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The effect of aryl hydrocarbon receptor ligands on the expression of *AhR*, *AhRR*, *ARNT*, *Hif1α*, *CYP1A1* and *NQO1* genes in rat liver

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Abstract

The aryl hydrocarbon receptor (AhR) mediates a variety of biological responses to ubiquitous environmental pollutants. AhR together with ARNT, AhRR, HIF1α represent a novel basic helix–loop–helix/PAS family of transcriptional regulators. Their interplay may affect the xenobiotic response. In this study, the effect of i.p. administration of different AhR ligands on the expression of AhR, AhRR, ARNT, HIF1α and CYP1A1 and NAD(P)H: quinone oxidoreductase (NQO1), the enzymes controlled by AhR were examined in Sprague–Dawley rat liver. Quantitative real-time RT-PCR analysis revealed no changes in the mRNA expression of ARNT and HIF1α following 3-methylcholanthrene (3-MC), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or β-naphthoflavone (BNF) treatment. AhRR expression was affected by TCDD but not by BNF and 3-MC. Expression of AhR mRNA and of the markers of its activation, CYP1A1 and NQO1, was significantly increased by administration of TCDD, 3-MC and, to lower extent, BNF.

These results indicate that binding of the ligands to AhR up-regulates the mRNA transcription not only of CYP1A1 and NQO1, but also of AhR itself. The level of AhR induction depends on the potency of xenobiotic metabolizing enzymes inducer.
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Keywords: AhR; ARNT; CYP1A1; β-Naphthoflavone; 3-Methylcholanthrene; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

1. Introduction

The aryl hydrocarbon receptor (AhR) mediates a variety of biological responses to ubiquitous environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and chlorinated dibenzo-*p*-dioxins. In what can be defined as an adaptive pathway, AhR binds its ligands and up-regulates a battery of xenobiotic-metabolizing enzymes (XMEs) (Nebert et al., 2000; Okey et al., 2005; Ramadoss et al., 2005). These enzymes metabolize many of their substrates to more soluble and excretable products, but also as a classic exam-

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator (synonym HIF1β); AhRR, AhR repressor; HIF1α, hypoxia inducible factor 1α; CYP1A1, cytochrome P450 1A1; NQO1, NAD(P)H: quinone oxidoreductase; BNF, β-naphthoflavone; ANF, α-naphthoflavone; 3-MC, 3-methylcholanthrene; PolR2a, RNA polymerase II; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH, polycyclic aromatic hydrocarbon; XMEs, xenobiotics metabolizing enzymes; bHLH/PAS, basic helix–loop–helix/PAS

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ple of benzo[*a*]pyrene shows, are responsible for their activation to ultimate carcinogenic metabolites leading to DNA adducts formation, sister chromatid exchanges and carcinogenesis (Pelkonen and Nebert, 1982; Nebert, 1989; Brauze et al., 1991, 1997). Beside the induction of XMEs, AhR ligands modulate the transcription of cell cycle-related genes (Puga et al., 2002) and some other recently described (Puga et al., 2000; Frueh et al., 2001; Sun et al., 2004; Tijet et al., 2006). Moreover, functional analysis of AhR knockout mice revealed that AhR is involved in teratogenesis, immunosupression due to thymic involution, hepatotoxicity and tumor promotion caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Poland and Knutson, 1982; Okey et al., 1994; Mimura et al., 1997; Viluksela et al., 2000). It is also possible that AhR is involved in adverse biological effects of environmental endocrine disruptants (Safe et al., 1998).

AhR can bind several structurally diverse classes of xenobiotic ligands, but their binding affinity differs to great extent (Denison et al., 2002; Denison and Nagy, 2003). Some, like α-naphthoflavone (ANF), are only partial AhR agonists and can modify AhR signaling induced by the other ligands like β-naphthoflavone (BNF) (Gasiewicz and Rucci, 1991; Santostefano et al., 1993; Gasiewicz et al., 1996; Harvey et al., 1998).

AhR resides in the cytoplasm as a complex with chaperone proteins: HSP90, XAP2 and p23 (Petrulis and Perdew, 2002). The receptor binds xenobiotics such as TCDD or 3-methylcholanthrene (3-MC) with extremely high affinity, and the complex subsequently translocates to the nucleus, where the liganded AhR binds the AhR nuclear translocator (ARNT) protein (McGuire et al., 1994; Heid et al., 2000). In the nucleus, the liganded AhR/ARNT heterodimer binds to xenobiotics responsive element sequences, which are enhancer DNA elements present in the 5′-flanking region of target genes. The aryl hydrocarbon receptor repressor (AhRR) is one of the genes identified as targets for the AhR/ARNT transcription factor. AhR and AhRR constitute a negative regulatory feedback loop of xenobiotic signal transduction, where the liganded AhR in a heterodimer with ARNT activates expression of the AhRR gene, while the expressed AhRR, in turn, inhibits the function of AhR (Mimura et al., 1999). The ARNT protein does not function exclusively in the AhR signaling pathway, but also forms a heterodimer with the hypoxia inducible factor 1α (HIF1α). The HIF1α ARNT complex binds to hypoxia responsive elements and activates transcription of a battery of hypoxia responsive genes (Kewley et al., 2004). AhR, AhRR, HIF1α and ARNT represent a novel basic helix–loop–helix/PAS (bHLH/PAS) family of transcriptional regulators and their interplay may affect the xenobiotic response. Potential competition for the recruitment of ARNT could lead to a functional interference between hypoxia and AhR signaling pathways. Indeed, a cross-talk between both pathways was demonstrated in some in vitro experimental models (Gradin et al., 1996; Chan et al., 1999; Nie et al., 2001). However, other in vitro studies indicated that the functional interference between hypoxia and AhR-mediated signaling pathways does not occur through the competition for ARNT protein (Pollenz et al., 1999).

In this study, we examined the effect of i.p. administration of different AhR ligands on the expression of bHLH/PAS transcription factors AhR, AhRR, ARNT and HIF1α, and of the enzymes controlled by AhR in rat liver, CYP1A1 and NAD(P)H: quinone oxidoreductase (NQO1). The results indicate that binding of the ligands to AhR up-regulates the transcription not only of CYP1A1 and NQO1, but also of AhR itself. The level of AhR induction depends on the potency of XMEs inducer.

2. Materials and methods

2.1. Chemicals

BNF, ANF, SYBR® Green I (10,000× concentration), agarose, JumpStart Taq DNA Polymerase, Enhanced Avian RT First Strand Synthesis Kit (STR-1), PCR Low Ladder Marker Set were supplied by Sigma–Aldrich Co. (St. Louis, MO). 3-MC was obtained from Eastman Kodak (Rochester, NY). TRIZOL® Reagent was provided by Gibco BRL (Gaithersburg, MD). Fluorescein was obtained from Bio-Rad Laboratories (Hercules, CA). Restriction endonucleases were purchased from Fermentas International Inc. (Burlington, Canada). Deoxyribonucleotides triphosphates: dATP, dGTP, aCTP, dTTP were provided by Roche Diagnostics (Mannheim, Germany). PCR primers were provided by Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland (oligo.pl). All the other compounds were readily available commercial products.

2.2. Animal treatment

Archival frozen liver samples were used, obtained from animals treated as described previously (Brauze and Malejka-Giganti, 2000; Brauze, 2004). Briefly, female Sprague–Dawley rats (Specific Pathogen Free from Harlan Sprague–Dawley, Indianapolis, IN, 50 ± 2 days old) were injected i.p. with a single dose (40 mg/kg body weight) of BNF, ANF or 3-MC dissolved in corn oil. One group of rats received BNF at the dose of 80 mg/kg body weight. A control group of animals received the vehicle only. Rats were sacrificed by decapitation under asphyxia with CO_2, 8–144 h after treatment. The livers were perfused, frozen in liquid nitrogen and stored at −80 °C. The livers from rats treated with TCDD at the dose of 25 μg/kg body weight and killed 72 h later, were provided by Dr. Karl K.

Rozman (University of Kansas Medical Center, Kansas City, KS).

All the experiments were conducted according to Local Ethics Committee Guidelines for animal experimentation.

2.3. RNA isolation

Total RNA was isolated from frozen rat liver samples using TRIZOL® Reagent (Gibco BRL/Life Technologies). Tissue samples (50–100 mg) were pulverized in a mortar under liquid nitrogen. Immediately after evaporation of nitrogen, 1 ml of TRIZOL was added and the frozen tissue powder was grinded. After melting the procedure was carried out according to the manufacturer's (Gibco BRL) instruction. The extracted total RNA dissolved in water was quantitated spectrophotometrically at 260 nm (A_{260}). The $A_{260/280}$ ratio > 1.8 was considered an acceptable measure of RNA purity. RNA integrity was estimated by visual examination of two distinct rRNA bands (28S and 18S) on denaturing 1% agarose gel stained with acridine orange. Only RNA samples with clear and sharp 28S band about twice as intense as that of 18S, were used for further experiments.

2.4. cDNA synthesis

Eight micrograms of total RNA were reverse-transcribed to cDNA in a total volume of 40 μl, using Enhanced Avian RT First Strand Synthesis Kit (Sigma) according to the manufacturer's instruction. mRNA from target genes contains a very long untranslated 3′-region, therefore, random nonamers were used as primers of the reaction. The amount of cDNA synthesized in a single reaction was sufficient to PCR-amplify all genes (targets and standards).

2.5. Primer design

PCR primers to published genes sequences (GenBank, accession numbers in Table 1) were designed with the Lasergene 5.05 (DNAStar Inc.) software and their specificity was verified with BLAST alignment search. To prevent amplification of sequences from the genomic DNA contamination, primers and/or amplicons were designed to cross the exon/exon boundaries (Table 1). To confirm amplification of the expected size fragment, amplification products were characterized by agarose gel electrophoresis. Identity of amplicons was further verified by the analysis of digestion products generated by restriction endonucleases (not shown).

2.6. SYBR® Green I real-time PCR

AhR, ARNT, AHRR, HIF 1α, CYP1A1 and NQO1 cDNA were amplified by real-time PCR in the iCycler iQ real-time PCR detection system with Optical System Software 3.1 (Bio-Rad Laboratories) using SYBR® Green I as the detection dye. Amplification was carried out in a total volume of 20 μl containing 0.2× SYBR® Green I, PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 3.5 mM $MgCl_2$, 10 nM fluorescein, 0.2 μM each primer, 0.2 mM each dNTPs, 0.5 U JumpStart Taq DNA Polymerase and 0.4 μl cDNA (undiluted reverse-transcription product derived from 8 μg RNA in 40 μl reaction). The reactions were cycled 45 times using the following parameters: 95 °C for 20 s, 56 °C (60 °C, Table 1) 30 s and 72 °C for 30 s during which the fluorescence data were collected. At the end of the PCR, a melting curve was generated by heating the samples from 50 to 95 °C in 0.5 °C increments with a dwell time at each temperature of 10 s, to verify the specificity of the product. A non template controls were run with every assay and no

Table 1
Sequence of primers used in real-time PCR, amplicon sizes, annealing temperatures and the amplification efficiencies

Targets accession no.	Sequences	Amplicon length (bp)	Annealing T_m (°C)	PCR efficiency
β-Actin, NM_031144	F: 5′ AACCCTAAGGCCAACCGTGAAAAG 3′; R: 5′ CGACCAGAGGCATACAGGGACAAC 3′	110-i	56	0.94
PolR2A, XM_343922	F: 5′ GACTGGGGGCCGCTGGAC-CTA 3′; R: 5′ GGCGAGTAACCTGGGCTGAAG 3′	214-i	60	0.98
CYP1A1, NM_012540	F: 5′ GTCCCGGATGTGGCC-CTTCTCAAA 3′; R: 5′ TAACTCTTCCCTGGATGCCTTCAA 3′	109	56	1.06
NQO1, NM_017000	F: 5′ ACATCACAG-GGGAGCCGAAGGACT3′; R: 5′ GGCACCCCAAACCAATACAATG 3′	166	56	1.02
AhR, NM_013149	F: 5′ TCACTGCGCAGAATCCCACATCC 3′; R: 5′ TCGCGTCCTTCTTCATCC-GTTAGC 3′	186-i	60	0.94
ARNT, NM_012780	F: 5′ AGAGACTTGCCAG-GGAAAATCATA 3′; R: 5′ TTTCGAGCCAGGGCACTACAGG 3′	115	56	0.99
AhRR, AY367561	F: 5′ CCTCCTCGGCTCTCCTTGTTTTG 3′; R: 5′ CTTTTGCC-CTTGAGTCCATCGTGA 3′	133	56	0.87
HIF1α, NM_024359	F: 5′ CGTGCCCCTACTATGTCGCTTTCT 3′; R: 5′ GGTTTCTGCTGCCTTGTATG 3′	205-i	56	1.04

The hyphen in the primer sequence denotes the exon/exon boundary. Letter "i" after the amplicon length indicates that exon/exon boundary was inside the amplified sequence.

Table 2
Expression levels of AhR, ARNT, AhRR, Hif1α, NQO1 and CYP1a1 mRNA in rat liver, following BNF administration

Time after BNF treatment (h)	AhR	ARNT	AhRR	Hif1α	NQO1	CYP1A1
0	1.00 ± 0.13	1.00 ± 0.24	1.00 ± 0.57	1.00 ± 0.17	1.00 ± 0.56	1.00 ± 0.17
8	1.29 ± 0.37	0.81 ± 0.19	0.85 ± 0.59	1.60 ± 0.61	9.94 ± 3.42[a]	843.23 ± 72.17[a]
24	1.80 ± 0.23	0.90 ± 0.19	0.31 ± 0.17	1.09 ± 0.26	2.61 ± 0.29	566.30 ± 88.96[a]
48	0.94 ± 0.16	0.75 ± 0.12	0.13 ± 0.05	0.83 ± 0.06	0.74 ± 0.10	43.14 ± 5.25
72	1.13 ± 0.14	0.91 ± 0.18	1.30 ± 1.07	0.92 ± 0.06	1.44 ± 0.21	83.60 ± 32.47
144	1.57 ± 0.13	2.09 ± 0.42	0.43 ± 0.11	1.00 ± 0.10	1.27 ± 0.0.23	150.95 ± 52.23

The level of mRNA expression (normalized to β-actin and PolR2A) is presented relative to that in untreated animals (time point "0"). Each value represents the mean ± S.E.M. of four rats; each measurement was performed in triplicates for two different cDNA samples.
[a] Significantly different from "0" time point as determined by one-way ANOWA followed by Tukey's post test, $p < 0.05$.

indication of PCR contamination was observed. Lack of PCR products from the non-reverse transcribed RNA control indicated that contamination of the genomic DNA did not serve as amplification template.

2.7. PCR data analysis and statistics

Expression levels of the target genes were normalized with respect to two reference genes, β-actin and PolR2a, using relative quantification method. The β-actin expression was reported not to be affected by treatment of rats with TCDD (Korkalainen et al., 2004), whereas PolR2a was recommended as the reference gene with the most constant expression in various tissues (Radonic et al., 2004). All calculations were performed using software Gene Expression MacroTM 1.10 (Bio-Rad).

To determine the efficiency of PCR amplification of reference and target genes, dilution series (1/2 dilution) of cDNA were prepared. Each dilution was amplified in real-time PCR and the obtained threshold cycle (C_t) values were used to construct a graph C_t versus log 10 of the cDNA sample dilution. The slope of the graph was used to determine the reaction efficiency according to the formula: efficiency = $[10^{(-1/\mathrm{slope})}] - 1$.

Two separate reverse transcriptions (RT) were performed on RNA from each animal and the products from each of these RT runs were analyzed in triplicate by real-time PCR. The relative gene expression was then calculated for the triplicate samples derived from each separate RT reaction by Gene Expression MacroTM 1.10 (Bio-Rad) software, then the average of the two values was carried forward as the value to be entered into calculation of the mean ± S.E.M. for each treatment group.

Statistical significance of differences was assessed by *t*-test (Table 4) or by one-way ANOVA (Tables 2 and 3) followed by Tukey's post test, using GraphPad Prism Version 4.03 for Windows (GraphPad Software, San Diego CA); $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Amplification kinetics

The effect of i.p. administered ligands, BNF, ANF, 3-MC and TCDD on the expression of AhR and Hif1α-mediated signaling pathways in rat liver was assessed by using quantitative real-time RT-PCR to measure changes in the mRNA level of AhR, ARNT, AhRR, Hif1α and CYP1A1 and NQO1. Normalization of the measurements was achieved by comparing with the expression of the control genes, β-actin and PolR2a, known to be invariant upon the treatment with tested compounds. The efficiencies of target and control genes amplification are shown in Table 1. The average threshold cycle (C_t) values determined for control rats were as follows: 23.6-β-actin, 26.1-PolR2A, 27.1-AhR, 27.4-ARNT, 27.9-Hif1α, 28.1-

Table 3
The effect of different Ah receptor ligands on the expression of AhR, ARNT, AhRR, Hif1α, NQO1 and CYP1a1 genes in rat liver

Treatment	*N*	AhR	ARNT	AhRR	Hif1α	NQO1	CYP1A1
Corn oil	4	1.00 ± 0.12	1.00 ± 0.28	1.00 ± 0.56	1.00 ± 0.18	1.00 ± 0.35	1.00 ± 0.15
BNF 40 mg/kg	4	1.40 ± 0.18	0.38 ± 0.03	0.21 ± 0.10	0.65 ± 0.09	3.03 ± 0.32[b]	414.89 ± 64.84[a,b]
BNF 80 mg/kg	3	1.71 ± 0.36	0.51 ± 0.10	0.22 ± 0.16	0.72 ± 0.19	3.80 ± 0.72	464.27 ± 88.03[a,b]
ANF	4	1.22 ± 0.16	0.82 ± 0.08	0.79 ± 0.28	0.96 ± 0.14	1.37 ± 0.33[b]	2.60 ± 0.91[b]
MC	4	2.56 ± 0.29[a]	1.08 ± 0.21	1.35 ± 0.69	1.20 ± 0.17	6.67 ± 1.50[a]	688.83 ± 102.21[a]

The level of mRNA expression (normalized to β-actin and PolR2a) is presented relative to that in animals treated with the corn oil. Each value represents the mean ± S.E.M. of *N* individual rats; each measurement was performed in triplicates for two different cDNA samples. Statistical significance was assessed by one-way ANOVA followed by Tukey's post test, $p < 0.05$ was considered as significant.
[a] Significantly different from corn oil treated control group.
[b] Significantly different from MC treated group.

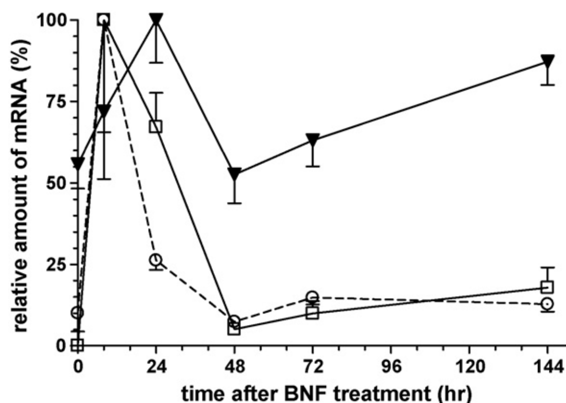

Fig. 1. Time-course of the induction of CYP1A1, NQO1 and AhR mRNA expression in the livers of BNF-treated rats. Expression levels of CYP1A1 (□), NQO1 (◯) or AhR (▼) relative to β-actin and PolR2A mRNA concentrations are presented as % of the highest value.

NQO1, 29.6-AhRR and 31.2-CYP1A1. In rats treated with BNF, C_t values for CYP1A1 and NQO1 dropped to 21.3 and 24.8, respectively.

3.2. *Time course of mRNA expression after treatment with BNF*

Table 2 and Fig. 1 present the mRNA levels of AhR, ARNT, AhRR and Hif1α as well as those of CYP1A1 and NQO1 throughout the 144 h time course following a single i.p. injection of BNF (40 mg/kg body weight). The only statistically significant changes in the expression level were observed for CYP1A1 and for NQO1. The maximal induction of CYP1A1 and NQO1 expression was observed after 8 h (843- and 10-fold increase, respectively). After that time point the expression of both genes gradually decreased. However 144 h later, 150-fold increase of CYP1A1 expression, although not statistically significant, was observed. The maximal (1.8-fold) increase of AhR mRNA expression was observed at 24 h, but the observed differences were not statistically significant. Changes in the expression level of the other transcription factors were also not significant (Table 2).

3.3. *The effect of various AhR ligands on the expression of AhR, ARNT, AhRR, Hif1α and CYP1A1 and NQO1*

The effect of different AhR ligands on the expression level of the investigated transcription factors and of CYP1A1 and NQO1 in rat livers was measured 24 h (BNF, ANF, 3-MC) or 72 h (TCDD) after ligand administration. Injection of BNF at 40 mg/kg body weight increased the level of CYP1A1, NQO1 and AhR expression, but only the former was statistically significant (Table 3). The increased dose of BNF (80 mg/kg body weight) did not induce expression of CYP1A1 further than the lower dose. No effect on the expression of ARNT and HIF1α was noted. BNF treatment of rats seemed to depress AhRR mRNA level, however, due to the marked inter-individual variations the effect was not statistically significant (Table 3).

Injection of ANF slightly increased the expression of CYP1A1 and NQO1, but no significance level of the changes was achieved at the dose used (40 mg/kg body weight).

3-MC at 40 mg/kg was more efficient inducer of the investigated genes expression than BNF. 24 h following its administration, the significant increase of the CYP1A1, NQO1 as well as of AhR genes was observed (689-, 6.7- and 2.6-fold, respectively; Table 3). No statistically significant effect on the expression of ARNT, AhRR and HIF1α was noted.

The effect of TCDD was evaluated 72 h after administration of the ligand at 25 μg/kg body weight (Table 4). At this dose TCDD caused the significant increase in the mRNA level of CYP1A1, NQO1, AhR and AhRR (1160-, 15-, 3- and 5-fold, respectively). No significant effect on ARNT or Hif1α expression was observed.

Interindividual variation of the AhRR mRNA expression remarkably exceeded these observed for all other

Table 4
The effect of TCDD on the expression of AhR, ARNT, AhRR, Hif1α, NQO1 and CYP1a1 genes in rat liver

Treatment	AhR	ARNT	AhRR	Hif1α	NQO1	CYP1A1
Corn oil	1.00 ± 0.29	1.00 ± 0.40	1.00 ± 0.25	1.00 ± 0.37	1.00 ± 0.18	1.00 ± 0.17
TCDD	3.00 ± 0.29[a]	1.02 ± 0.13	4.93 ± 0.92[a]	1.54 ± 0.73	15.19 ± 1.42[a]	1161.60 ± 244.72[a]

The level of mRNA expression (normalized to β-actin and PolR2a) is presented relative to that in animals treated with the corn oil. Each value represents the mean ± S.E.M. of three individual rats; each measurement was performed in triplicates for two different cDNA samples. Statistical significance was assessed by t-test, $p < 0.05$ was considered as significant.

[a] Significantly different from corn oil treated control group.

studied genes, although the same cDNA samples were used for expression measurements.

4. Discussion

Planar polycyclic aromatic compounds are potent ligands for the AhR and, through this interaction, they activate the transcription of the XME genes battery, which includes *CYP1A1* and Phase II enzyme, *NQO1*.

The focus of the present study was to evaluate the relationship between the effects of ligands on the expression of bHLH/PAS family of transcription factors mediating the AhR response and on the CYP1A1 and NQO1 induction. For this purpose, three different classes of ligands were used: chlorinated dibenzo-*p*-dioxin TCCD, polycyclic aromatic hydrocarbon 3-MC and naphthoflavones, ANF and BNF. Among them, TCDD binds with the highest affinity to AhR and is recognized as the most powerful XMEs inducer (Huff et al., 1994; Okey et al., 1994). 3-MC is a carcinogen, which exerts its activity by inducing enzymes that activate parent compound to ultimate reactive metabolite reacting with DNA (Riddick et al., 1994; Moorthy, 2002). Naphthoflavones are known as either inhibitors or enhancers of chemical carcinogenesis and these effects are related to induction and modulation of XMEs (Gasiewicz and Rucci, 1991; McKillop and Case, 1991; Miller et al., 1991).

Quantitative real-time RT-PCR measurement of mRNA in rats following i.p. administration of ligands showed that exposure to XMEs inducers provoked up-regulation of the AhR receptor. This effect was related to inducer potency. TCDD and 3-MC caused the significant increase in the CYP1A1 and NQO1 mRNA levels, as well as the increase in the level of AhR mRNA. BNF, in the doses comparable to 3-MC, also increased levels of CYP1A1 and NQO1 mRNAs, albeit at lower extent as compared to 3-MC. ANF, considered as specific inhibitor of CYP1A isoenzymes, only slightly increased the levels of CYP1A1 and NQO1 mRNA, and did not affect the AhR mRNA. BNF at 80 mg/kg body weight affected the mRNA level of AhR and CYP1A1 and NQO1 in the extent comparable to 40 mg/kg dose.

Similar conclusion on the relationship of AhR and CYP1A1 gene expression in Sprague–Dawley rats, but only for TCDD, was formulated by Franc et al. (2001). These authors measured ligand binding, immunoreactive protein level and mRNA levels of AhR and of CYP1A1 as a biomarker of the inducer's efficiency. Since increases in the AhR protein level were invariably accompanied by substantial increases of the AhR mRNA level, they suggested that up-regulation of AhR by its own ligand occurs (at least in part) at the pre-translational level. The results of our study, indicating the positive relationship between the mRNA levels of CYP1A1 and AhR after i.p. administration of TCDD and also other AhR ligands, support this suggestion. This is also consistent with some earlier reports. For example, Sloop and Lucier (1987) reported significant increase in cytosolic AhR levels measured by radioligand binding in rat liver after exposure to TCDD. Interestingly, we have found that the time course of induction of mRNA by BNF treatment of rats followed the same pattern for CYP1A1 and NQO1. However, results of our previous work indicated that CYP1A1 enzyme activity was induced more rapidly than that of NQO1 (Brauze, 2004). It seems that some post-transcriptional mechanisms were responsible for delayed induction of NQO1 protein. We observed that the level of NQO1 mRNA induction was much lower than that of CYP1A1. It is believed that besides being regulated by AhR pathway, this enzyme, as well as several other enzymes of Phase II metabolism, is controlled by the transcription factor Nrf2. This transcription factor interacts with the antioxidant response elements, consensus sequence upstream of the promoter of many Phase II genes (Talalay et al., 2003). Our results indicate positive relationship between treatment of rats with agonists of AhR and the levels of receptor mRNA. This indirectly confirms the suggestion that the observed rapid and prolonged depletion of AhR protein in cell cultures treated with AhR agonists is due to its proteolytic degradation (Prokipcak and Okey, 1991; Reick et al., 1994; Giannone et al., 1995, 1998; Pollenz, 1996; Ma and Baldwin, 2000).

In our study, AhR levels were altered as the result of in vivo treatment with TCDD, 3-MC and BNF or ANF. However, these compounds had little influence on the receptor's dimerization partner, ARNT. Similar observation was reported Franc et al. (2001) in rats exposed to TCDD. Contrary to our findings, Nishihashi et al. (2006) reported significant induction of ARNT mRNA after treatment of male Wistar rats with 3-MC. It cannot be completely excluded that induction of ARNT by AhR ligands is gender specific, as female rats were used in both, ours and Franc et al. (2001) experiments.

The AhRR is a negative regulator of AhR, which mediates most of the toxic and biochemical effects of TCDD. The studies of (Korkalainen et al., 2004) have shown that AhRR is not the auxiliary contributing factor to the rat strain difference in TCDD sensitivity, but simultaneous measurement of CYP1A1 mRNA indicated that AhRR may be an important determinant of tissue-specific responsiveness to TCDD. In our study, significant induction of AhRR mRNA level was seen

when TCDD was used as AhR agonist. Contrary to TCDD, treatment of Sprague–Dawley rats with BNF did not induce, but rather depressed, expression of AhRR mRNA in the liver. However, due to large inter-individual variations depression was not statistically significant. The observation that BNF treatment of rats could reduce expression of the AhRR was unexpected and definitely needs further studies. Treatment of rats with 3-MC did not induce expression of AhRR mRNA as well. However, because effect of 3-MC treatment on AhRR mRNA expression was tested at one time point only (24 h), we cannot completely exclude the possibility that the difference in the effect of 3-MC versus TCDD was due to the difference in the duration of exposure to these AhR agonists. The relative lack of effect of 3-MC treatment on AhRR mRNA levels in liver was reported recently by Nishihashi et al. (2006) who also found that 3-MC did not significantly induce AhRR mRNA in Wistar rats 24 h after treatment.

The large inter-individual variation in AhRR mRNA expression was not due to poor quality of cDNA, because when expression of other tested genes was measured using the same cDNA samples, the inter-individual variation was remarkably smaller. Similar variation of AhRR mRNA levels among individuals was observed also by Korkalainen et al. (2004). Our PCR primers and those used by Korkalainen et al. (2004) were designed to amplify distinct parts of AhRR cDNA. In this way, the probability that large inter-individual variation of AhRR mRNA expression is not due to experimental artifacts but reflect a real phenomenon is increased. The Sprague–Dawley rat is not an inbred strain and it is possible that variation of AhRR expression represents natural physiological difference among individuals.

Potential competition for ARNT binding exists between AhR and Hif1α. Cross-talk between the AhR and Hif1α signaling pathways was reported in several cell culture models (Gradin et al., 1996; Chan et al., 1999; Nie et al., 2001; Kewley et al., 2004). This raises the possibility that activation of the AhR-dependent pathway by AhR ligands may lead to interference with hypoxia signaling pathway by sequestering of ARNT. The AhR agonists used in the current study did not affect the Hif1α mRNA levels. In order to better evaluate the relationship between the AhR and Hif1α, the agents inducing hypoxia should be introduced to experimental protocol. On the other hand, analysis of the AhR-mediated signaling during physiological hypoxia revealed lack of competition for ARNT transcription factor (Pollenz et al., 1999). Moreover, the study of Hofer et al. (2004) showed that exposure of rats to CO inhibited the xenobiotic response, while TCDD administration had no significant negative impact on hypoxia-mediated gene transcription. The results of our current study, although performed in normoxia conditions, seem to confirm the latter observation.

In summary, our study extends previous basically limited to TCDD, in vivo studies on the AhR mediated signaling pathway. While expression of AhR mRNA was induced by Ah receptor ligands, expression of ARNT and Hif1α remained unchanged. Treatment of rats with TCDD caused induction of AhRR mRNA, whereas different AhR ligands, BNF and 3-MC, were ineffective as inducers of AhRR expression in rat liver. However, BNF and 3-MC demonstrated to be very effective as inducers of CYP1A1 and NQO1 mRNAs.

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