

Exposure of rats to exogenous endocrine disruptors 17 α -ethinylestradiol and benzo(a)pyrene and an estrogenic hormone estradiol induces expression of cytochromes P450 involved in their metabolism

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Abstract

OBJECTIVES: The term “endocrine disruptor” (ED) is used for compounds that mimic or antagonize the effects of endogenous hormones. Synthetic estrogen 17 α -ethinylestradiol (EE2) and a human carcinogen benzo[a]pyrene (BaP) are assigned as exogenous endocrine disruptors and an estrogenic hormone estradiol is a natural endogenous disruptor. Here, the potency of these three disruptors administered to rats individually and in combination to induce expression of cytochrome P450 (CYP) enzymes involved in their own metabolism (CYP1A1, 2C and 3A) *in vivo* was investigated.

METHODS: Changes in CYP protein expression after exposure of rats to BaP, EE2 or estradiol were analyzed by Western blotting. Using the HPLC method, CYP1A1, 2C and 3A specific activities in hepatic microsomes isolated from exposed rats were analyzed.

RESULTS: Whereas exposure to BaP induces expression of CYP1A1 protein and its marker activity (Sudan I oxidation) in liver, kidney and lung of rats, no significant induction of this CYP and its enzyme activity was produced by EE2 and estradiol. Treatment of BaP in combination with EE2 and/or estradiol decreased the BaP-mediated CYP1A1 induction in liver of exposed rats. BaP also induces CYP2C11 protein in rat liver and kidney, but does not increase its enzyme activity measured as testosterone 16 α -hydroxylation. The enzyme activity of another enzyme of the 2C subfamily, CYP2C6, diclofenac 4'-hydroxylation, is even decreased by BaP. The CYP2C11 protein expression and/or its activity are also increased in liver of rats treated with EE2 and estradiol, but its expression is significantly decreased in lung. The CYP2C6 activity is also elevated by treatment of rats with EE2 and estradiol administered individually as well as in their combination. Whereas only a slight increase in CYP3A protein expression was found by BaP in rat liver, its enzyme activity, testosterone 6 β -hydroxylation, increased significantly in this organ. In contrast, no effect or even a decrease in CYP3A expression and its enzyme activity was produced by EE2 and estradiol in rats exposed to these compounds.

CONCLUSIONS: The results found indicate that three EDs tested in this study, a carcinogen BaP, an estrogenic hormone estradiol and its synthetic derivative, EE2, might, due to their potential to induce expression of CYPs involved in their own metabolism, modulate their biological efficiencies.

Abbreviations:

AhR	- aryl hydrocarbon receptor
ARNT	- aryl hydrocarbon receptor nuclear translocator
BaP	- benzo[a]pyrene
BPDE	- BaP-7,8-dihydrodiol-9,10-epoxide
CAR	- constitutive androstane receptor
CO	- carbon monoxide
CYP	- cytochrome P450
dG-N ² -BPDE	- 10-(deoxyguanosin-N ² -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo-[a]pyrene
DNA	- deoxyribonucleic acid
ED	- endocrine disruptor
EE2	- 17 α -ethinylestradiol
GADPH	- glyceraldehyde 3-phosphate dehydrogenase
HPLC	- high performance liquid chromatography
IARC	- International Agency for Research on Cancer
mEH	- microsomal epoxide hydrolase
OCs	- oral contraceptives
PVDF	- polyvinylidene fluoride
PXR	- pregnane X receptor
RXR	- retinoid X receptor
SDS-PAGE	- sodium dodecylsulfate polyacrylamide gele electrophoresis
TBST-Tween buffer	- Tris-buffered saline with Tween 20
XRE	- xenobiotic response element

INTRODUCTION

The term “endocrine disruptor” (ED) is used for compounds that mimic or antagonize the effects of endogenous hormones, alter the synthesis and metabolism of natural hormones, or modify hormone receptor levels. Synthetic estrogen 17 α -ethinylestradiol (EE2) and a carcinogenic environmental pollutant benzo[a]pyrene (BaP), belong to the group of chemicals assigned as exogenous endocrine disruptive compounds, while an estrogenic hormone estradiol, or more precisely, 17 β -estradiol, is a natural endogenous endocrine disruptor.

BaP is a polycyclic aromatic hydrocarbon (PAH) that has been classified as human carcinogen (Group 1) by the International Agency for Research on Cancer IARC (IARC 2010). BaP and other PAHs are produced mainly by incomplete combustion of organic matter and are ubiquitous in the environment, leading to measurable background levels of exposure in the general population (IARC 2010). Beside the inhalation of polluted air, the main sources of exposure are tobacco smoke and diet (Phillips and Castegnaro 1999; Phillips 2002). BaP has been shown to cause cytotoxic, genotoxic, mutagenic and carcinogenic effects in various tissues and cell types (Labib *et al.* 2012; Lemieux *et al.* 2012; Sidden *et al.* 2012). Chronic exposure of laboratory animals

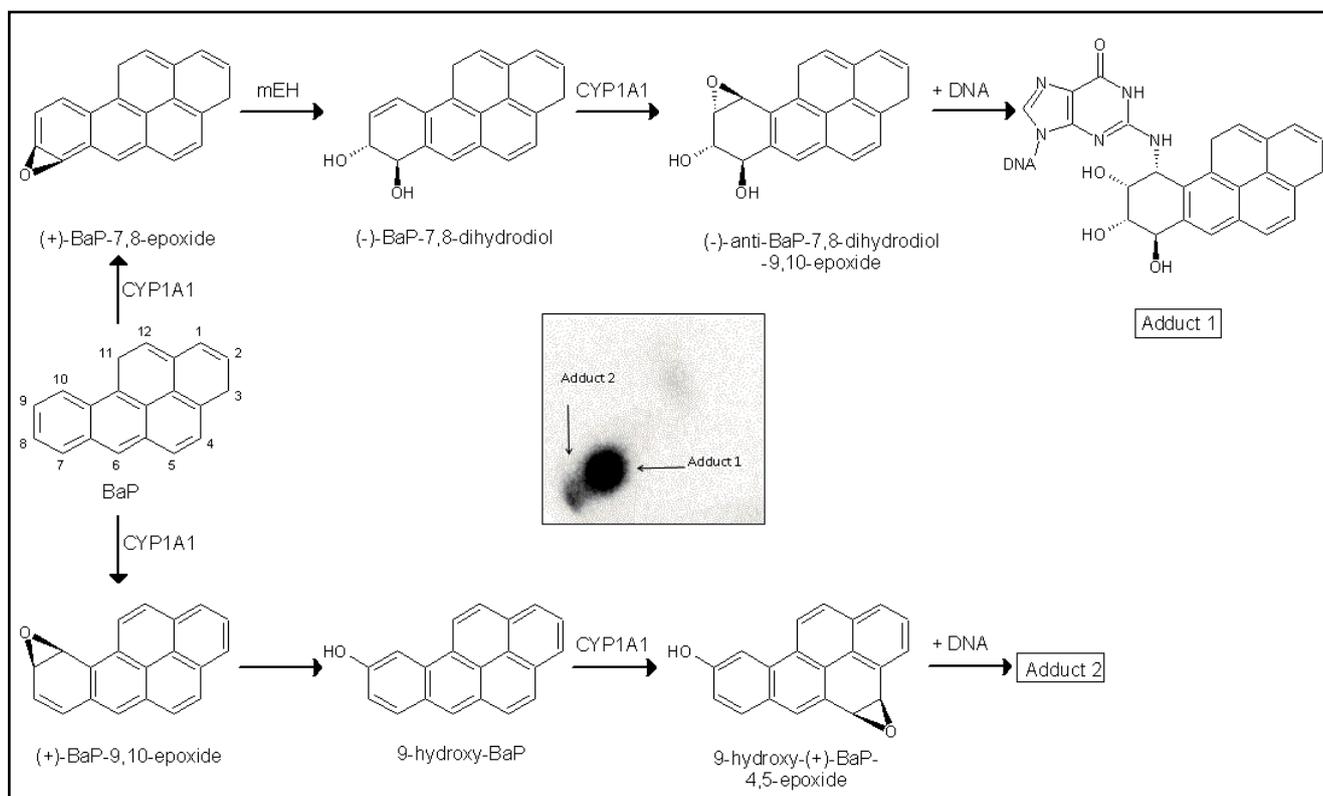


Fig. 1. Proposed pathways of biotransformation and DNA adduct formation of BaP catalyzed by CYP1A1 and mEH. *Upper*, the typical three-step activation process by P450 1A1 followed by hydrolysis by mEH leads to BPDE which forms dG-N²-BPDE (adduct 1). *Lower*, the two-step activation process by P450 1A1 leads to the formation of 9-hydroxy-BaP-4,5-epoxide that can react with deoxyguanosine in DNA (adduct 2). *Insert*: Autoradiographic profile of BaP-DNA adducts formed by human P450 1A1 in Supersomes™ with mEH in the presence of NADH and cytochrome b₅ as evaluated by TLC ³²P-postlabeling as described previously (Stiborova *et al.* 2016c).

to BaP has been associated with the development of cancer, primarily in the skin, stomach and lungs (IARC 2010).

BaP requires metabolic activation prior to reaction with DNA (Baird *et al.* 2005; Phillips 2005). Cytochrome P450 (CYP) enzymes, mainly CYP1A1 and 1B1, are the most important enzymes involved in this process (Baird *et al.* 2005; Phillips 2005; Hamouchene *et al.* 2011; Stiborova *et al.* 2014; 2016a; Kraus *et al.* 2016), in combination with microsomal epoxide hydrolase (*mEH*) (Figure 1). First, CYP1A1 enzyme oxidizes BaP to an epoxide that is then converted to a dihydrodiol by *mEH* (*i.e.* BaP-7,8-dihydrodiol); then further bio-activation by CYP1A1 leads to the ultimately reactive species, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) that can react with DNA, forming adducts preferentially at guanine residues (Figure 1). The 10-(deoxyguanosin-*N*²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-*N*²-BPDE) adduct is the major product of the reaction of BPDE with DNA *in vitro* and *in vivo* (Phillips 2005). However, BaP is also oxidized to other metabolites such as other dihydrodiols, BaP-diones and further hydroxylated metabolites (Baird *et al.* 2005; Indra *et al.* 2013; 2014; Stiborova *et al.* 2014; 2016a; Sulc *et al.* 2016). Although most of these metabolites are detoxification products, BaP-9-ol (BaP-hydroxy-BaP) is the precursor of 9-hydroxy-BaP-4,5-epoxide that can form another adduct with deoxyguanosine in DNA (Figure 1). The expression of CYP enzymes of the family 1 metabolizing BaP (CYP1A1, 1A2 and 1B1) is known to be up-regulated by the aryl hydrocarbon receptor (AhR); BaP itself can bind to and activate AhR thereby enhancing its own metabolic activation (Hockley *et al.* 2007; Arlt *et al.* 2008; 2012).

Synthetic estrogen 17 α -ethinylestradiol (Figure 2) is an additional studied endocrine disruptor. This compound is widely used as the major component in oral

contraceptives (OCs) (Bolt 1979). Incorporation of an acetylenic moiety into the estradiol molecule resulted in an increase in the oral availability of the drug. EE2 undergoes hydroxylation at the 2, 4, 6, and 16 α position of the steroid nucleus (Back *et al.* 1984; Rogers *et al.* 1987; Stanczyk *et al.* 2013; Zhang *et al.* 2007). Of the hydroxylation reactions, 2-hydroxylation, is clearly the major oxidative reaction (Ball *et al.* 1990) (Figure 2). A 2-hydroxy-EE2 derivative can be subsequently methylated *in vivo* to give 2-methoxyethinylestradiol (Back *et al.* 1984; Rogers *et al.* 1987). The CYP enzymes predominantly contributing to the 2-hydroxylation of EE2 in human liver microsomes are CYP2C9 and 3A4, whereas CYP2C8, 2C19, and 1A2 contribute to this reaction to a lesser extent. Wang *et al.* (2004) showed that recombinant CYP1A1, a predominantly extrahepatic CYP enzyme, exhibited higher intrinsic catalytic activity than recombinant CYP3A4 and/or 2C9. EE2 is also a substrate of various rat hepatic CYPs. Of them the CYP2C6 and 2C11 are most efficient in 2-hydroxylation of EE2, forming the major EE2 metabolite 2-hydroxy-EE2, whereas rat CYP2A and 3A catalyze EE2 hydroxylation predominantly to its minor hydroxylation metabolite, whose structure remains to be identified (Borek-Dohalska *et al.* 2014; 2015).

There are relatively few reports describing potency of EE2 to induce expression of CYP enzymes. An increase in levels of CYP2B1/2 mRNAs (Kocarek *et al.* 1994) and CYP3A9 activities (Reilly *et al.* 1991; Jager *et al.* 1999) by EE2 has been reported in rats. However, no study investigating the effect of EE2 on induction of the CYP enzymes participating in its own metabolism has been carried out as yet.

Metabolism of the other tested ED, the hormone estradiol (Figure 2), has been extensively studied in large numbers of studies. It undergoes extensive oxidative metabolism at various positions (namely, the

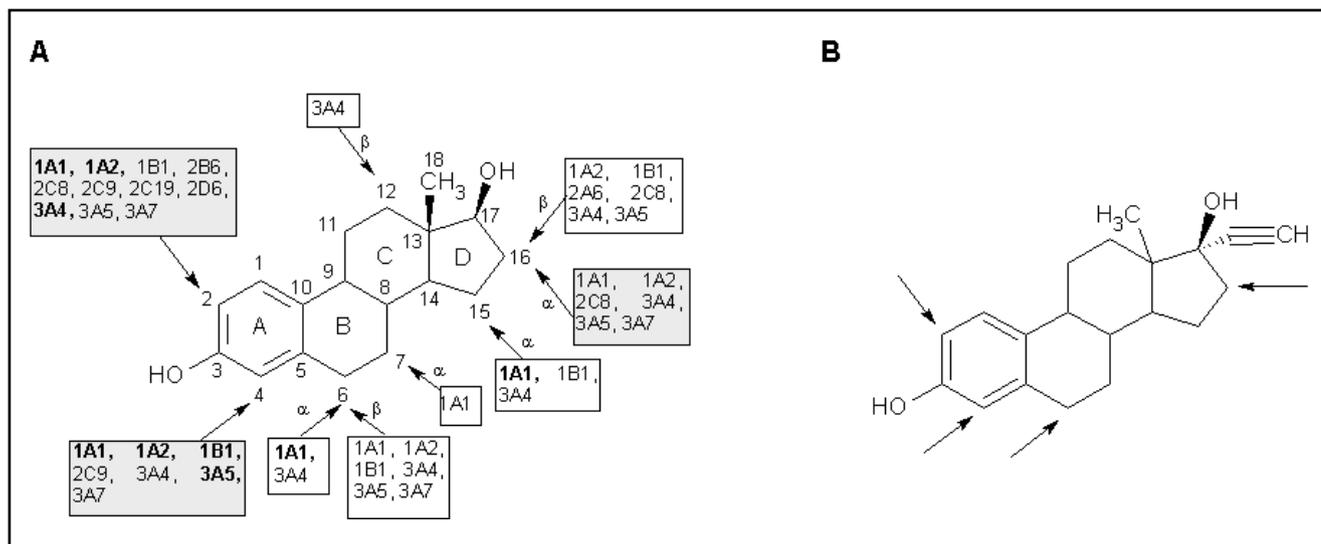


Fig. 2. Positions of estradiol (A) and 17 α -ethinylestradiol (B) hydroxylation catalyzed by human cytochromes P450 (A: adopted from Zhu & Lee 2005).

formation of various hydroxylated or keto metabolites catalyzed by several CYP enzymes present in liver and in extrahepatic estrogen target organs (Figure 2) (reviewed in Zhu & Lee 2005). Among them, the CYP1 family (CYP1A1, 1A2 and 1B1), the CYP3A subfamily (CYP3A4/5/7) and CYPs of the 2C subfamily are mainly effective in estradiol oxidation (see Figure 2). Aromatic hydroxylation at either the C2 or C4 position is a major route of estradiol metabolism in humans and other mammals, although there is less 4-hydroxylation than 2-hydroxylation. The 2-hydroxyestradiol metabolite is considered as a non-toxic metabolite, whereas 4-hydroxyestradiol, primarily forming by the extrahepatic CYP, CYP1B1, as the genotoxic estradiol metabolite (Lee *et al.* 2003). Several CYPs including CYPs of a subfamily 1A (CYP1A1/2), CYP1B1, CYPs of a subfamily 2C (CYP2C8/9/19), CYPs of a subfamily 3A (CYP3A4/5) and CYP2D6 were shown to catalyze hydroxylation of estradiol to 2-hydroxyestradiol and/or 4-hydroxyestradiol (Jin *et al.* 1993; Hayes *et al.* 1996; Badawi *et al.* 2001; Chen *et al.* 2001; Dawling *et al.* 2001; Lee *et al.* 2003; Lepine *et al.* 2004). The CYP1A2 and 3A4 also catalyzes 16 α -hydroxylation of estradiol to estriol (Badawi *et al.* 2001). Interestingly, the expression of CYP1 family is regulated by estradiol (Zhu & Lee 2005). But, the induction potential of estradiol on further CYP enzymes is still rather enigmatic.

Here, we examined the effect of treatment of rats with BaP, EE2 and estradiol individually and in their combination on expression of CYP1A1, 2C and 3A, which are the main enzymes metabolizing the examined EDs.

MATERIALS AND METHODS

Chemicals

17 α -ethinylestradiol, glucose-6-phosphate, NADP⁺, NADPH, 17 β -estradiol, benzo[a]pyrene were obtained from Sigma Chemical Co. (St. Louis, MO, USA); Sudan I from BDH (Poole, UK). Testosterone, 16 α -hydroxyprogesterone and 6 β -hydroxytestosterone were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate dehydrogenase was from Serva (Heidelberg, Germany). Bicinchoninic acid was from Pierce (Rockford, IL, USA). All chemicals were of a reagent grade or better. All other chemicals were of analytical purity or better.

Treatment of rats and preparation of microsomes

All animal experiments were conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Male Wistar rats (150 g, AnLab, Czech Republic), were housed in groups of 3 in wire cages at 22°C with a 12 h light/dark period and *ad libitum* diet (ST-1 diet from Velaz, Czech Republic) and water access.

Rats were treated with BaP (150 mg/kg), estradiol (20 mg/kg) and 17 α -ethinylestradiol (20 mg/kg) diluted in sunflower oil by gavage in one dose. The control group received oil.

Microsomes were isolated from livers, kidneys and lungs of untreated rats and those of pretreated with selected endocrine disruptors by procedures as described (Aimova *et al.* 2007; Stiborova *et al.* 2002; 2012a; 2013a; 2013b). Protein concentrations were assessed using the bicinchoninic acid protein assay with serum albumin as a standard (Wiechelman *et al.* 1988). Total CYP content was measured based on a complex of reduced CYP with carbon monoxide (CO) (Omura & Sato 1964).

Western blot analysis

For the detection of cytochrome P450, 75 μ g of total protein was separated via sodium dodecylsulfate polyacrylamide gele electrophoresis (SDS-PAGE) (10% acryl amide, Bio-Rad).

The polyvinylidene fluoride (PVDF) membrane after the electrotransfer was blocked in a solution of 5% skim milk in TBST-Tween buffer (20 mM Tris/HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The CYP1A1 was detected with a rabbit anti-rat CYP1A1 primary antibody (BioTech, Czech Republic) (dilution 1:2500), a rabbit anti-rat CYP2C11 primary antibody (BioTech, Czech Republic) (dilution 1:1500) and a rabbit anti-rat CYP3A1/2 primary antibody (BioTech, Czech Republic) (dilution 1:2500) diluted in 5% skim milk in Tris-buffered saline with Tween 20 (TBST-Tween buffer) over night at 4°C. After washing in TBST-Tween buffer, membrane was incubated with alkaline phosphatase-conjugated rabbit IgG anti-rabbit IgG in 5% skim milk in TBST-Tween buffer (dilution 1:1430) for 1 h at room temperature. Protein bands were visualized with the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet. For densitometric evaluation of the intensity of the protein bands, we used ImageJ. To assure comparable protein amount and expression, we routinely use anti-GAPDH for normalization of the Western blot data. This normalization is already included in the presented evaluation.

Sudan I oxidation

The incubation mixtures for measuring the Sudan I oxidation contained in a final volume of 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.4, 100 μ M Sudan I (10 μ l of stock methanol solution per incubation), 1 mM NADPH, and 0.5 mg/ml of protein.

The mixtures were incubated for 30 min, at 37°C in a shaking incubator. The reaction was terminated by addition of 1 ml of ethylacetate and then, twice extracted with 1 ml of ethylacetate. The extracts were evaporated to dryness. The residues were dissolved in the mobile phase for high performance liquid chromatography (HPLC) (see below). *HPLC analysis: Sudan I*

and the metabolites were separated on Nucleosil (C18) HPLC column (4.6×25 mm, 5 mm, Macherey-Nagel, Germany). The flow rates, mobile phases and detection wavelengths were 0.7 ml/min, 10:90 bicarbonate buffer/H₂O (v/v), and 480 nm, respectively (Stiborova *et al.* 2002; 2005).

Testosterone hydroxylation

The incubation mixtures for measuring the testosterone metabolism contained in a final volume of 0.5 ml: 0.1 M

potassium phosphate buffer, pH 7.4, 50 μM testosterone (2.5 μl of stock methanol solution per incubation), 10 mM MgCl₂, 10 mM D-glucose 6-phosphate, 1 mM NADP⁺, 1 unit/ml D-glucose 6-phosphate dehydrogenase and 0.5 mg/ml of protein.

The mixtures were incubated for 15 min, at 37°C in a shaking incubator. The reaction was terminated by addition of 0.1 ml of 1 M aqueous Na₂CO₃ containing 2M NaCl. The metabolites were extracted with 2×1 ml of ethylacetate and the extracts were evaporated to dry-

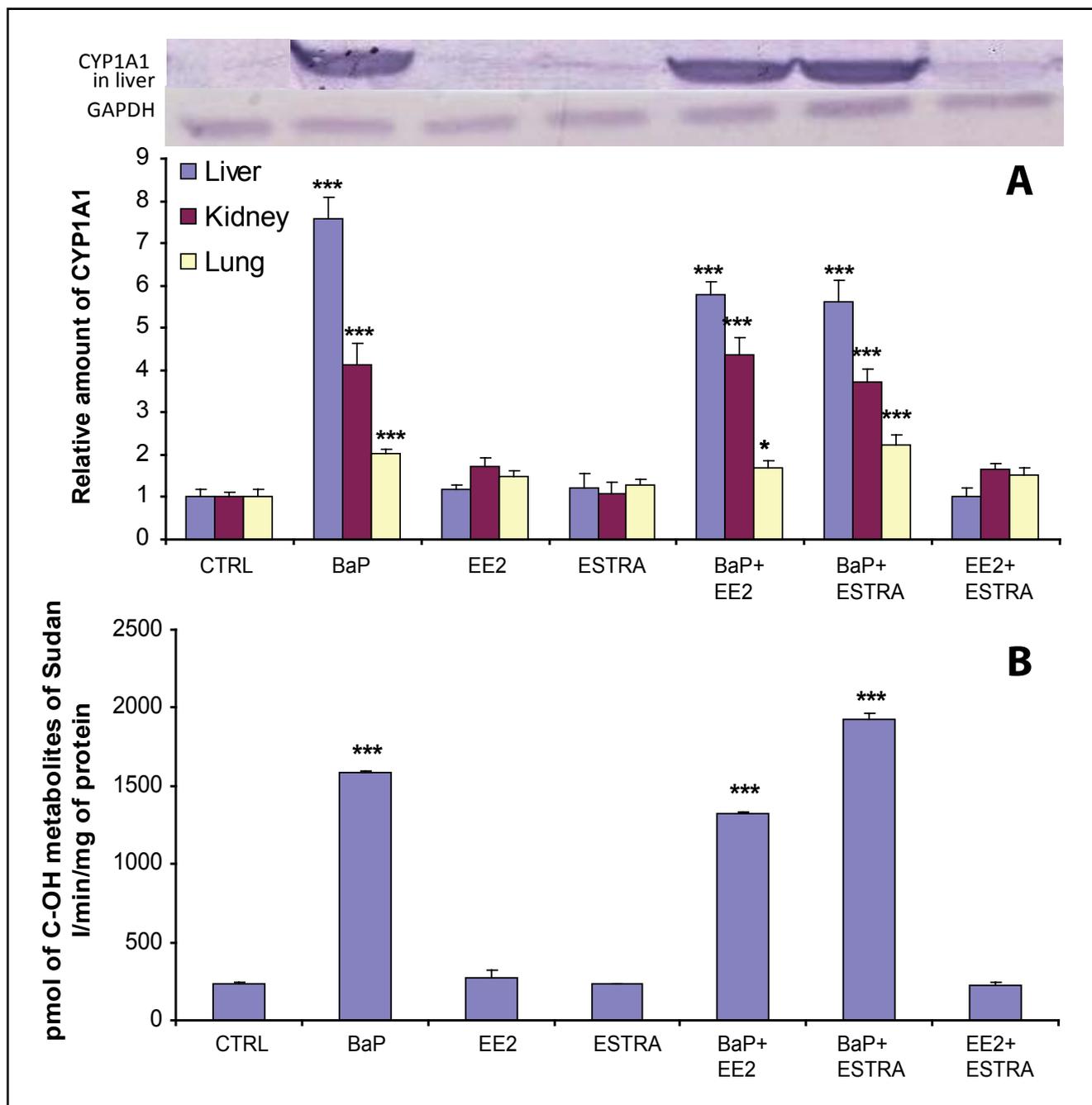


Fig. 3. Expression of CYP1A1 protein in liver, kidney and lung microsomal samples isolated from untreated rats (CTRL) and rats treated with BaP, EE2, estradiol (ESTRA) alone and in their combinations (A) and CYP1A1 specific activity measured as Sudan I oxidation (B) in liver microsomal samples. Insert: representative Western blots of rat liver CYP1A1. GAPDH was used as loading control and representative blots are shown.

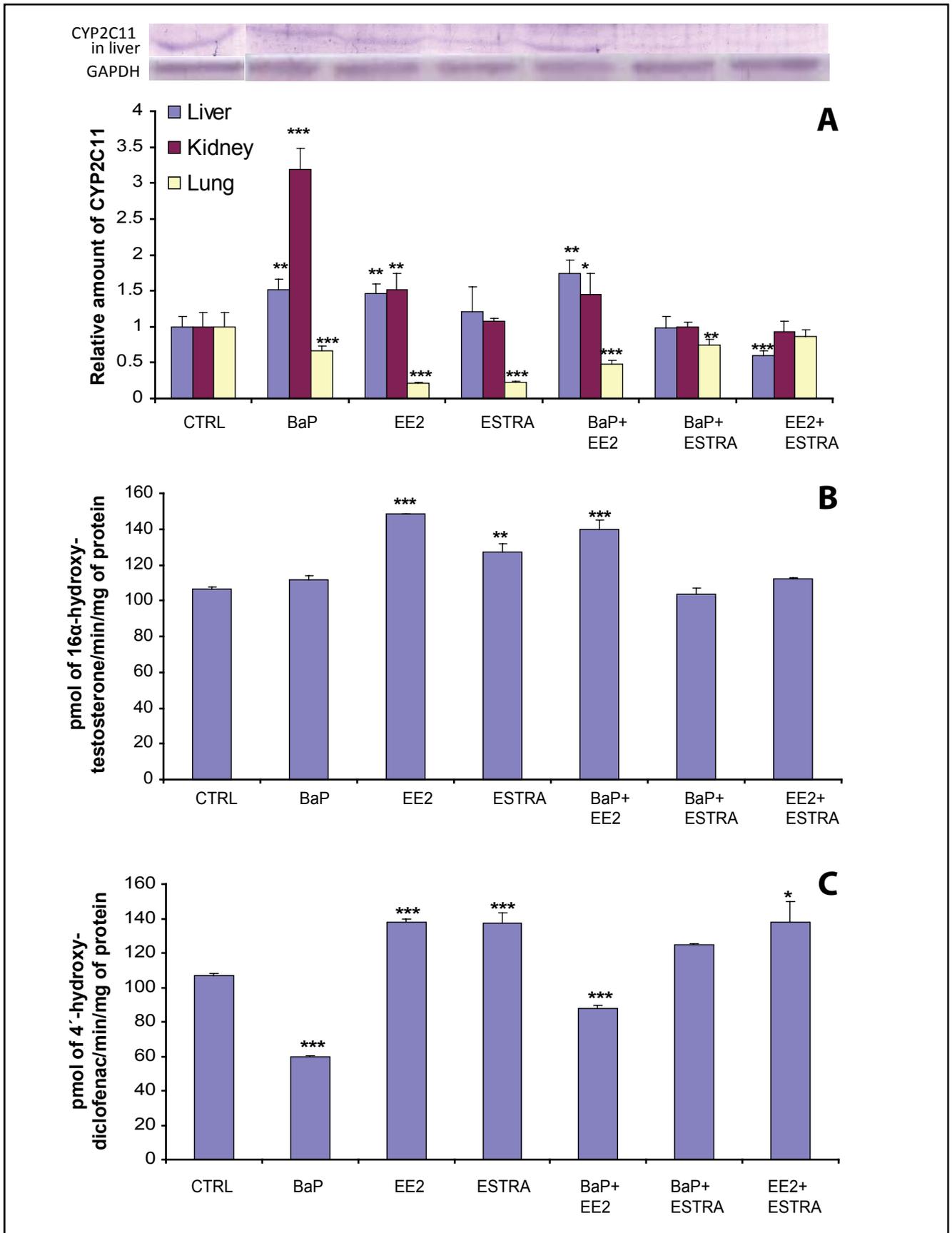


Fig. 4. Expression of CYP2C11 protein in liver, kidney and lung microsomal samples isolated from untreated rats (CTRL) and rats treated with BaP, EE2, estradiol (ESTRA) alone and in their combinations (A), CYP2C11 specific activity measured as testosterone 16α -hydroxylation (B) and CYP2C6 specific activity measured as diclofenac 4'-hydroxylation (C) in liver microsomal samples. Insert: representative Western blots of rat liver CYP2C11. GAPDH was used as loading control and representative blots are shown.

ness. The residues were dissolved in the mobile phase for HPLC (see below). *HPLC analysis:* testosterone and their metabolites were separated on Nucleosil (C18) HPLC column (4.6×25 mm, 5 mm, Macherey-Nagel, Germany). The flow rates, mobile phases and detection wavelengths for were 0.7 ml/min, 65:35 methanol/H₂O (v/v), and 254 nm, respectively (Borek-Dohalska *et al.* 2001; 2010).

Diclofenac 4'-hydroxylation

The incubation mixtures for measuring the diclofenac metabolism contained in a final volume of 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.4, 50 μM diclofenac (5 μl of stock methanol solution per incubation), 10 mM MgCl₂, 10 mM D-glucose 6-phosphate, 1 mM NADP⁺, 1 unit/ml D-glucose 6-phosphate dehydrogenase and 0.5 mg/ml of protein.

The mixtures were incubated for 15 min, at 37 °C in a shaking incubator. The reaction was terminated by addition of 0.1 ml of ice cold acetonitrile. The metabolites were extracted with 1 ml of diethylether and the extracts were evaporated to dryness. The residues were dissolved in the mobile phase for HPLC (see below). *HPLC analysis:* diclofenac and its metabolite were separated on Nucleosil (C18) HPLC column (4.6×25 mm, 5 mm, Macherey-Nagel, Germany). The flow rates, mobile phases and detection wavelengths were 0.5 ml/min, 1,5:2 acetonitrile/acetate buffer (v/v), and 280 nm, respectively (Transon *et al.* 1996).

RESULTS

The effect of treatment of rats with BaP, EE2 and estradiol either alone or in their combinations on expression and specific activity of CYP1A1, CYP2C6/11 and CYP3A

Because CYP1A1, 2C and 3A enzymes were found to be the major enzymes metabolizing tested EDs (BaP, EE2 and estradiol), their effects on expression of these CYP enzymes were investigated in rats *in vivo*.

In the case of BaP, which is known to act as a strong inducer of CYP1A1 (Hockley *et al.* 2007; Arlt *et al.* 2008; 2012; 2015; Hodek *et al.* 2014; Stiborova *et al.* 2014; 2016b), exposure of rats to this carcinogenic ED led to induction of CYP1A1 protein expression in all tested organs (liver, kidney and lung) (Figure 3A). The highest induction effect of BaP on CYP1A1 was found in the liver (almost 8-times as compared to control, untreated, rats). Whereas BaP strongly induced rat CYP1A1, no significant induction of this CYP enzyme was produced by EE2 and estradiol. When these disruptors were administered to rats with BaP, they decreased the degree of the BaP-mediated CYP1A1 induction in rat livers (see Figure 3A). These results essentially correspond to the CYP1A1 specific activity (Sudan I hydroxylation) (Stiborova *et al.* 2002), which was also increased in hepatic samples of rat treated with BaP alone (6.9-times) and together with EE2 (5.7-times). However, treatment of rats with BaP together with

estradiol increased Sudan I oxidation, by 8.4-times as compared to control, untreated rats (Figure 3B).

Since CYP2C11/2C6 are major enzymes catalyzing conversion of EE2 to 2-hydroxyEE2 (Borek-Dohalska *et al.* 2014; 2015) and they also oxidize estradiol (Zhu & Lee 2005), the effect of tested EDs on induction of these enzymes was also investigated. The results found indicate that expression of CYP2C11 was slightly increased in liver of rats exposed to all EDs either administered individually or in combination of BaP with EE2. An increase in CYP2C11 protein expression by tested EDs in rat livers correlated with an increase in its specific enzyme activity, testosterone 16α-hydroxylation. Both EE2 and estradiol also elevated enzyme activity of CYP2C6, diclofenac 4'-hydroxylation (Figure 4C). On the contrary, treatment of rats with all EDs down-loaded expression of CYP2C11 in lungs (Figure 4A). EE2 together with estradiol did not change the levels of CYP2C11 in rat kidney and lung, but led to a decrease in CYP2C11 protein expression in liver (Figure 4A). These findings correspond to the results of Laurenzana *et al.* (2002), who postulated that in EE2-treated rats, this compound may covalently bind to CYP2C11, resulting in degradation of the enzyme, rather than regulating of its expression.

Surprisingly, BaP also induces CYP2C11 protein in liver and kidney of rats exposed to this ED, but does not increase its enzyme activity measured as testosterone 16α-hydroxylation. The enzyme activity of CYP2C6, diclofenac 4'-hydroxylation, was even decreased by BaP (Figures 4B and 4C).

The CYP3A enzyme was detectable only in kidney and liver of untreated rats and rats exposed to EDs. A slight, but non-significant, increase in CYP3A protein expression was produced by BaP in rat liver (Figure 5A). However, CYP3A enzyme activity, testosterone 6β-hydroxylation, was significantly increased by treatment of rats with this carcinogenic ED alone or in combination with estradiol (Figure 5B). The CYP3A expression levels and its enzyme activity, testosterone 6β-hydroxylation, in liver decreased after treatment of rats with two other EDs (EE2 and estradiol) alone or in combinations (Figure 5). This finding corresponds to results found by Lin *et al.* (2002), who found that EE2 inactivated testosterone 6β-hydroxylation catalyzed by a human CYP3A orthologue, CYP3A4, in a mechanism-based manner. Recently we found that EE2 acts either as a reversible inhibitor of CYP3A-mediated progesterone 6β-hydroxylation or inactivates CYP3A- and CYP2C-catalyzed testosterone 6β-hydroxylation and progesterone 21- or 16α-hydroxylation, respectively, in a mechanism-based manner (Borek-Dohalska *et al.* 2010; 2014).

In kidney, CYP3A level increased after treatment of rats with BaP combined with EE2. Other EDs did not affect its expression level in kidney, except of BaP and combination of EE2 with estradiol, which decreased expression of this enzyme (Figure 5A).

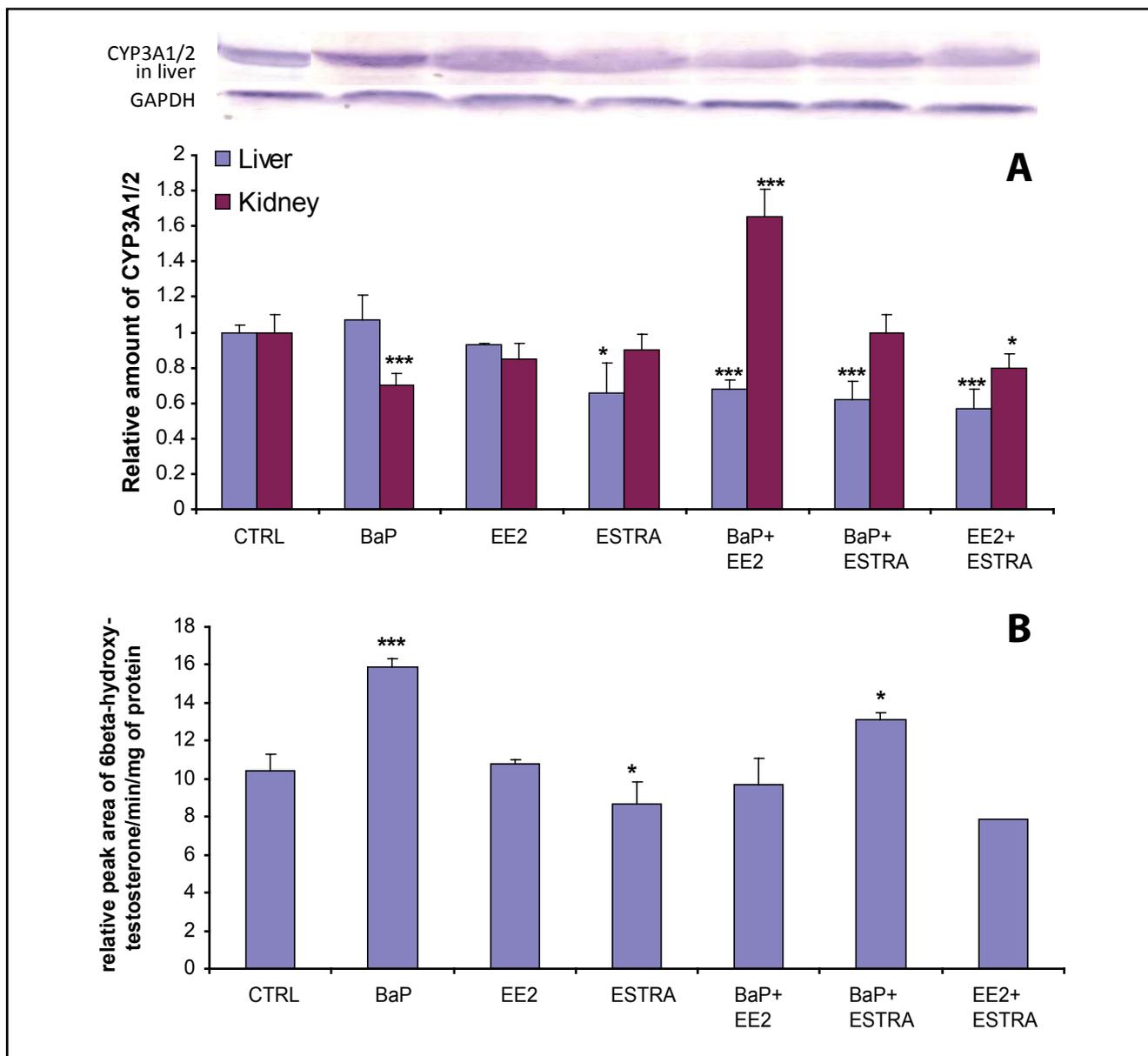


Fig. 5. Expression of CYP3A1/2 in microsomal samples isolated from untreated rats (CTRL) and rat premedicated with BaP, EE2, ESTRA and their combinations (A) and CYP3A specific activity measured as testosterone β -hydroxylation (B) in these samples. Insert: representative Western blots of rat liver CYP3A1/2. GAPDH was used as loading control and representative blots are shown.

DISCUSSION

The results showing that BaP induces expression of CYP1A1 enzyme corresponds well to a known potency of this compound to act as the activator of AhR, which finally leads to elevated expression of CYPs of the 1A subfamily and CYP1B1. Namely, expression of these CYP enzymes is regulated by the xenobiotic response element (XRE), involving ligand-activated AhR (Nebert and Jones 1989; Nebert *et al.* 2000). AhR binds variety exogenous ligands such as PAHs including BaP. AhR is a cytosolic transcription factor that is normally inactive, bound to several co-chaperones (Cox & Miller 2004). Upon ligand binding to chemicals such as BaP, the

chaperones dissociate, which results in AhR translocating into the nucleus and dimerizing with AhR nuclear translocator (ARNT), leading to changes in gene transcription (Denison & Nagy 2003).

The increase in protein expression of CYP2C11 by exposure of rats to BaP and BaP with EE2 in liver and lung was, however, rather surprising. The enzymes of the CYP2C subfamily are the constitutive CYPs, nevertheless, they might also be slightly induced by the mechanism that involves activation of nuclear constitutive androstane receptor (CAR) (Kodama & Negishi 2007). Examples for CAR-regulated genes are members of the CYP2B, CYP2C, and CYP3A subfamilies (Ueda *et al.* 2002). This transcriptional regulator is constitutively

active in the absence of ligand and is regulated by both agonists and inverse agonists. Ligand binding results in translocation of CAR from the cytosol into the nucleus, where the protein can bind to specific DNA sites, called response elements. Binding occurs both as a monomer and together with the retinoid X receptor (RXR) resulting in activation or repression of target gene transcription. Several eobiotics (i.e. steroid hormones) and xenobiotics (i.e. many drugs, but not PAH such as BaP) act as ligands binding to CAR (Kawamoto *et al.* 1999). Concerning the other EDs investigated in this work, both steroid hormone estradiol and its synthetic derivative increase expression of CYP2C11 protein and its enzyme activity, which is in accordance with the mechanism of CAR activation by steroid hormones. Because BaP increases an induction potential of EE2, the interaction between CAR and AhR seems to occur in this process. However, this suggestion needs to be investigated in further studies.

Induction of CYP3A, which is regulated mainly by the pregnane X receptor (PXR) (Rendic and Di Carlo 1997), is essentially not mediated by the tested EDs, estradiol and EE2 even decreased its expression. An increase in CYP3A activity measured as testosterone 6 β -hydroxylation was, however, induced by BaP and BaP in combination with estradiol. Based on these results, this increase should result from the mechanism(s) that is different from the direct induction of the CYP protein; a stimulation of CYP3A activity by these compounds might be one of the reasons. Indeed, CYP3A enzyme activity is known to be stimulated by a variety of endogenous and exogenous compounds (Rendic and Di Carlo 1997; Rendic & Guengerich 2015; Stiborova *et al.* 2012b). The investigation of effects of BaP and BaP with estradiol on CYP3A enzyme activity *in vitro* is hence planned to be carried out in future studies.

CONCLUSIONS

The results found in this work demonstrate that three EDs tested in this study, a carcinogenic pollutant BaP, an estrogenic hormone estradiol and its synthetic derivative, EE2, might, due to their potential to induce expression of CYPs involved in their own metabolism, modulate their biological efficiencies. Further, even though BaP has been found to induce several CYPs in previous studies, the induction potency of EE2 and estradiol or these compounds with BaP is shown in this work for the first time. Because of the complex interactions of tested EDs on expression of CYP1A1, 2C and 3A and their enzyme activities, these compounds might participate in drug-drug interactions that may result in problems of their clinical significance.

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REFERENCES

- 1 Aimova D, Svobodova L, Kotrbova V, Mrazova B, Hodek P, Hudecek J, Vaclavikova R, Frei E and Stiborova M (2007) The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism. *Drug Metab Dispos* **35**: 1926–34.
- 2 Arlt VM, Stiborova M, Henderson CJ, Thiemann M, Frei E, Aimova D, Singhs R, da Costa GG, Schmitz OJ, Farmer PB, Wolf CR and Phillips DH (2008) Metabolic activation of benzo[a]pyrene *in vitro* by hepatic cytochrome P450 contrasts with detoxification *in vivo*: experiments with hepatic cytochrome P450 reductase null mice. *Carcinogenesis* **29**: 656–665.
- 3 Arlt VM, Poirier MC, Sykes SE, John K, Moserova M, Stiborova M, Wolf CR, Henderson CJ and Phillips DH (2012) Exposure to benzo[a]pyrene of Hepatic Cytochrome P450 Reductase Null (HRN) and P450 Reductase Conditional Null (RCN) mice: Detection of benzo[a]pyrene diol epoxide-DNA adducts by immunohistochemistry and ³²P-postlabelling. *Toxicol Lett* **213**: 160–6.
- 4 Arlt VM, Kraus AM, Godschalk RW, Ruffo-Vasquez Y, Mrizova I, Roufosse CA, Corbin C, Shi Q, Frei E, Stiborova M, van Schooten FJ, Phillips DH and Spina D. (2015) Pulmonary inflammation impacts on CYP1A1-mediated respiratory tract DNA damage induced by the carcinogenic air pollutant benzo[a]pyrene. *Toxicol Sci* **146**: 213–225.
- 5 Back DJ, Maggs JL, Purba HS, Newby S and Park B K (1984) 2-Hydroxylation of ethinyloestradiol in relation to the oxidation of sparteine and antipyrine. *Br J Clin Pharmacol* **18**: 603–607.
- 6 Badawi AF, Cavalieri EL and Rogan EG (2001) Role of human cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16 α -hydroxylation of 17 β -estradiol. *Metabolism: clinical and experimental* **50**: 1001–3.
- 7 Ball SE, Forrester LM, Wolf CR and Back DJ (1990) Differences in the cytochrome P-450 isoenzymes involved in the 2-hydroxylation of oestradiol and 17 α -ethinyloestradiol. Relative activities of rat and human liver enzymes. *Biochem J* **267**: 221–226.
- 8 Baird WM, Hooven LA and Mahadevan B (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environ Mol Mutagen* **45**: 106–114.
- 9 Bolt HM (1979) Metabolism of estrogens-natural and synthetic. *Pharmacol Ther* **4**: 155–181.
- 10 Borek-Dohalská L, Hodek P, Sulc M and Stiborova M (2001) α -Naphthoflavone acts as activator and reversible or irreversible inhibitor of rabbit microsomal CYP3A6. *Chem Biol Interact* **138**: 85–106.
- 11 Borek-Dohalska L and Stiborova M (2010) Cytochrome P450 3A activities and their modulation by α -naphthoflavone *in vitro* are dictated by the efficiencies of model experimental systems. *Collect Czech Chem Commun* **75**: 201–220.
- 12 Borek-Dohalska L, Valaskova P, Cerna V and Stiborova M (2014) Role of rat cytochromes P450 in the oxidation of 17 α -ethinyloestradiol. *Environ Toxicol Pharmacol* **38**: 852–60.
- 13 Borek-Dohalska L, Valaskova P, Kubickova B, Sulc M, Kresinova Z, Cajthaml T and Stiborova M (2015) A study on 17 α -ethinyloestradiol metabolism in rat and *Pleurotus ostreatus*. *Neuro Endocrinol Lett* **36**(1): 5–12.
- 14 Cheng ZN, Shu Y, Liu ZQ, Wang LS, Ou-Yang DS and Zhou HH (2001) Role of cytochrome P450 in estradiol metabolism *in vitro*. *Acta Pharmacol Sin* **22**: 148–154.
- 15 Cox MB and Miller CA (2004) Cooperation of heat shock protein 90 and p23 in aryl hydrocarbon receptor signaling. *Cell Stress Chaperones* **9**: 4–20.
- 16 Dawling S, Roodi N, Mernaugh RL, Wang X, Parl FF (2001) Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens: comparison of wild-type and variant COMT isoforms. *Cancer research* **61**: 6716–22
- 17 Denison MS and Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* **43**: 309–334
- 18 Hamouchene H, Arlt VM, Giddings I and Phillips DH (2011) Influence of cell cycle on responses of MCF-7 cells to benzo[a]pyrene. *BMC Genomics* **12**: 333.

- 19 Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ and Sutter TR (1996) 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc Natl Acad Sci USA* **93**: 9776–9781.
- 20 Hockley SL, Arlt VM, Brewer D, Te Poelle R, Workman P, Giddings I and Phillips DH (2007) AHR- and DNA-damage-mediated gene expression responses induced by benzo(a)pyrene in human cell lines. *Chem Res Toxicol* **20**: 1797–810.
- 21 Hodek P, Fousova P, Brabcncova E, Moserova M, Pavek P, Anzenbacherova E, Brotanek J, Hudecek J, Frei E and Stiborova M (2014) Effect of dihydromyricetin on benzo[a]pyrene activation in rats. *Neuro Endocrinol Lett* **35**: 158–68.
- 22 IARC (International Agency for Research on Cancer) (2010) Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. In IARC Monogr. Eval. Carcinog. Risks Hum. Vol. 92, IARC, Lyon, France, pp. 1–853.
- 23 Indra R., Moserova M., Sulc M, Frei E. and Stiborova M (2013) Oxidation of carcinogenic benzo[a]pyrene by human and rat cytochrome P450 1A1 and its influencing by cytochrome *b*₅ – a comparative study. *Neuro Endocrinol Lett* **34**: 55–63.
- 24 Indra R, Moserova M, Kroftova N, Sulc M, Martinkova M, Adam V, Eckschlager T, Kizek R, Arlt VM and Stiborova M (2014) Modulation of human cytochrome P450 1A1-mediated oxidation of benzo[a]pyrene by NADPH:cytochrome P450 oxidoreductase and cytochrome *b*₅. *Neuro Endocrinol Lett* **35**: 105–113.
- 25 Jäger W, Correia MA, Bornheim LM, Mahnke A, Hanstein WG, Xue L and Benet LZ (1999) Ethynylestradiol-mediated induction of hepatic CYP3A9 in female rats: implication for cyclosporine metabolism. *Drug Metab Dispos* **27**: 1505–1511.
- 26 Jin CJ, Miners JO, Lillywhite KJ and Mackenzie PI (1993) cDNA cloning and expression of two new members of the human liver UDP-glucuronosyltransferase 2B subfamily. *Biochem Biophys Res Commun* **194**: 496–503.
- 27 Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* **19**: 6318–6322.
- 28 Kocarek TA, Schuetz EG and Guzelian PS (1994) Regulation of cytochrome P450 2B1/2 mRNAs by Kepone (chlordecone) and potent estrogens in primary cultures of adult rat hepatocytes on Matrigel. *Toxicol Lett* **71**: 183–196.
- 29 Kodama S and Negishi M (2006) Phenobarbital confers its diverse effects by activating the orphan nuclear receptor CAR. *Drug Metab Rev* **38**(1–2): 75–87.
- 30 Kraus AM, Speksnijder EN, Melis JP, Indra R, Moserova M, Godschalk RW, van Schooten FJ, Seidel A, Kopka K, Schmeiser HH, Stiborova M, Phillips DH, Luijten M and Arlt VM (2016) The impact of p53 on DNA damage and metabolic activation of the environmental carcinogen benzo[a]pyrene: effects in Trp53(+/+), Trp53(+/-) and Trp53(-/-) mice. *Arch Toxicol* **90**: 839–851.
- 31 Labib S, Yauk C, Williams A, Arlt VM, Phillips DH, White PA and Halappanavar S (2012) Subchronic oral exposure to benzo(a)pyrene leads to distinct transcriptomic changes in the lungs that are related to carcinogenesis. *Toxicol Sci* **129**: 213–224.
- 32 Laurenzana EM, Weis CC, Bryant CW, Newbold R and Delclos KB (2002) Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P-450 enzymes, and estrogen receptor alpha expression. *Food Chem Toxicol* **40**: 53–63.
- 33 Lee AJ, Cai MX, Thomas PE, Conney AH and Zhu BT (2003) Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology* **144**: 3382–3398.
- 34 Lemieux CL, Douglas GR, Gingerich J, Phonethepswath S, Torous DK, Dertinger SD, Phillips DH, Arlt VM and White PA (2011) Simultaneous measurement of benzo[a]pyrene-induced Pig-a and lacZ mutations, micronuclei and DNA adducts in Muta™ Mouse. *Environ Mol Mutagen* **52**: 756–765.
- 35 Lepine J, Bernard O, Plante M, Tetu B, Pelletier G, Labrie F, Belanger A and Guillemette C (2004) Specificity and regioselectivity of the conjugation of estradiol, estrone, and their catecholestrogen and methoxyestrogen metabolites by human uridine diphospho-glucuronosyltransferases expressed in endometrium. *J Clin Endocrinol Metab* **89**: 5222–5232.
- 36 Lin HL, Kent UM and Hollenberg PF (2002) Mechanism-based inactivation of cytochrome P450 3A4 by 17 alpha-ethynylestradiol: evidence for heme destruction and covalent binding to protein. *J Pharmacol Exp Ther* **301**: 160–167.
- 37 Omura T and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. II. solubilization, purification and properties. *J Biol Chem* **239**: 2379–2385.
- 38 Phillips DH and Castegnaro M (1999) Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trials and production of recommended protocols. *Mutagenesis* **14**: 301–315.
- 39 Phillips DH (2002) Smoking-related DNA and protein adducts in human tissues. *Carcinogenesis* **23**: 1979–2004.
- 40 Phillips DH (2005) DNA adducts as markers of exposure and risk. *Mutat Res* **577**: 284–292.
- 41 Reilly PE, Mason SR and Hooper WD (1991) Effects of ethinylestradiol and testosterone implants on hepatic microsomal cytochrome P450 monooxygenases of birth gonadectomized male and female Dark Agouti rats. *J Steroid Biochem Mol Biol* **39**: 741–749.
- 42 Rendic S and DiCarlo FJ (1997) Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* **29**: 413–480.
- 43 Rendic S and Guengerich FP (2015) Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem Res Toxicol* **28**: 38–42.
- 44 Rogers SM, Back DJ and Orme ML (1987) Intestinal metabolism of ethinylestradiol and paracetamol *in vitro*: studies using Ussing chambers. *Br J Clin Pharmacol* **23**: 727–734.
- 45 Siddens LK, Larkin A, Krueger SK, Bradfield CA, Waters KM, Tilton SC, Pereira CB, Löhr CV, Arlt VM, Phillips DH, Williams DE and Baird WM (2012) Polycyclic aromatic hydrocarbons as skin carcinogens: comparison of benzo[a]pyrene, dibenzo[def,p]chrysene and three environmental mixtures in the FVB/N mouse. *Toxicol Appl Pharmacol* **264**: 377–386.
- 46 Stanczyk FZ, Archer DF and Bhavnani BR (2013) Ethinyl estradiol and 17β-estradiol in combined oral contraceptives: pharmacokinetics, pharmacodynamics and risk assessment. *Contraception* **87**: 706–727.
- 47 Stiborova M, Martinek V, Rydlova H, Hodek P and Frei E (2002) Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. *Cancer Res* **62**: 5678–5684.
- 48 Stiborova M, Martinek V, Rydlova H, Koblas T and Hodek P (2005) Expression of cytochrome P450 1A1 and its contribution to oxidation of a potential human carcinogen 1-phenylazo-2-naphthol (Sudan I) in human livers. *Cancer Lett* **220**: 145–154.
- 49 Stiborova M, Cechova T, Borek-Dohalska L, Moserova M, Frei E, Schmeiser HH, Paca J and Arlt VM (2012a) Activation and detoxification metabolism of urban air pollutants 2-nitrobenzanthrone and carcinogenic 3-nitrobenzanthrone by rat and mouse hepatic microsomes. *Neuro Endocrinol Lett* **33**: 8–15.
- 50 Stiborova M, Indra R, Moserova M, Cerna V, Rupertova M, Martinek V, Eckschlager T, Kizek R and Frei E (2012b) Cytochrome *b*₅ increases cytochrome P450 3A4-mediated activation of anticancer drug ellipticine to 13-hydroxyellipticine whose covalent binding to DNA is elevated by sulfotransferases and N,O-acetyltransferases. *Chem Res Toxicol* **25**: 1075–1085.
- 51 Stiborova M, Cerna V, Moserova M, Arlt VM and Frei E (2013a) The effect of benzo[a]pyrene on metabolic activation of anticancer drug ellipticine in mice. *Neuro Endocrinol Lett* **34**: 43–54.
- 52 Stiborova M, Dracinska H, Martinek V, Svaskova D, Hodek P, Milichovsky J, Hejdukova Z, Brotanek J, Schmeiser HH and Frei E (2013b) Induced expression of cytochrome P450 1A and NAD(P)H:quinone oxidoreductase determined at mRNA, protein, and enzyme activity levels in rats exposed to the carcinogenic azo dye 1-phenylazo-2-naphthol (Sudan I). *Chem Res Toxicol* **26**: 290–299.

- 53 Stiborova M, Moserova M, Cerna V, Indra R, Dracinsky M, Šulc M, Henderson CJ, Wolf CR, Schmeiser HH, Phillips DH, Frei E and Arlt VM (2014) Cytochrome b_5 and epoxide hydrolase contribute to benzo[a]pyrene-DNA adduct formation catalyzed by cytochrome P450 1A1 under low NADPH:P450 oxidoreductase conditions. *Toxicology* **318**: 1–12.
- 54 Stiborova M, Indra R, Moserova M, Sulc M, Hodek P, Frei E, Schmeiser HH and Arlt VM (2016a) NADPH- and NADH-dependent metabolism of and DNA adduct formation by benzo[a]pyrene catalyzed with rat hepatic microsomes and cytochrome P450 1A1. *Monatsh. Chem.* **147**: 847–855.
- 55 Stiborova M, Moserova M, Mrizova I, Dracinska H, Martinek V, Indra R, Frei E, Adam V, Kizek R, Schmeiser HH, Kubackova K and Arlt VM (2016b) Induced expression of microsomal cytochrome b_5 determined at mRNA and protein levels in rats exposed to ellipticine, benzo[a]pyrene, and 1-phenylazo-2-naphthol (Sudan I). *Monatsh Chem* **147**: 897–904.
- 56 Stiborova M, Indra R, Moserova M, Frei E, Schmeiser HH, Kopka K, Phillips DH, Arlt VM (2016c) NADH:cytochrome b_5 reductase and cytochrome b_5 can act as sole electron donors to human cytochrome P450 1A1-mediated oxidation and DNA adduct formation by benzo[a]pyrene. *Chem Res Toxicol* **29**: 1325–1334.
- 57 Sulc M, Indra R, Moserova M, Schmeiser HH, Frei E, Arlt VM and Stiborova M (2016) The impact of individual cytochrome P450 enzymes on oxidative metabolism of benzo[a]pyrene in human livers. *Environ Mol Mutagen* **57**: 229–235.
- 58 Transon C, Leemann T and Dayer P (1996) *In vitro* comparative inhibition profiles of major human drug metabolising cytochrome P450 isozymes (CYP2C9, CYP2D6 and CYP3A4) by HMG-CoA reductase inhibitors. *Eur J Clin Pharmacol* **50**: 209–215.
- 59 Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM and Negishi M (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Molec Pharmacol* **61**: 1–6.
- 60 Wang B, Sanchez RI, Franklin RB, Evans DC and Huskey SE (2004) The involvement of CYP3A4 and CYP2C9 in the metabolism of 17 alpha-ethinylestradiol. *Drug Metab Dispos* **32**: 1209–1212.
- 61 Wiechelmann KJ, Braun RD and Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal Biochem* **75**: 231–237.
- 62 Zhang H, Cui D, Wang B, Han YH, Balimane P, Yang Z, Sinz M and Rodrigues AD (2007) Pharmacokinetic drug interactions involving 17alpha-ethinylestradiol: a new look at an old drug. *Clin Pharmacokinet* **46**: 133–157.
- 63 Zhu BT and Lee AJ (2005) NADPH-dependent metabolism of 17beta-estradiol and estrone to polar and nonpolar metabolites by human tissues and cytochrome P450 isoforms. *Steroids*: 225–44.