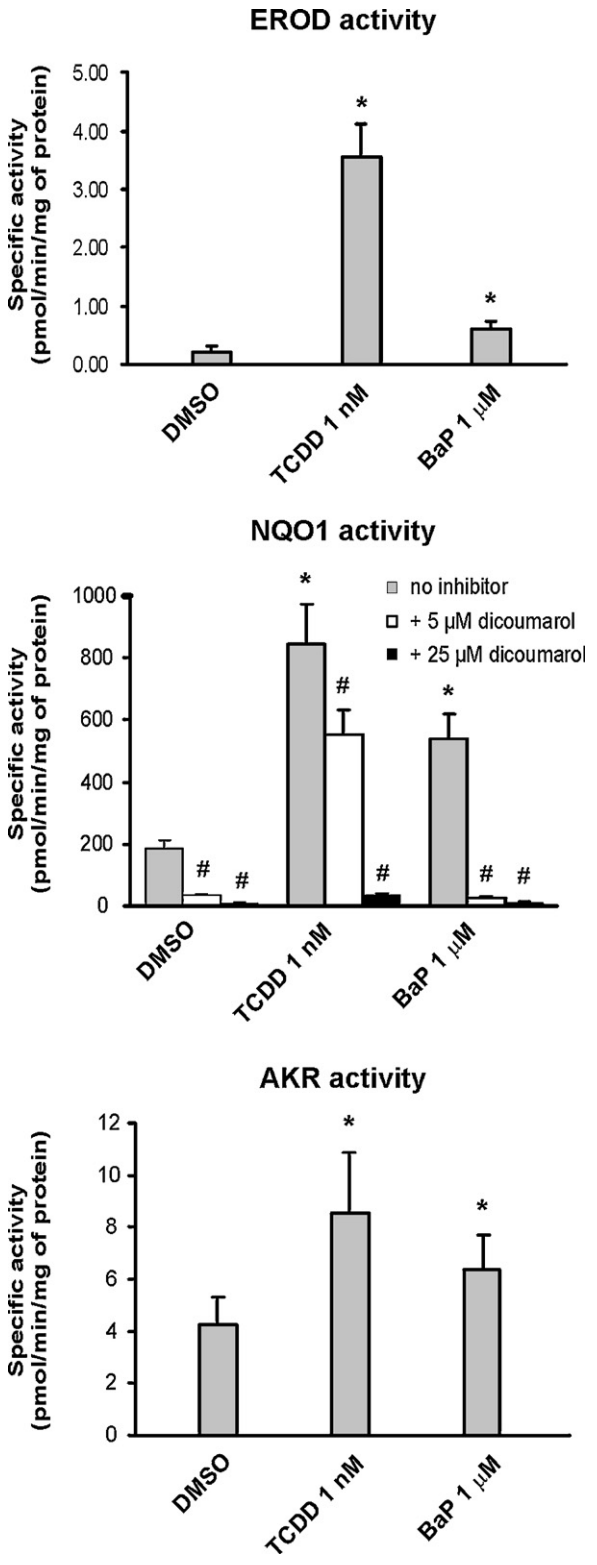


Fig. 2. Induction of activities of 7-ethoxyresorufin-*O*-deethylase (EROD) in microsomal fractions, and quinone reductase (NQO1) or aldo–keto reductases (AKRs) in cytosolic fractions of WB-F344 cells. The fractions were prepared from cells treated for 24 h with BaP (1 μM), or with a prototypical AhR ligand, TCDD (1 nM). The enzymatic assays were carried out as described in Section 2. The results were expressed as mean ± S.D. of three independent experiments. *Significant difference between control (0.1% DMSO) and treated samples ($p < 0.05$). #Significant difference between samples treated with dicoumarol and the respective treatment with DMSO, TCDD or BaP ($p < 0.05$).

ertheless, the induction of CYP1B1 was reduced only approximately by 50%, thus perhaps suggesting that the residual AhR is still sufficient for induction of *Cyp1b1* gene expression. In contrast, NQO1 mRNA levels were not affected by the presence of anti-AhR shRNA. The present data suggested that AKR1C9 induction depends on the presence of functional AhR.

3.4. Neither AKR1C9 nor AKR1A1 are induced in rat 5L hepatoma cells, and none of the enzymes are up-regulated either by TCDD or by BaP in BP8 cells

In order to compare the pattern of enzyme induction in WB-F344 cells with hepatoma-derived cells, we next employed two

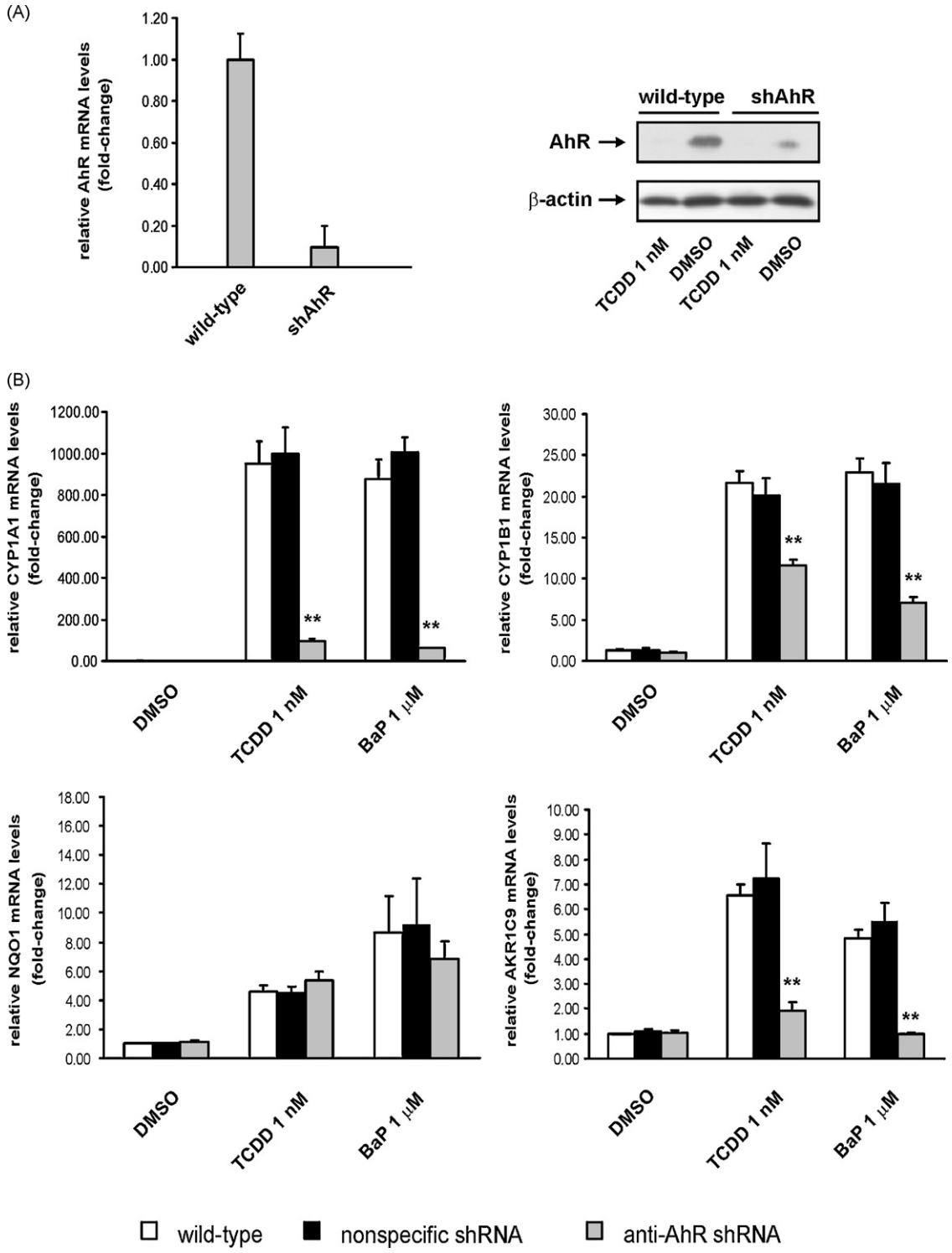


Fig. 3. Effects of AhR knock-down on the induction of CYP1A1, CYP1B1, AKR1C9, and NQO1 mRNAs in WB-F344 cells. (A) Expression of construct coding for shRNA targeted against AhR resulted in a notable reduction of both AhR mRNA and protein. (B) Wild-type WB-F344 cells, cells expressing anti-AhR shRNA or non-specific control shRNA were treated with DMSO (negative control), TCDD (1 nM) or BaP (1 μM) for 24 h. Total RNA was isolated and quantitative real-time RT-PCR was performed as described in Section 2. The results were expressed as mean ± S.D. of two independent experiments each performed in triplicates. **Significant difference between wild-type and shAhR cells ($p < 0.01$).

rat hepatoma cell lines, derived from rat hepatoma H4IIEC3 cell line, as a model: 5L cells, expressing functional AhR, and their variant clone, BP8 cell line, which lacks AhR protein [30]. Again, cells were treated for 24 h with either 1 nM TCDD or 1 μM BaP. Unlike in WB-F344 cells, we found highly inducible CYP1A2 expression in 5L cells (Fig. 4). Induction of CYP1A1, CYP1A2, CYP1B1 and NQO1 mRNA, all representing the AhR battery genes, was completely absent in BP8 cells. In contrast to WB-F344 cells, we observed no induction of AKR1C9 expression either in 5L or in BP8 cells (Fig. 4). AKR1C9 mRNA was even slightly decreased in 5L cells upon TCDD treatment. No inducibility of AKR1A1 was observed either. Nevertheless, the absence of AhR in BP8 was associated with approximately five times lower constitutive levels of AKR1C9 mRNA (Table 1), suggesting that AhR might participate in constitu-

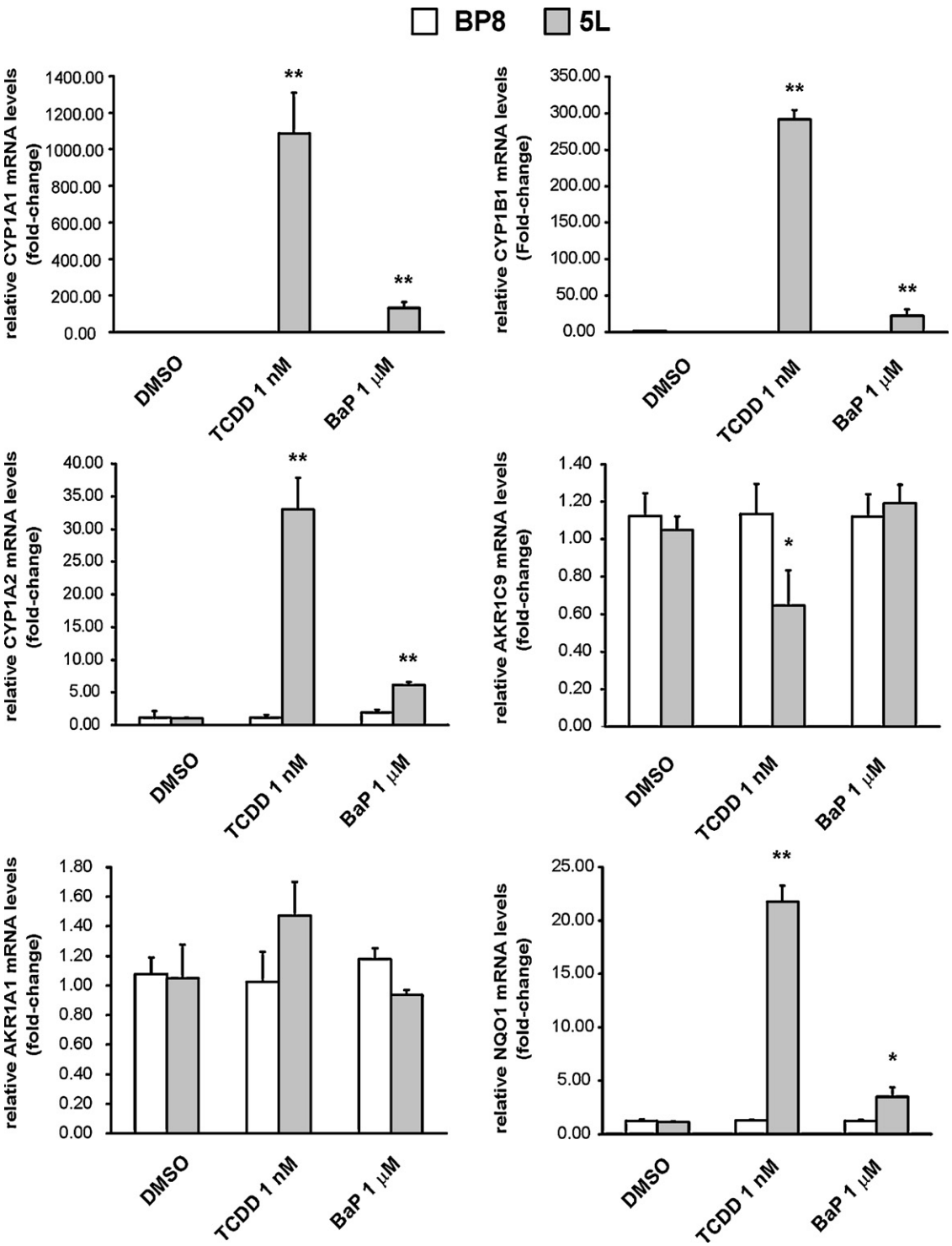


Fig. 4. Induction of CYP1A1, CYP1A2, CYP1B1, AKR1C9, AKR1A1 and NQO1 mRNAs in rat hepatoma 5L (grey columns; AhR-positive) and BP8 (white columns; AhR-negative) cells following 24-h treatment with BaP (1 μM), as compared to a prototypical AhR ligand, TCDD (1 nM). Total RNA was isolated and quantitative real-time RT-PCR was performed as described in Section 2. The results were expressed as mean ± S.D. of three independent experiments. *Significant difference between control (0.1% DMSO) and treated samples ($p < 0.05$). **Significant difference between control (0.1% DMSO) and treated samples ($p < 0.01$).

Table 1
A comparison of basal mRNA levels of AhR-regulated enzymes and AKRs in 5L (AhR-positive) and BP8 (AhR-negative) cells.

Enzyme	BP8	5L
CYP1A1	**0.00168** ± 0.00026[a]	0.97 ± 0.13
CYP1A2	1.18 ± 0.09	1.05 ± 0.04
CYP1B1	**0.66** ± **0.09**	1.05 ± 0.05
NQO1	1.11 ± 0.15	1.07 ± 0.07
AKR1C9	**0.19** ± **0.02**	1.05 ± 0.07
AKR1A1	**2.36** ± **0.24**	1.05 ± 0.06

Total RNA was isolated and quantitative real-time RT-PCR was performed as described in Section 2. Values in bold indicate a significant difference ($p < 0.05$) from the respective 5L cells value.

[a] The data were expressed as an amount of mRNA relative to 5L cells and they represent means ± S.D. of three independent experiments.

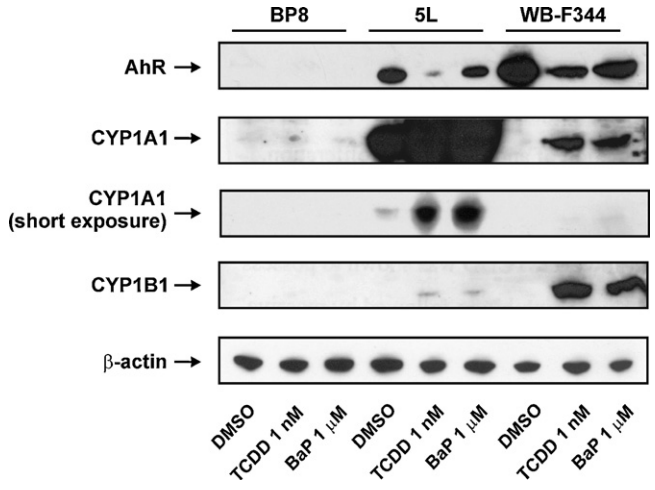


Fig. 5. Comparison of effects of BaP and TCDD on AhR, CYP1A1 and CYP1B1 protein levels in rat hepatoma BP8 cells (AhR-negative), rat hepatoma 5L cells (AhR-positive) and rat liver epithelial WB-F344 cells. Cells were treated for 24 h with BaP (1 μM), or with a prototypical AhR ligand, TCDD (1 nM). Cell lysates were prepared as described in Section 2 and subjected to Western blot analysis. The results are representative of three experiments. β-actin served as a loading control.

tive AKR1C9 expression in hepatoma cells. This trend was similar as for some other AhR battery genes, namely CYP1A1 and CYP1B1 (Table 1). In contrast, AKR1A1 levels were slightly higher in BP8 cells.

Next, we compared also the protein levels of AhR, CYP1A1 and CYP1B1 in rat hepatoma cells vs. the WB-F344 model of liver progenitor cells. As shown in Fig. 5, WB-F344 cell line expressed significantly higher AhR protein levels than 5L cells. The AhR levels in WB-F344 were even somewhat higher than in another frequently used model for studies of enzymes metabolizing PAHs–mouse Hepa-1 cells (data not shown). As expected, neither AhR protein nor CYP1A1/1B1 induction was observed in BP8 cells. There were also marked differences in the expression of CYP1A1 and CYP1B1 proteins between 5L and WB-F344 cells. Induction of CYP1A1 was much higher in 5L cells, whereas WB-F344 cells expressed significantly higher CYP1B1 protein levels after both BaP and TCDD treatment.

As shown in Fig. 6A, both BaP and TCDD induced AKR1C9 protein expression in WB-F344. In rat hepatoma cells, no induction of AKR1C9 mRNA was observed and, moreover, AKR1C9 protein levels were significantly higher in the AhR-positive 5L cells (Fig. 6B). These results corresponded with the results of real-time RT-PCR detection of AKR1C9 mRNA in all three cell lines. Taken together, there were significant differences in the pattern of induction of enzymes, which may contribute to PAH activation, between the liver progenitor and hepatoma cell lines.

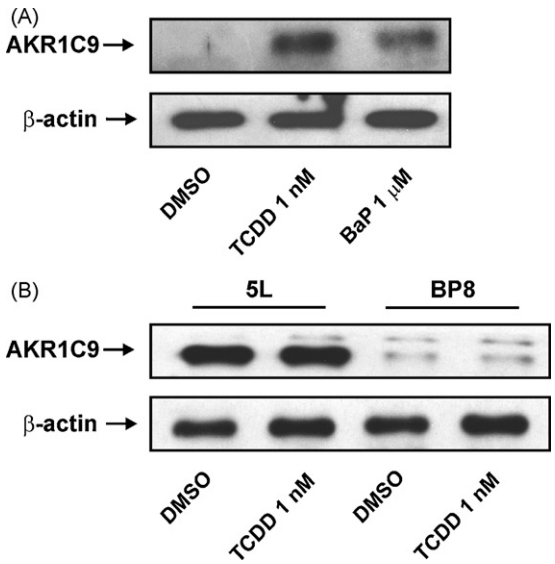
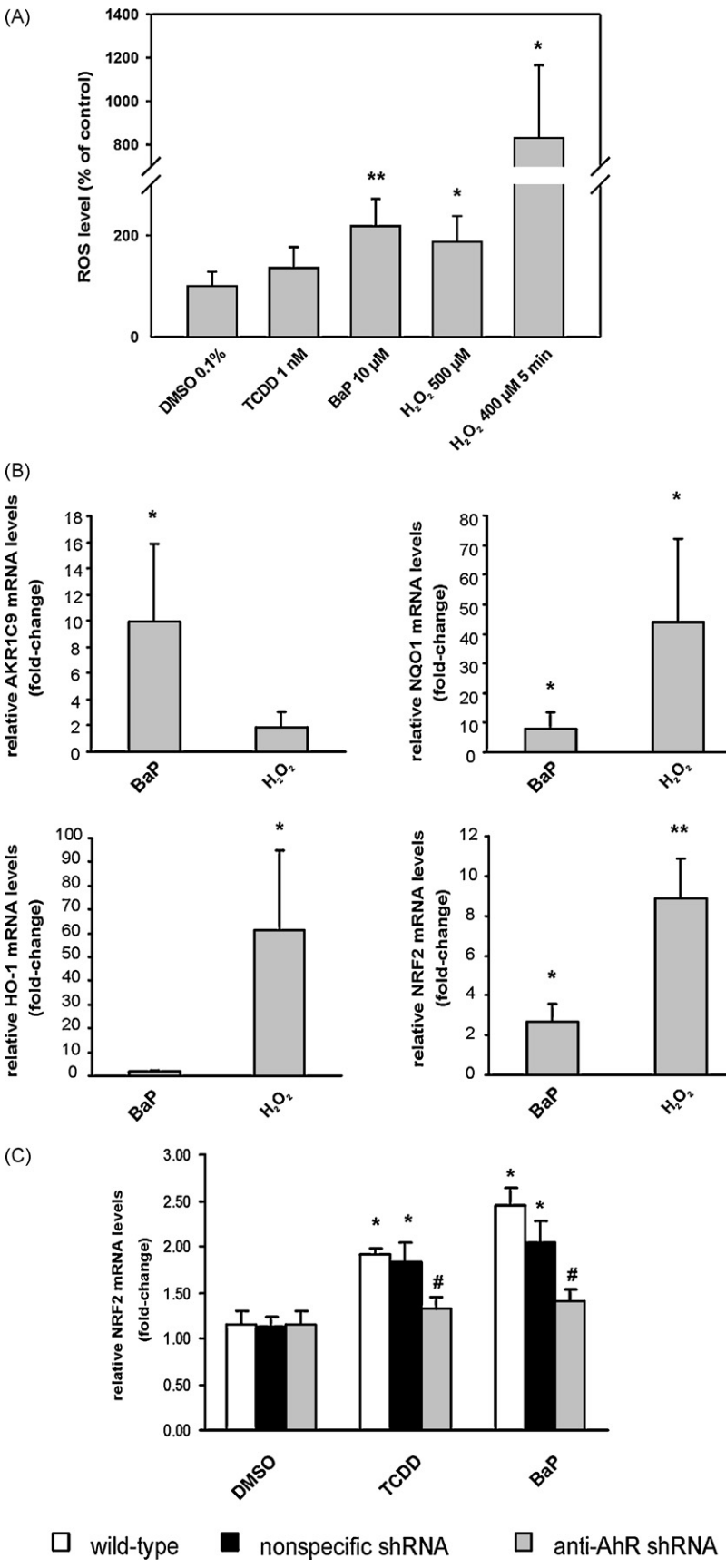


Fig. 6. Effects of AhR ligands on AKR1C9 protein levels in rat liver epithelial WB-F344 cells (A) or in rat hepatoma 5L (AhR-positive) and BP8 (AhR-negative) cells (B). Cells were treated for 24 h with BaP (1 μM), or with a prototypical AhR ligand, TCDD (1 nM). Cell lysates were prepared as described in Section 2 and subjected to Western blot analysis. The results are representative of three independent experiments. β-actin served as a loading control.

3.5. BaP induces formation of ROS and the AhR-dependent expression of NRF2 in WB-F344 cells

The oxidative stress induced by PAHs or their mixtures may contribute to up-regulation of both cellular defense against oxidative stress, including induction of NQO1 [42,43], and to up-regulation of genes further contributing to cellular and DNA damage, such as human AKR1C enzymes [16]. Therefore, we next examined effects of BaP on ROS formation in WB-F344 cells. As shown in Fig. 7A, BaP was able to elicit increased ROS production after 6 h. Therefore, we further investigated effects of model oxidative stress inducer, hydrogen peroxide, and compared them with BaP, following 6 h incubation. The shorter exposure period was selected based on preliminary experiments revealing significant hydrogen peroxide toxicity after longer treatments. As shown in Fig. 7B, hydrogen peroxide was a potent inducer of NQO1 mRNA expression. It also increased levels of HO-1 mRNA, an enzyme, which has been shown to act in cellular defense towards ROS [44], and which induction is considered to be a marker of oxidative stress [42]. Although BaP induced ROS formation, we did not find a significant increase in HO-1 expression, suggesting that the BaP-induced ROS production might be too low to elicit significant early oxidant production and activation of proteins involved in cellular defense towards oxidants. Contrary to the above results, hydrogen peroxide did not induce AKR1C9 mRNA significantly. These data seem to indicate that, at least in WB-F344 cells, AKR1C9 induction proceeds through an AhR-dependent mechanism, which does not require increased ROS levels.

The transcription factor NRF2 plays a key role in induction of expression of genes activated by oxidative stress, including NQO1 or HO-1 [45]. Apart from its activation at the protein level, following its dissociation from Keap1 [46], its expression has been shown to be induced by AhR ligands, thus providing a direct link between the AhR activity and induction of ARE-regulated genes [22]. As shown in Fig. 7B, BaP was found to induce NRF2 expression in WB-F344 cells. Therefore, we next investigated if the induction of NRF2 was dependent on the presence of AhR. As shown in Fig. 7C both TCDD and BaP induced NRF2 mRNA and this effect was prevented in cells expressing anti-AhR shRNA. This seems to indicate that the AhR-


(A)
ROS level (% of control)
1400
1200
1000
800
200
0
**
*
*
DMSO 0.1%
TCDD 1 nM
BaP 10 μM
H2O2 500 μM
H2O2 400 μM 5 min
(B)
relative AKR1C9 mRNA levels (fold-change)
relative NQO1 mRNA levels (fold-change)
relative HO-1 mRNA levels (fold-change)
relative NRF2 mRNA levels (fold-change)
BaP
H2O2
(C)
relative NRF2 mRNA levels (fold-change)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
#
DMSO
TCDD
BaP
wild-type
nonspecific shRNA
anti-AhR shRNA

dependent NRF2 induction might also contribute to BaP control of expression of enzymes participating in its metabolic activation and/or detoxification in WB-F344 cells.

4. Discussion

For many years, the very existence of liver progenitor cells was a matter of an intensive debate. However, the existence of both rodent and human liver progenitor cells is now generally acknowledged and they are observed in many liver disease conditions, including e.g. chronic viral hepatitis, liver cirrhosis or alcoholic and non-alcoholic fatty liver diseases [7]. As many of these chronic liver diseases have been linked to hepatocellular carcinoma or cholangiocarcinoma development, it seems likely that liver progenitor cells may participate in hepatocarcinogenesis [7]. However, little is known about the expression and/or activity of enzymes participating in metabolic activation of environmental carcinogens, such as PAHs. As shown recently, environmental PAH exposure may enhance the hepatocarcinogenicity of other critical factors involved in etiology of hepatocellular carcinoma, such as chronic hepatitis B virus infection [47]. Therefore, it is important to understand the regulation of PAH metabolism not only in hepatocytes, but also in other liver cell populations, such as liver progenitor cells. The principal aim of the present study was therefore to analyze the impact of BaP, a model PAH, on regulation of the xenobiotic-metabolizing enzymes in a cell-specific context of model liver progenitor cell line and to evaluate the role of AhR in their regulation.

The CYP1 family enzymes play a crucial role both in detoxification and in metabolic activation of PAHs into their respective dihydrodiol epoxide metabolites [8,15]. Previous studies have reported that in freshly isolated or undifferentiated liver progenitor cells, there seems to be very little, if any, CYP1A1 or CYP1A2 expression. This increases with their differentiation into hepatocyte-like cells [2,10–12,14]. The present data confirmed that WB-F344 cells contain inducible CYP1A1 and CYP1B1 proteins, as well as the corresponding EROD activity. On the other hand, we found that they have barely inducible CYP1A2 mRNA, a principal CYP1 family member found in mature hepatocytes or hepatocyte-like cells [48]. This seems to indicate that their enzyme profile does not resemble mature hepatocyte-like cells. The lack of CYP1A1 and CYP1B1 expression in oval cells and oval cell lines reported previously is intriguing, as even less differentiated cells, such as e.g. murine embryonic stem cells, seem to possess inducible CYP1A1 and/or CYP1B1 expression [49,50]. However, some of the early studies employed either pan-specific P450 antibodies or used AhR inducers acting simultaneously as CYP1 inhibitors. They also often concentrated only on CYP1A2 as a principal liver CYP1 form. Therefore, it cannot be excluded that e.g. CYP1B1 is indeed inducible in oval cells. This CYP1 enzyme is a potent metabolic activator of PAHs and we have recently observed that its up-regulation might be critical for formation of DNA adducts under conditions of CYP1A1 suppression [26]. Moreover, CYP1B1 has been shown to metabolize a variety of other genotoxic compounds, such as heterocyclic amines, aromatic amines, steroid hormones, etc. [51]. Therefore, it might play a significant role in metabolic activation of PAHs and related genotoxins in liver progenitor cells.

As the CYP1-dependent metabolic activation of PAHs to dihydrodiol epoxides is functional in the present liver progenitor cell line, we next concentrated on further enzymes contributing to metabolic activation of PAHs. Rat liver AKR1C9 is constitutively expressed at high levels in rat liver [52] and it catalyzes the second step in the metabolism of nearly all circulating steroid hormones, being simultaneously essential for the biosynthesis of bile acids [53]. It is its dihydrodiol dehydrogenase activity, which activates PAH *trans*-dihydrodiols by forming reactive and redox-active *o*-quinones [19]. As shown in Figs. 1, 2 and 6, AKR1C9 mRNA and protein expression, as well as AKR activity, were inducible in WB-F344 cells by both types of AhR agonists, persistent TCDD and the readily metabolized BaP, which can also induce production of ROS. As the induction of AKR1C9 was largely prevented by the shRNA-mediated AhR knock-down, effects of PAHs seem to be mediated by this transcription factor. Although AKR1C9 was not induced by AhR ligands in rat hepatoma 5L cells, its levels in AhR-positive 5L cells were significantly higher than in AhR-negative BP8 cells, thus suggesting a possible role of AhR in AKR1C9 regulation. Moreover, we have recently observed a significant induction of AKR1C9 mRNA expression by TCDD or BaP in rat lung epithelial cells (data not shown), which again indicates that AhR is involved in regulation of AKR1C9 expression. The increased AKR activity might perhaps contribute to the observed induction of ROS formation by BaP. However, oxidative stress did not seem to contribute to AKR1C9 induction by BaP. This conclusion is based on the observation that hydrogen peroxide did not increase AKR1C9 mRNA levels significantly. The AhR-dependent regulation of *AKR1C9* transcription is likely to be indirect, as no XREs have been found in the 5′-flanking region of the rat *AKR1C9* gene [54]. It has been suggested that activated AhR may also act as a co-activator for some yet unidentified transcriptional regulators [55], and such a mechanism might provide the necessary link between AhR activation and AKR1C9 regulation. The induction of AKR1C9 might further depend on cell-specific factors. It seems plausible to hypothesize that it can be more significant in liver progenitor cells, and not e.g. in hepatocytes, which have high constitutive AKR1C9 levels. The differential inducibility and relatively low basal expression of AKR1C9 in the present model of liver progenitors might be also related to the fact that WB-F344 do not express a full set of transcription factors found in differentiated hepatocytes, such as e.g. hepatocyte nuclear factors [56]. The mRNA of the second AKR enzyme investigated in the present study, AKR1A1, which human counterpart has been shown to catalyze *o*-quinone formation [18], was constitutively expressed in WB-F344 and it was not up-regulated in rat hepatoma cells either.

In the present study, BaP was found to increase ROS levels in WB-F344 cells. Induction of oxidative stress by BaP or related

Fig. 7. Induction of oxidative stress in WB-F344 cells by BaP and its impact on regulation of mRNA expression of enzymes involved in BaP metabolism. (A) WB-F344 cells were treated with DMSO (negative control), TCDD (1 nM), BaP (10 μM) or with hydrogen peroxide for 6 h and the level of reactive oxygen species in cells was determined by flow cytometry as fluorescence of dichlorofluorescein (DCF). Hydrogen peroxide (400 μM, 5-min exposure) was used as a positive control. The results are expressed as mean ± S.D. of results of at least three independent experiments. *Significant difference between control (0.1% DMSO) and treated samples ($p < 0.05$). **Significant difference between control (0.1% DMSO) and treated samples ($p < 0.01$). (B) Induction of AKR1C9, NQO1, HO-1 and NRF2 mRNAs in rat liver epithelial WB-F344 cells following 6-h treatment with BaP (1 μM), as compared to a hydrogen peroxide (500 μM). Total RNA was isolated and quantitative real-time RT-PCR was performed as described in Section 2. The results were expressed as mean ± S.D. of three independent experiments, relative to control treated with DMSO. *Significant difference between control (0.1% DMSO) and treated samples ($p < 0.05$). **Significant difference between control (0.1% DMSO) and treated samples ($p < 0.01$). (C) Effects of shRNA knock-down on induction of NRF2 by BaP and TCDD. Wild-type WB-F344 cells, cells transfected with anti-AhR shRNA or with non-specific shRNA were treated with DMSO (negative control), TCDD (1 nM) or with BaP (1 μM) for 24 h. Total RNA was isolated and quantitative real-time RT-PCR was performed as described in Section 2. The results were expressed as mean ± S.D. of two independent experiments each performed in triplicates. *Significant difference between control (0.1% DMSO) and treated samples ($p < 0.05$). #Significant difference between wild-type and shAhR cells ($p < 0.01$).

compounds, either through dihydrodiol dehydrogenases, or other mechanisms, has been shown to be linked to oxidative DNA damage both *in vitro* and *in vivo*, and it has been hypothesized to be associated with carcinogenic properties of PAHs [57–61]. NQO1 is an enzyme belonging to the battery of AhR-regulated genes, which has been suggested to contribute to cellular protection from oxidative stress by at least three different mechanisms, including reduction of quinones, maintenance of endogenous antioxidants in their reduced forms or stabilization of p53 tumor suppressor [62–65]. In the present study, both AhR ligands and hydrogen peroxide have been found to be potent inducers of NQO1 expression and activity. Although the AhR knock-down did not prevent either BaP- or TCDD-induced up-regulation of NQO1 mRNA, it should be noted that the residual AhR expression might be sufficient to drive NQO1 induction. As shown in Fig. 5, WB-F344 cells express relatively high levels of AhR protein and also CYP1B1 induction was not fully suppressed in cells expressing anti-AhR. In contrast, both NQO1 and CYP1B1 induction were fully prevented, when AhR was completely absent, in the AhR-negative BP8 hepatoma cells.

Despite increasing intracellular ROS formation, BaP failed to induce HO-1 mRNA expression. HO-1 is an inducible mammalian heme oxygenase, which has been shown to be activated in various organs in response to oxidative stress, and which has been suggested to play potentially a hepatoprotective role [66], e.g. in protection against redox-active environmental toxicants, such as quinones [67,68]. In contrast to BaP, hydrogen peroxide was a potent inducer of HO-1 expression, which might indicate that the BaP-induced production of ROS did not reach a threshold necessary for HO-1 up-regulation. The transcription factor NRF2 regulates both NQO1 and HO-1 expression [21,66]. It has been shown that NRF2 and AhR cooperate in NQO1 regulation, possibly through a direct or indirect interaction between those two transcription factors [69,70], and that AhR activation may lead to induction of NRF2 expression. The interplay between AhR and NRF2 could be even more complex, as another recent study suggested that NRF2 is involved in control of AhR expression and may thus modulate downstream events of the AhR signaling, including induction of CYP1 enzymes [71]. In WB-F344, both BaP and TCDD induced NRF2 mRNA expression in the AhR-dependent manner, thus suggesting that they might activate expression of ARE-regulated genes in this manner. Nevertheless, induction of NQO1 probably did not rely upon this mechanism, as it was not affected by inhibition of NRF2 induction in cells expressing anti-AhR shRNA.

Taken together, the above data seem to indicate that at least two major pathways contributing to genotoxic effects of PAHs, the CYP1A1/1B1-dependent metabolic pathway catalyzing production of PAH dihydrodiol epoxides forming covalent DNA adducts, and the AKR1C9-dependent pathway leading to an increased ROS formation and oxidative DNA damage, can be induced in rat liver progenitor cell line. Of particular note is the role of AhR in AKR1C9 induction; AhR might also play some role in the constitutive AKR1C9 expression, as found in hepatoma cells. In contrast, increased levels of ROS do not seem to play a major role in AKR1C9 up-regulation, at least in the WB-F344 cell model. Future studies should establish the precise role of AhR in regulation of AKR1C9. BaP also activated cellular defense mechanisms against oxidative stress, such as induction of NQO1 expression and activity. This *in vitro* model of liver progenitor cells might provide a further interesting insight into possible impact of genotoxic compounds on liver progenitor cells; nevertheless, further studies should confirm, whether the observed effects also apply to oval cells *in vivo*.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

Authors thank Carsten Weiss (Institute of Toxicology and Genetics, Research Center Karlsruhe, Germany) for providing 5L and BP8 cell lines, and Trevor M. Penning (University of Pennsylvania School of Medicine, Philadelphia, PA) for providing antibody against rat AKR1C9. The expert technical assistance of Iva Lišková is gratefully acknowledged. This study was supported by the Czech Science Foundation (grant no. 524/06/0517). The institutional support was provided by the Academy of Sciences of the Czech Republic (Research Plans AV0Z50040507 and AV0Z50040702) and the Czech Ministry of Agriculture (MZE0002716202).

References

[1] G.K. Michalopoulos, Liver regeneration, J. Cell. Physiol. 213 (2007) 286–300.
[2] G.J. Gordon, W.B. Coleman, J.W. Grisham, Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats, Am. J. Pathol. 157 (2000) 771–786.
[3] B. Knight, V.B. Matthews, J.K. Olynyk, G.C. Yeoh, Jekyll and Hyde: evolving perspectives on the function and potential of the adult liver progenitor (oval) cell, Bioessays 27 (2005) 1192–1202.
[4] S.S. Thorgeirsson, J.W. Grisham, Overview of recent experimental studies on liver stem cells, Semin. Liver Dis. 23 (2003) 303–312.
[5] M.H. Walkup, D.A. Gerber, Hepatic stem cells: in search of, Stem Cells 24 (2006) 1833–1840.
[6] M.R. Alison, M.J. Lovell, Liver cancer: the role of stem cells, Cell Prolif. 38 (2005) 407–421.
[7] T. Roskams, Liver stem cells and their implication in hepatocellular and cholangiocarcinoma, Oncogene 25 (2006) 3818–3822.
[8] D.W. Nebert, T.P. Dalton, The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis, Nat. Rev. Cancer 6 (2006) 947–960.
[9] T. Shimada, Y. Fujii-Kuriyama, Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1, Cancer Sci. 95 (2004) 1–6.
[10] M. Golding, C.E. Sarraf, E.N. Lalani, T.V. Anilkumar, R.J. Edwards, P. Nagy, S.S. Thorgeirsson, M.R. Alison, Oval cell differentiation into hepatocytes in the acetylaminofluorene-treated regenerating rat liver, Hepatology 22 (1995) 1243–1253.
[11] D. Schrenk, I. Eisenmann-Tappe, R. Gebhardt, D. Mayer, M. el Mouelhi, E. Rohrdanz, P. Münzel, K.W. Bock, Drug metabolizing enzyme activities in rat liver epithelial cell lines, hepatocytes and bile duct cells, Biochem. Pharmacol. 41 (1991) 1751–1757.
[12] P. Steinberg, R. Steinbrecher, D. Schrenk, P. Münzel, M. Bruck, H. Gschaidmaier, F. Oesch, K.W. Bock, Drug-metabolizing enzyme activities in freshly isolated oval cells and in an established oval cell line from carcinogen-fed rats, Cell Biol. Toxicol. 10 (1994) 59–65.
[13] H. Ooe, J. Kon, S. Miyamoto, Y. Ozone, S. Ninomiya, T. Mitaka, Cytochrome P450 expression of cultured rat small hepatocytes after long-term cryopreservation, Drug Metab. Dispos. 34 (2006) 1667–1671.
[14] L. Yin, M. Sun, Z. Ilic, H.L. Leffert, S. Sell, Derivation, characterization, and phenotypic variation of hepatic progenitor cell lines isolated from adult rats, Hepatology 35 (2002) 315–324.
[15] W. Xue, D. Warshawsky, Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review, Toxicol. Appl. Pharmacol. 206 (2005) 73–93.
[16] M.E. Burczynski, H.K. Lin, T.M. Penning, Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase, Cancer Res. 59 (1999) 607–614.
[17] M.E. Burczynski, T.M. Penning, Genotoxic polycyclic aromatic hydrocarbon ortho-quinones generated by aldo-keto reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor, Cancer Res. 60 (2000) 908–915.
[18] N.T. Palackal, M.E. Burczynski, R.G. Harvey, T.M. Penning, The ubiquitous aldehyde reductase (AKR1A1) oxidizes proximate carcinogen trans-dihydrodiols to o-quinones: potential role in polycyclic aromatic hydrocarbon activation, Biochemistry 40 (2001) 10901–10910.
[19] T.M. Penning, M.E. Burczynski, C.F. Hung, K.D. McCoull, N.T. Palackal, L.S. Tsuruda, Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active o-quinones, Chem. Res. Toxicol. 12 (1999) 1–18.
[20] D.W. Nebert, A.L. Roe, M.Z. Dieter, W.A. Solis, Y. Yang, T.P. Dalton, Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis, Biochem. Pharmacol. 59 (2000) 65–85.
[21] P. Nioi, J.D. Hayes, Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors, Mutat. Res. 555 (2004) 149–171.

[22] W. Miao, L. Hu, P.J. Scrivens, G. Batist, 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, J. Biol. Chem. 280 (2005) 20340–20348.
[23] M.S. Tsao, J.D. Smith, K.G. Nelson, J.W. Grisham, A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells, Exp. Cell Res. 154 (1984) 38–52.
[24] J. Topinka, S. Marvanová, J. Vondráček, O. Sevastyanova, Z. Nováková, P. Krčmář, K. Pěnčíková, M. Machala, DNA adducts formation and induction of apoptosis in rat liver epithelial 'stem-like' cells exposed to carcinogenic polycyclic aromatic hydrocarbons, Mutat. Res. 638 (2008) 122–132.
[25] J. Zatloukalová, L. Švihálková-Šindlerová, A. Kozubík, P. Krčmář, M. Machala, J. Vondráček, Beta-naphthoflavone and 3′-methoxy-4′-nitroflavone exert ambiguous effects on Ah receptor-dependent cell proliferation and gene expression in rat liver 'stem-like' cells, Biochem. Pharmacol. 73 (2007) 1622–1634.
[26] L. Umannová, M. Machala, J. Topinka, Z. Nováková, A. Milcová, A. Kozubík, J. Vondráček, Tumor necrosis factor-alpha potentiates genotoxic effects of benzo[a]pyrene in rat liver epithelial cells through upregulation of cytochrome P450 1B1 expression, Mutat. Res. 640 (2008) 162–169.
[27] Z. Andrysík, J. Vondráček, M. Machala, P. Krčmář, L. Švihálková-Šindlerová, A. Kranz, C. Weiss, D. Faust, A. Kozubík, C. Dietrich, The aryl hydrocarbon receptor-dependent deregulation of cell cycle control induced by polycyclic aromatic hydrocarbons in rat liver epithelial cells, Mutat. Res. 615 (2007) 87–97.
[28] C. Weiss, D. Faust, I. Schreck, A. Ruff, T. Farwerck, A. Melenberg, S. Schneider, B. Oesch-Bartlomowicz, J. Zatloukalová, J. Vondráček, F. Oesch, C. Dietrich, TCDD deregulates contact inhibition in rat liver oval cells via Ah receptor, JunD and cyclin A, Oncogene 27 (2008) 2198–2207.
[29] T.E. Smithgall, T.M. Penning, Electrophoretic and immunochemical characterization of 3alpha-hydroxysteroid/dihydrodiol dehydrogenases of rat tissues, Biochem. J. 254 (1988) 715–721.
[30] C. Weiss, S.K. Kolluri, F. Kiefer, M. Göttlicher, Complementation of Ah receptor deficiency in hepatoma cells: negative feedback regulation and cell cycle control by the Ah receptor, Exp. Cell Res. 226 (1996) 154–163.
[31] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[32] M.D. Burke, S. Thompson, R.J. Weaver, C.R. Wolf, R.T. Mayer, Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver, Biochem. Pharmacol. 48 (1994) 923–936.
[33] R.W. Nims, R.A. Prough, R.A. Lubet, Cytosol-mediated reduction of resorufin: a method for measuring quinone oxidoreductase, Arch. Biochem. Biophys. 229 (1984) 459–465.
[34] M.D. Burke, S. Thompson, C.R. Elcombe, J. Halpert, T. Haaparanta, R.T. Mayer, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450, Biochem. Pharmacol. 34 (1985) 3337–3345.
[35] N.T. Palackal, S.H. Lee, R.G. Harvey, I.A. Blair, T.M. Penning, Activation of polycyclic aromatic hydrocarbon trans-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells, J. Biol. Chem. 277 (2002) 24799–24808.
[36] J. Vondráček, L. Švihálková-Sindlerová, K. Pěnčíková, P. Krčmář, Z. Andrysík, K. Chramostová, S. Marvanová, Z. Valovičová, A. Kozubik, A. Gábelová, M. Machala, 7H-Dibenzo[c,g]carbazole and 5,9-dimethyldibenzo[c,g]carbazole exert multiple toxic events contributing to tumor promotion in rat liver epithelial 'stem-like' cells, Mutat. Res. 596 (2006) 43–56.
[37] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ method, Methods 25 (2001) 402–408.
[38] A.M. Gorman, A. Samali, A.J. McGowan, T.G. Cotter, Use of flow cytometry techniques in studying mechanisms of apoptosis in leukemic cells, Cytometry 29 (1997) 97–105.
[39] M. Till, D. Riebniger, H.J. Schmitz, D. Schrenk, Potency of various polycyclic aromatic hydrocarbons as inducers of CYP1A1 in rat hepatocyte cultures, Chem. Biol. Interact. 117 (1999) 135–150.
[40] T. O'Connor, L.S. Ireland, D.J. Harrison, J.D. Hayes, Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members, Biochem. J. 343 (Pt 2) (1999) 487–504.
[41] A.K. Jaiswal, Regulation of genes encoding NAD(P)H:quinone oxidoreductases, Free Radic. Biol. Med. 29 (2000) 254–262.
[42] N. Li, J. Alam, M.I. Venkatesan, A. Eiguren-Fernandez, D. Schmitz, E. Di Stefano, N. Slaughter, E. Killeen, X. Wang, A. Huang, M. Wang, A.H. Miguel, A. Cho, C. Sioutas, A.E. Nel, Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals, J. Immunol. 173 (2004) 3467–3481.
[43] C. Xu, C.Y. Li, A.N. Kong, Induction of phase I, II and III drug metabolism/transport by xenobiotics, Arch. Pharm. Res. 28 (2005) 249–268.
[44] S.W. Ryter, A.M. Choi, Heme oxygenase-1: redox regulation of a stress protein in lung and cell culture models, Antioxid. Redox Signal. 7 (2005) 80–91.
[45] J.M. Lee, J. Li, D.A. Johnson, T.D. Stein, A.D. Kraft, M.J. Calkins, R.J. Jakel, J.A. Johnson, Nrf2, a multi-organ protector? FASEB J. 19 (2005) 1061–1066.
[46] T. Nguyen, P.J. Sherratt, P. Nioi, C.S. Yang, C.B. Pickett, Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1, J. Biol. Chem. 280 (2005) 32485–32492.
[47] H.C. Wu, Q. Wang, L.W. Wang, H.I. Yang, H. Ahsan, W.Y. Tsai, L.Y. Wang, S.Y. Chen, C.J. Chen, R.M. Santella, Polycyclic aromatic hydrocarbon- and aflatoxin-albumin adducts, hepatitis B virus infection and hepatocellular carcinoma in Taiwan, Cancer Lett. 252 (2007) 104–114.
[48] A. Guillouzo, A. Corlu, C. Aninat, D. Glaise, F. Morel, C. Guguen-Guillouzo, The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics, Chem. Biol. Interact. 168 (2007) 66–73.
[49] K. Maezawa, K. Miyazato, T. Matsunaga, Y. Momose, T. Imamura, K. Johkura, K. Sasaki, S. Ohmori, Expression of cytochrome P450 and transcription factors during in vitro differentiation of mouse embryonic stem cells into hepatocytes, Drug Metab. Pharmacokinet. 23 (2008) 188–195.
[50] T. Neri, V. Merico, S. Garagna, C.A. Redi, M. Zuccotti, Expression of phase I and phase II genes in mouse embryonic stem cells cultured in the presence of 2,3,7,8-tetrachlorodibenzo-para-dioxin, Biochim. Biophys. Acta 1780 (2008) 826–836.
[51] F.P. Guengerich, Y.J. Chun, D. Kim, E.M.J. Gillam, T. Shimada, Cytochrome P4501B1: a target for inhibition in anticarcinogenesis strategies, Mutat. Res. 523 (2003) 173–182.
[52] T.E. Smithgall, T.M. Penning, Indomethacin-sensitive 3 alpha-hydroxysteroid dehydrogenase in rat tissues, Biochem. Pharmacol. 34 (1985) 831–835.
[53] C.F. Hung, T.M. Penning, Members of the nuclear factor 1 transcription factor family regulate rat 3alpha-hydroxysteroid/dihydrodiol dehydrogenase (3alpha-HSD/DD AKR1C9) gene expression: a member of the aldo-keto reductase superfamily, Mol. Endocrinol. 13 (1999) 1704–1717.
[54] H.K. Lin, T.M. Penning, Cloning, sequencing, and functional analysis of the 5'-flanking region of the rat 3 alpha-hydroxysteroid/dihydrodiol dehydrogenase gene, Cancer Res. 55 (1995) 4105–4113.
[55] P.C. Boutros, I.D. Moffat, M.A. Franc, N. Tijet, J. Tuomisto, R. Pohjanvirta, A.B. Okey, Dioxin-responsive AHRE-II gene battery: identification by phylogenetic footprinting, Biochem. Biophys. Res. Commun. 321 (2004) 707–715.
[56] M.P. Piechocki, R.M. Toti, M.J. Fernström, R.D. Burk, R.J. Ruch, Liver cell-specific transcriptional regulation of connexin32, Biochim. Biophys. Acta 1491 (2000) 107–122.
[57] K. Frenkel, L. Wei, H. Wei, 7,12-Dimethylbenz[a]anthracene induces oxidative DNA modification in vivo, Free Radic. Biol. Med. 19 (1995) 373–380.
[58] K.B. Kim, B.M. Lee, Oxidative stress to DNA, protein, and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo(a)pyrene, Cancer Lett. 113 (1997) 205–212.
[59] S.A. Leadon, M.R. Stampfer, J. Bartley, Production of oxidative DNA damage during the metabolic activation of benzo[a]pyrene in human mammary epithelial cells correlates with cell killing, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 4365–4368.
[60] J.H. Park, S. Gopishetty, L.M. Szewczuk, A.B. Troxel, R.G. Harvey, T.M. Penning, Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) by PAH o-quinones: involvement of reactive oxygen species and copper(II)/copper(I) redox cycling, Chem. Res. Toxicol. 18 (2005) 1026–1037.
[61] K. Seike, M. Murata, K. Hirakawa, Y. Deyashiki, S. Kawanishi, Oxidative DNA damage induced by benz[a]anthracene dihydrodiols in the presence of dihydrodiol dehydrogenase, Chem. Res. Toxicol. 17 (2004) 1445–1451.
[62] G. Asher, J. Lotem, B. Cohen, L. Sachs, Y. Shaul, Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 1188–1193.
[63] L. Landi, D. Fiorentini, M.C. Galli, J. Segura-Aguilar, R.E. Beyer, DT-Diaphorase maintains the reduced state of ubiquinones in lipid vesicles thereby promoting their antioxidant function, Free Radic. Biol. Med. 22 (1997) 329–335.
[64] D. Siegel, E.M. Bolton, J.A. Burr, D.C. Liebler, D. Ross, The reduction of alpha-tocopherolquinone by human NAD(P)H:quinone oxidoreductase: the role of alpha-tocopherolhydroquinone as a cellular antioxidant, Mol. Pharmacol. 52 (1997) 300–305.
[65] P. Talalay, A.T. Dinkova-Kostova, Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates, Methods Enzymol. 382 (2004) 355–364.
[66] E.O. Farombi, Y.J. Surh, Heme oxygenase-1 as a potential therapeutic target for hepatoprotection, J. Biochem. Mol. Biol. 39 (2006) 479–491.
[67] N. Li, M.I. Venkatesan, A. Miguel, R. Kaplan, C. Gujuluva, J. Alam, A. Nel, Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element, J. Immunol. 165 (2000) 3393–3401.
[68] T. Xia, P. Korge, J.N. Weiss, N. Li, M.I. Venkatesen, C. Sioutas, A. Nel, Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity, Environ. Health Perspect. 112 (2004) 1347–1358.
[69] Q. Ma, K. Kinneer, Y. Bi, J.Y. Chan, Y.W. Kan, 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, Biochem. J. 377 (2004) 205–213.
[70] C. Köhle, K.W. Bock, Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2, Biochem. Pharmacol. 73 (2007) 1853–1862.
[71] S. Shin, N. Wakabayashi, V. Misra, S. Biswal, G.H. Lee, E.S. Agoston, M. Yamamoto, T.W. Kensler, NRF2 modulates aryl hydrocarbon receptor signaling: influence on adipogenesis, Mol. Cell. Biol. 27 (2007) 7188–7197.