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ABSTRACT

Many endogenous and xenobiotic compounds are substrates and regulators of human placental ABC transporters. ABCG2 is protecting fetus against foreign chemicals. Environmental xenoestrogens, like bisphenol A (BPA) and *p*-nonylphenol (*p*-NP), mimic natural estrogens and can affect hormonal systems.

Effects of BPA, *p*-NP, DES (diethylstilbestrol) and estradiol (E2), on ABCG2 expression were studied using human first trimester and term placental explants. Role of estrogen receptors (ER) in the effects of chemicals was studied by ER antagonist.

Term placenta expressed less ABCG2 protein. In term placentas BPA ($p < 0.05$), *p*-NP ($p < 0.01$) and E2 ($p < 0.05$) decreased the ABCG2 protein expression after 48 h exposure while after 24 h exposure, only E2 decreased the expression ($p < 0.05$). The chemicals did not affect ABCG2 in first trimester placentas. The ER antagonist affected differently the responses of chemicals.

In conclusion, environmental xenoestrogens downregulate placental ABCG2 protein expression depending on gestational age.

1. Introduction

Human placental ATP binding cassette (ABC) transporters facilitate the transfer of various compounds, both endogenous compounds and xenobiotics, through membranes maintaining pregnancy and normal fetal development (Hutson et al., 2010; Vähäkangas and Myllynen, 2009). ABCG2 is an efflux ABC transporter localized in the apical brush border membrane of syncytiotrophoblast in human placenta (Maliepaard et al., 2001; Ni and Mao, 2011). ABCG2 transfers a large variety of compounds and has been shown to protect fetus against foreign chemicals present in maternal circulation (Hutson et al., 2010; Ni and Mao, 2011). Furthermore, inhibition of ABCG2 has been shown to affect the transfer of chemicals in human placental perfusions leading to increased materno-fetal transfer of drugs and environmental toxins (Myllynen et al., 2008; Pollex et al., 2008).

Several factors affect the expression and function of ABCG2 during pregnancy. In humans ABCG2 is expressed throughout the pregnancy with lower expression in term placenta (Meyer zu Schwabedissen et al., 2006), although some controversies exist in the literature (Mathias et al., 2005; Yeboah et al., 2006). Genetic polymorphisms affect the expression and function of ABC transporters, and hence placental fate and permeability of compounds (Hutson et al., 2010; Ni and Mao, 2011). In addition, ABC transporters are regulated by other factors, like hormones, growth factors, nuclear receptors and transcription factors, cytokines, oxygen tension and epigenetic factors as well as pathological conditions

Abbreviations: ABC transporter, ATP-binding cassette transporter; ABCG2, ATP-binding cassette sub-family G member 2; BCRP, breast cancer resistance protein; ATP, adenosine triphosphate; BPA, bisphenol A; DES, diethylstilbestrol; E2, estradiol; EDC, endocrine disrupting chemical; ER-α, estrogen receptor alpha; ERR-γ, estrogen related receptor gamma; GPER, g-protein-coupled estrogen receptor; hCG, human chorionic gonadotropin; ICI 182780, fulvestrant (antiestrogen); *p*-NP, *para*-nonylphenol.

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(Evseenko et al., 2007; Hutson et al., 2010; Lye et al., 2013; Mathias et al., 2005; Meyer zu Schwabedissen et al., 2006; Ni and Mao, 2011). Xenobiotics may also modulate the expression and function of transporters (Arias et al., 2014; Dankers et al., 2013; Hanet et al., 2008), although there are very few studies concerning human placenta (Huuskonen et al., 2013; Prouillac et al., 2009).

Endocrine disrupting chemicals (EDCs) are defined as chemicals being biologically active in the endocrine system with an effect on normal endocrine system and its regulation (Robins et al., 2011; Welshons et al., 2003). People are increasingly exposed to EDCs through environment during their whole lifespan (Robins et al., 2011; Rochester, 2013), major exposure sources being food, water and plastics. Dose-response curves of EDCs often differ from conventional toxicological dose-response curves, and they have been shown to have significant effects already with low environmental concentrations (Welshons et al., 2003). Some of the effects may induce acute responses while other effects emerge later in life leading, for instance, to a higher susceptibility to certain health disorders and even cancer (Rochester, 2013; Vähäkangas, 2011; Welshons et al., 2003). Human placenta is a hormonally regulated endocrine organ and its development and function may be negatively affected by EDCs (Robins et al., 2011).

Bisphenol A (BPA) is produced mainly by plastic industry and known to leach significantly from consumable plasticware (Rochester, 2013). Para-nonylphenol (*p*-NP) is used extensively as surfactant in industry (industrial detergents, synthesis of pesticides) (Chen et al., 2008). Both BPA and *p*-NP, are known to cross human placenta (Balakrishnan et al., 2010; Mørck et al., 2010). They have been detected in blood samples of pregnant women (BPA ~3.1 ng/ml, *p*-NP 5.68 ng/g), in fetal serum (BPA 2.2–2.3 ng/ml, *p*-NP 2.95 ng/g) and in placenta (BPA 12.7 ng/g) (Cao et al., 2012; Chen et al., 2008; Ikezuki et al., 2002; Schönfelder et al., 2002; Zhang et al., 2011). Diethylstilbestrol (DES) is a synthetic nonsteroidal estrogen prescribed in the past to pregnant women to avoid adverse clinical outcomes during pregnancy but was found to be teratogenic and to cause cancer in following generations (Hilakivi-Clarke, 2014). Xenoestrogens can affect many placental functions, excretion of human placental hormones (Bechi et al., 2006, 2013; Robins et al., 2011), implantation and placentation in mice (Tachibana et al., 2007; Takai et al., 2000) and ABC transporters in human placental choriocarcinoma cells (Huuskonen et al., 2013; Jin and Audus, 2005). However, very little is currently known of the effects of BPA and *p*-NP on human placenta.

The aim of this study was to determine the effects of xenoestrogens, bisphenol A (BPA), *para*-nonylphenol (*p*-NP) and diethylstilbestrol (DES), on the expression of ABCG2 transporter in the first trimester and term human placenta using chorionic villous explant cultures.

2. Materials and methods

This study was conducted as a collaboration between the laboratories of the University of Siena, Italy (first trimester placental cultures, protein and hCG analysis of those samples), and the University of Oulu, Finland (term placental cultures and all analysis concerning term placentas).

2.1. Chemicals

Bisphenol A (BPA, CAS registry number: 80-05-7), *para*-nonylphenol (*p*-NP, CAS registry number: 104-40-5), diethylstilbestrol (DES, CAS registry number: 56-53-1, a known teratogen and used for comparison in this study (Hilakivi-Clarke, 2014)), 17β-Estradiol (E2, CAS registry number: 50-28-2, used as a positive control for estrogenic responses) and ICI 182780 (ER antagonist, also called Fulvestrant, CAS registry number: 129453-61-8) were all purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA or Steinheim, Germany). Studied chemicals share structural similarities (Supplementary Fig. 1). Ethanol (EtOH) was used as a solvent for all compounds.

2.2. Human first trimester and term placental villous explant cultures

Altogether 16 first trimester placentas (number of placentas used for different treatments and time points varies from 3 to 10 between experiments, for details see figures) and 8 term placentas (number of placentas used for different treatments and time points varies from 3 to 4 between experiments, for details see figures) were used in this study. Placentas were obtained with the approval of the local ethics committees in Siena (Siena, Italy) and the Northern Ostrobotnia Hospital District (Oulu, Finland). Volunteers donating their placentas for the study signed an informed consent. First trimester placentas were collected only in Italy after elective termination of pregnancy at the Obstetrics and Gynecology Division, USL 7, Hospital, Campostaggia, Siena, Italy. The first trimester placentas were between 7 and 9 weeks of gestation age. A few term placentas were also collected in Italy to compare the expression levels of ABCG2 in first trimester and term placentas. Full-term placentas, to study the effects of xenoestrogens on ABCG2 expression, were collected after elective caesarean section from the Department of Gynecology and Obstetrics, University Hospital of Oulu, Finland. All collected placentas in Finland were from healthy non-smoking mothers. Placentas were handled anonymously.

Placental explant cultures were done according to previously published studies (Caniggia et al., 1997). Placentas were taken into laboratory in cold PBS or HBSS solution within 2 h. For the explant cultures first trimester placentas were used as a whole while only a couple of small cross-sectional central pieces near umbilical cord were taken from term placentas. The tissues were washed with cold PBS and sectioned under stereo microscope to separate the villous branches from villus trees. Selected fragments from tip branches of villi were put into wells of 24-well plates (one explant/well). Culture medium was DMEM/F12 –medium free of phenol red and serum but including L-glutamine (Gibco, Invitrogen) and supplemented with 1% antibiotics (penicillin-streptomycin, Euroclone).

In floating cultures (both first trimester and term placentas) 500 μl of medium was put into well with an explant. After overnight incubation in an incubator (at 37 °C, 95% air/5% carbon dioxide), old medium was replaced by exposure medium. Control sample (triplicate) was vehicle treated (0.1% ethanol in every well). Explants were exposed to either 1 nM or 100 nM of BPA, DES or *p*-NP as triplicates. As a positive control for estrogenic responses, explants were exposed also to 1 nM E2 alone. To see possible synergistic effect of chemicals with estradiol, explants were exposed to the combination of E2 and BPA, *p*-NP or DES (each at 1 nM). In addition, term placental explants were exposed to the antiestrogen fulvestrant (ICI 182780, 100 nM) alone or in combination with each of the studied chemicals mentioned above (1 nM). Explants for each exposure within the series (control, xenoestrogens, estradiol, antagonist) were taken from the same placenta and cultured as triplicates. Explants were exposed for 24 h or 48 h.

The function and the endocrine integrity of all cultured explants were analyzed by measuring the concentration of human chorionic gonadotropin (hCG) from culture medium. Measurement was done according to the instructions of the manufacturer of the used kit (β-hCG IEMA Well, Radim, Pomezia, Italy, or hCG + beta ELISA, IBL International GMBH, Hamburg, Germany). OD values were measured by ELISA reader (Dynex Technologies MRX TC Revelation) at the wavelength of 405 nm. Results of the samples were

normalized using external standards and protein concentrations.

2.3. RNA extraction and real time-PCR

Total RNA extraction from explant cultures was done with a commercial RNA extraction kit (RNeasy® Plus Mini kit, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Briefly, explants were collected and three explants were pooled. All steps were performed at room temperature and all reagents used were provided by the kit. Explants were homogenized into RTL® buffer containing 1% β-mercaptoethanol and RNA protecting agents using glass homogenizer or ceramic beads containing Magna lyser tubes (Magna Lyser Green tubes, Roche Diagnostics GmbH, Mannheim, Germany). When using tubes containing ceramic beads, explants were homogenized by Magna Lyser (7500 rpm at +4 °C). To extract RNA, lysate was centrifuged and washed several times with different buffers using different filter spin columns. Finally RNA was eluted from the column by sterile RNAase free water and stored at −70 °C. RNA concentrations were determined by NanoDrop ND-1000 spectrophotometer. Due to the low RNA content in the samples, as much RNA as possible was taken into reaction mixture to be converted into cDNA. A commercial kit (High Capacity cDNA Reverse Transcription kit, Applied Biochem, Foster City, CA, USA) was used for reverse transcription.

Quantitative real time-PCR analysis was used to analyse the ABCG2 mRNA expression. Analysis was done using TaqMan chemistry and a commercial kit supplying master mix for the reactions (FastStart Universal Probe Master (Rox), Roche Diagnostics GmbH, Mannheim, Germany). The used primers (forward and reverse) and the fluorogenic probes (Supplementary Table I) were purchased from Sigma. Samples were analyzed by the 7300 Real Time PCR System (Applied Biosystems). Results were normalized with 18S mRNA. Samples were analyzed as duplicates and a reaction without a template as negative control. Results were analyzed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

2.4. Protein extraction and immunoblotting

Total protein fractions were extracted from tissue explants as previously described (Bechi et al., 2010). Explants were collected into ice cold RIPA homogenization buffer (50 mM Tris-HCl; 150 mM NaCl; 1% Triton x-100; 1% Na-Deoxycholate; 0.1% SDS, pH 7.5) supplemented with protease inhibitor cocktail (from Sigma or Complete EDTA-free from Roche Diagnostics GmbH, Mannheim, Germany) and 0.1 mM Na-orthovanadate (Sigma). Explants were homogenized on ice with a glass homogenizer or by using Magna Lyser. Homogenates were centrifuged before concentrating supernatants by lyophilization. Samples were resolubilized into ice cold PBS and stored at −70 °C. Protein concentrations were determined by Bradford analysis. The exposed triplicates of the same placenta were pooled for one sample before extraction to get enough sample for analysis.

Immunoblotting was performed as previously described (Myllynen et al., 2008). Details of the used immunoblotting protocol are listed in Supplementary Table II. Protein samples (20–50 μg of protein) were incubated in sample buffer (with 150 mM DTT) at room temperature at least for 20 min. Samples were run into 7.5–10% SDS-PAGE gels in electrophoresis at 200 V for 40–90 min. Protein standards were used to see separation and molecular sizes of the protein bands. Proteins were transferred into methanol pre-treated membrane by blotting. Membranes were washed and blocked at room temperature for 1–3 h before antibody treatments. All antibodies were diluted in TBS/BPS-T with 0.5–5% milk powder depending on the antibody. Primary antibody for ABCG2 transporter was ABCG2 (1:500/1000 dilution; clone BXP-21, Alexis Biochemicals or the same clone from Chemicon International/Millipore, Temecula, California, USA). Anti-mouse IgG (1:1000/2000 dilution; v/v, Cell Signaling Technologies) was used as the secondary antibody. Detection of protein bands was performed by commercial chemiluminescent kits. Membranes were strip-washed if more than one protein was analyzed from the same membrane. Intensities of the protein bands were quantified by QuantityOne-software (BIO-RAD, Hercules, CA, USA). The extent of protein loading on membranes was analyzed with loading controls and the detected intensities of analyzed ABC transporter proteins were normalized to the loading controls. In addition, all the samples were compared to the control samples of their own data series.

2.5. Statistical analysis

Data was analyzed to be normally distributed by SPSS using Shapiro-Wilk test ($p > 0.05$) for normality and visual analysis of graphics. For statistical analysis variances between different groups of independent samples were analyzed by One-Way ANOVA. If there were statistically significant differences in variances between the groups, the *t*-test was used as a test for statistical significances; otherwise One-way ANOVA (Tukey as a post-hoc test) was used (for details of used test for each experiment, see figure legends). Data are presented as mean ± standard deviation (SD) and the p-value <0.05 considered as statistically significant. IBM SPSS statistics software (version 19) was used for the calculations.

3. Results

3.1. Expression of ABCG2 protein in human placenta

All human placental explant tissue samples, both first trimester and term, expressed the ABCG2 transporter protein (Fig. 2). There was interindividual variation in the expression of ABCG2. Term placentas expressed statistically significantly less ABCG2 protein than first trimester placentas ($p = 0.03$).

3.2. Effect of bisphenol A on the expression of ABCG2 mRNA and protein in first trimester and term placental explant cultures

BPA had only minor effects on mRNA expression of ABCG2. However, after 24 h in term placentas, BPA decreased the expression of ABCG2 mRNA with both of the used concentrations and the effect of 100 nM BPA was statistically significant ($P < 0.05$) (Fig. 3A). E2 alone or in combination with BPA did not have an effect on ABCG2 mRNA expression. After 48 h BPA or E2 did not have statistically significant effects. In the first trimester explants BPA or E2 alone or in combination did not change ABCG2 mRNA expression after 24 h or 48 h (data not shown).

In term placental explants, 1 nM concentration of BPA ($P < 0.05$) downregulated the expression of ABCG2 protein statistically significantly after 48-h exposure (Fig. 3C). Higher concentration of BPA (100 nM) did not have significant effect on the ABCG2 protein expression in any studied time point. E2 alone decreased the expression of ABCG2 protein (statistically significantly, $P < 0.05$) both after 24 and 48 h. Combination of BPA and E2 did not have significant effects on the expression of ABCG2 and no synergism was seen.

In the first trimester explants no statistically significant effects either by BPA or E2 alone or by their combination were seen at any studied time points (Fig. 3B). All explant cultures (first trimester and term) released β-hCG. There were no statistically significant differences in hCG release between the cultures treated by the chemicals compared to the untreated control cultures (data not shown).

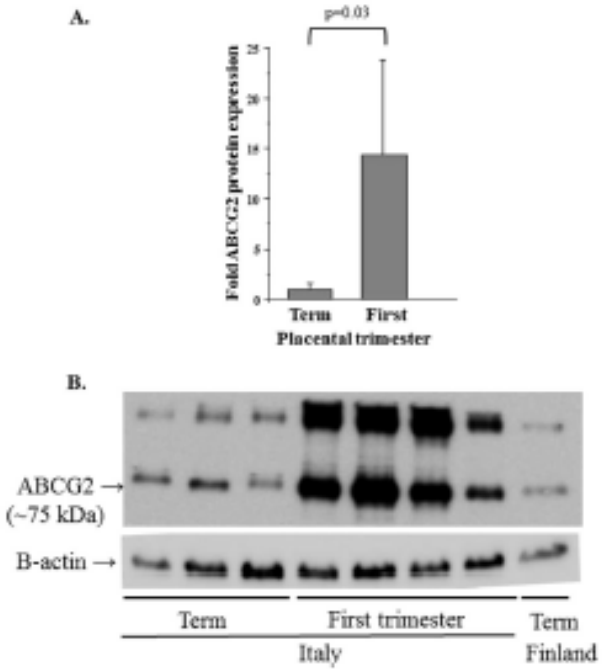


Fig. 2. The expression of the ABCG2 transporter protein in human placental choronic villous explants. A) Densitometry of immunoblotting of first trimester (weeks 7–9) and term (>38 weeks) placental explants ($n = 4$ in both groups, all collected after altogether 48 h culture). Values represent mean ± SD. Statistically significant differences between groups were analyzed by One-way ANOVA using SPSS. B) A representative immunoblot showing the expression of the ABCG2 protein in first trimester and term placentas. β-actin was used as a loading control and results were normalized with it (analyzed in Finland).

3.3. *The effects of para-nonylphenol and diethylstilbestrol on the expression of ABCG2 in first trimester and term placental explant cultures*

In the term placental explants, *p*-NP decreased the expression of the ABCG2 protein at 48 h, especially exposure to 1 nM *p*-NP causing statistically significant decrease ($p < 0.01$) (Fig. 4B). After the exposure for 24 h the studied chemicals displayed no statistically significant effects. E2 alone decreased the expression of the ABCG2 protein at both time points ($P < 0.05$). Addition of E2 in combination with either DES or *p*-NP did not have an effect on the responses. No statistically significant effects were seen by studied chemicals on ABCG2 mRNA levels (data not shown).

In the first trimester explants, chemicals (BPA, DES or *p*-NP) or estradiol alone or in combination did not have statistically significant effects on the expression of ABCG2 mRNA or protein at the studied time points (Fig. 4A). The release of β-hCG was detected in all explants cultures and there were no statistically significant differences between the cultures treated with different study chemicals compared to the control cultures (data not shown).

3.4. *The reversion of xenoestrogen induced ABCG2 downregulation by ER antagonist in term placentas*

The estrogen receptor (ER) antagonist fulvestrant (ICI 182780) was used to clarify whether the effects of chemicals on ABCG2 are mediated via ER. The antagonist alone caused no statistically significant effects on the expression of the ABCG2 protein (Fig. 5). BPA ($p < 0.05$), *p*-NP ($p < 0.01$) and E2 ($p < 0.05$) decreased the expression of ABCG2 after 48 h as previously shown. E2, when added in combination with chemical, reversed partly the effect of BPA and *p*-NP. The antagonist affected the responses of BPA, E2 and *p*-NP on ABCG2 implying involvement of ERs in actions of these chemicals. The effect of DES on ABCG2 protein was not significant and the antagonist did not affect the effects of DES.

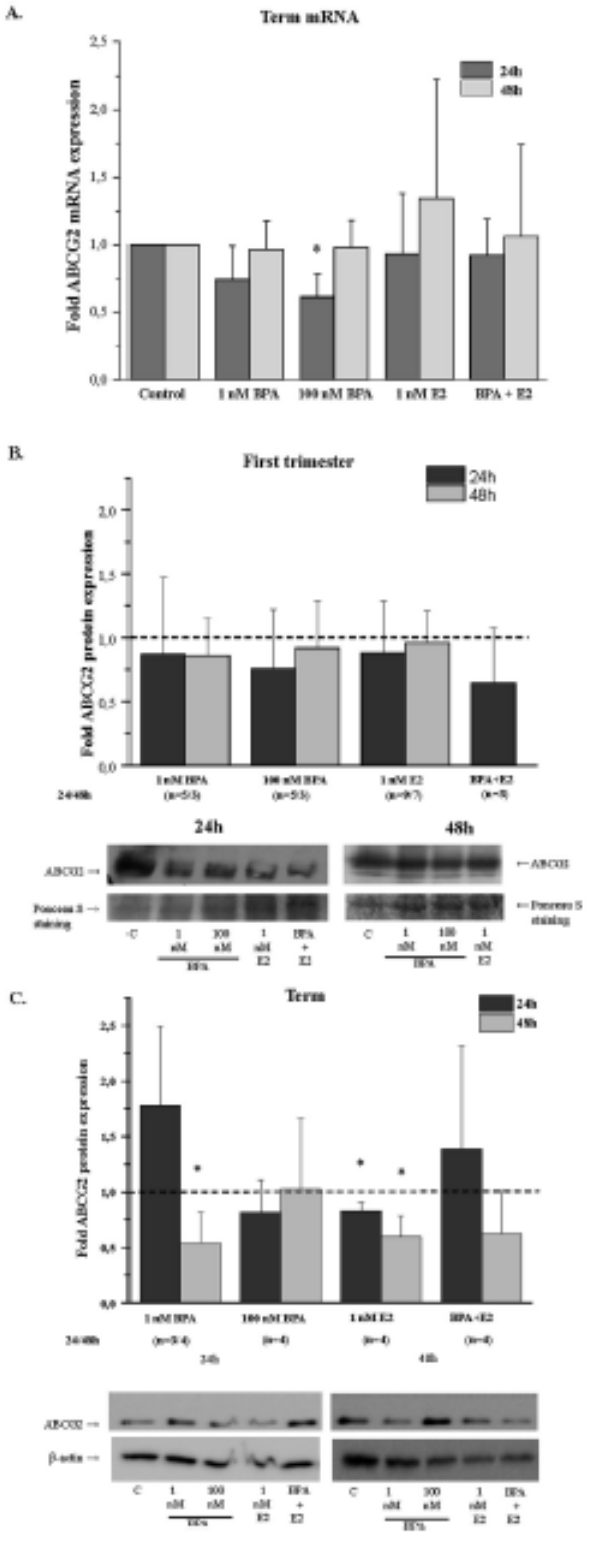
4. Discussion

This is the first study showing downregulation of ABCG2 transporter protein by environmental xenoestrogens, bisphenol A and *para*-nonylphenol, in human placenta. It was already known that physiological changes, such as syncytialization/differentiation and the amount of endogenous hormones like estradiol and progesterone, affect the expression of the ABC transporters in human placenta (Evseenko et al., 2007; Yashwanth et al., 2006). However, data in the literature are controversial and further studies are needed to determine the most important factors behind the differences.

According to the literature, very little is known of the effects of estrogens and xenoestrogens on the expression and function of ABCG2, and data vary between the individual tissues and the models used (Table II). Previously, BPA has been shown to downregulate ABCG2 expression in blood brain barrier of rat (Nickel and Mahringer, 2014) but there are no published studies about the effects in placenta and nothing of the effects of *p*-NP on ABCG2. In our study, both compounds downregulated ABCG2 expression in human placenta. Diethylstilbestrol (DES) did not have a significant effect on ABCG2 expression in human placenta in our study. Previously in the literature DES has been reported to decrease the ABCG2 protein expression in human breast cancer cells (Imai et al., 2005). It has been found that estradiol downregulates ABCG2 protein in human placenta (Wang et al., 2006, 2008) as seen also in our study. Contradictory studies however exist (Table II). The effect of the downregulation in transporter expression on actual transfer or accumulation of compounds, needs to be confirmed in future studies.

We found the effects of xenoestrogens on the ABCG2 transporter at protein level, while mRNA seemed to be mostly unaffected. This agrees with the notion that the regulation of ABCG2 is post-transcriptional, at least to some extent, as reported by Imai et al. (2005). However, some papers have reported downregulation also at mRNA level in human placental (Wang et al., 2006, 2008) or breast cancer cells (Honorat et al., 2008; Zhang et al., 2006). On the contrary, Zhang et al. (2006) reported transcriptional induction of ABCG2 by estradiol in human breast cancer cells. In our study the effects of chemicals on ABC transporter expression were more pronounced and consistent after longer exposure (48 h) as reported also by other studies (Evseenko et al., 2007; Yasuda et al., 2006; Wang et al., 2006). This may be due to the long half-life of the ABC transporters as well as posttranslational modifications (Imai et al., 2005).

The basal expression level of placental ABCG2 transporter protein differs during gestation and our results showing lower expression of the ABCG2 protein in term placental explants compared to first trimester placentas are in line with the previous study in the literature (Meyer zu Schwabedissen et al., 2006). In addition, a dependency of responses on gestational age was seen in our study, which may be due to variation of oxygen tension, first trimester placenta being under lower oxygen tension than term placenta (Schneider, 2011). In the literature, hypoxia has been shown to increase the protein expression of ABCG2 while the mRNA expression of ABCG2 was decreased in human first trimester placental explants (Lye et al., 2013). Oxygen tension did not significantly affect the expression of ABCG2 in term placentas



(Javam et al., 2014). Thus, the higher basal expression of ABCG2 protein in first trimester placentas may partly mask the responses of chemicals compared to term placenta. Also the hormonal status changes during pregnancy (Hill et al., 2014) as estradiol concentration (1 nM) in first trimester placenta increases towards the term trimester (~50–100 nM) (O'Leary et al., 1991). Differences in hormonal status may affect the responses. However, the concentrations of xenoestrogens used in this study correspond with concentrations measured from biological fluids, such as maternal serum/plasma, umbilical cord serum/plasma, amniotic and ovarian follicular fluids, during pregnancy (Cao et al., 2012; Chen et al., 2008; Ikezuki et al., 2002; Schönfelder et al., 2002; Zhang et al., 2011) and thus represent actual exposure concentrations during pregnancy. Responses of chemicals were seen mainly with lower concentrations, which may be due to an inverted, U-shape dose-response curve common for estrogens and xenoestrogens (Vandenberg et al., 2009; Welshons et al., 2003). Hence smaller concentrations may cause stronger responses and make low dose effects of chemicals more significant as seen also in this study. Low and environmental dose effects of xenoestrogens differ in this sense from conventional toxicological effects and they are especially relevant when considering placental and prenatal exposure during the sensitive time of pregnancy.

Mechanisms of action for xenoestrogens in tissues are under research. DES, p-NP and especially BPA have been shown to act via several receptors, including the estrogen receptors (ER-α/β), estrogen-related receptor gamma (ERR-γ) and g-protein-coupled estrogen receptor (GPER) depending on the study and target tissue (Gutendorf and Westendorf, 2001; Morice et al., 2011; Takayanagi et al., 2006; Vandenberg et al., 2009). Placental expression of these receptors varies during gestation (Fujimoto et al., 2005; Morice et al., 2011). Based on our results it is likely that ERs are involved in the effects of BPA and p-NP on ABCG2 in term placenta. At this point, only limited and partially controversial, information on mechanism of actions of studied chemicals can be found in literature suggesting differences between species and cell types in the regulation of ABCG2. The actions are probably tissue- and species-specific acting via different receptors with or without specific coactivators *in vitro* and *in vivo* (Routledge et al., 2000) including genomic and non-genomic mechanisms, depending also on the concentration (Vandenberg et al., 2009). The binding of various co-activators to ERs, or binding to various receptors probably determinates whether xenoestrogens act as agonists or antagonists in different tissues (Routledge et al., 2000). Changes in the expression levels of receptors during pregnancy, may at least partly cause the differences in the responses to chemicals at different gestational stages and also between individuals. Higher receptor expression in term placenta (Fujimoto et al., 2005) possibly enables stronger responses by chemicals acting via them.

Human placenta has unique anatomy and function compared to those of other species (Myllynen et al., 2007). Human placental chorionic villous explant cultures mimic the *in vivo* situation better than placental cell lines because the paracrinic relations between

Fig. 3. The effect of BPA on the expression of ABCG2 in human chorionic villous placental explants. Expression of ABCG2 mRNA in term (n = 4) (A) and the protein in first trimester (B) and term (C) placental explants. Explants were exposed to vehicle (C = control, 0.1% EtOH in all samples; n = 10 for 24 h and n = 7 for 48 h for first trimester, n = 4 for term), bisphenol A (BPA, 1 or 100 nM), estradiol (E2, 1 nM) or to the combination of BPA and E2 (both 1 nM) for 24 or 48 h (n = 3–10 for details see figure). Densitometry values (upper part of B and C) represent mean ± SD. Statistically significant differences between the groups were analyzed by Student's *t*-test using SPSS. *P < 0.05. Representative immunoblots show ABCG2 (~75 kDa) protein expression in analyzed samples. Ponceau S staining or β-actin was used as loading control in immunoblotting.

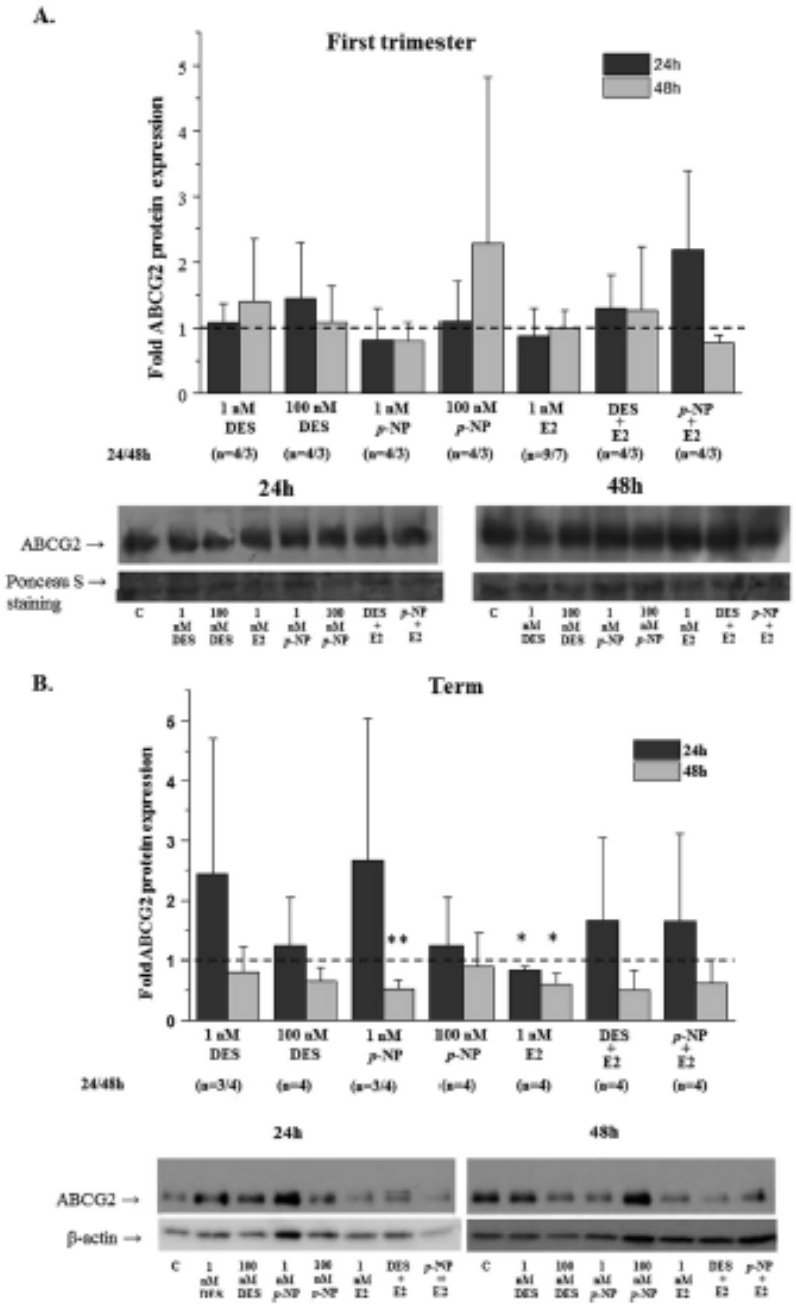


Fig. 4. The effect of DES and p-NP on the expression of ABCG2 protein in human chorionic villous placental explants. Expression of ABCG2 protein in first trimester (A) and term (B) placental explants. Explants were exposed to vehicle (C = control, 0.1% EtOH in all samples; n = 10 for 24 h and n = 7 for 48 h for first trimester, n = 4 for term), diethylstilbestrol (DES, 1 or 100 nM), para-nonylphenol (p-NP, 1 or 100 nM), estradiol (E2, 1 nM) or to the combination of either DES or p-NP and E2 (both 1 nM) for 24 or 48 h (n = 3–10 for details see figure). Densitometry values (upper part of A and B) represent mean ± SD (analyzed by Student's t-test using SPSS). *P < 0.05, **P < 0.01. Representative immunoblots of the ABCG2 protein (~75 kDa) are shown. Ponceau S staining or β-actin was used as loading control in immunoblotting.

different cells remain more intact in explants. In addition, by using explants placenta can be studied at different gestational age (Miller et al., 2005). Selection of the study model as well as cell culture conditions may have significant effects on the detected responses of xenoestrogens and chemicals, especially as to weak estrogenic responses (Immonen et al., 2009). Careful consideration of study design may improve the use of *in vitro* methods in chemical toxicity testing, including reproductive toxicology. Another challenge in the

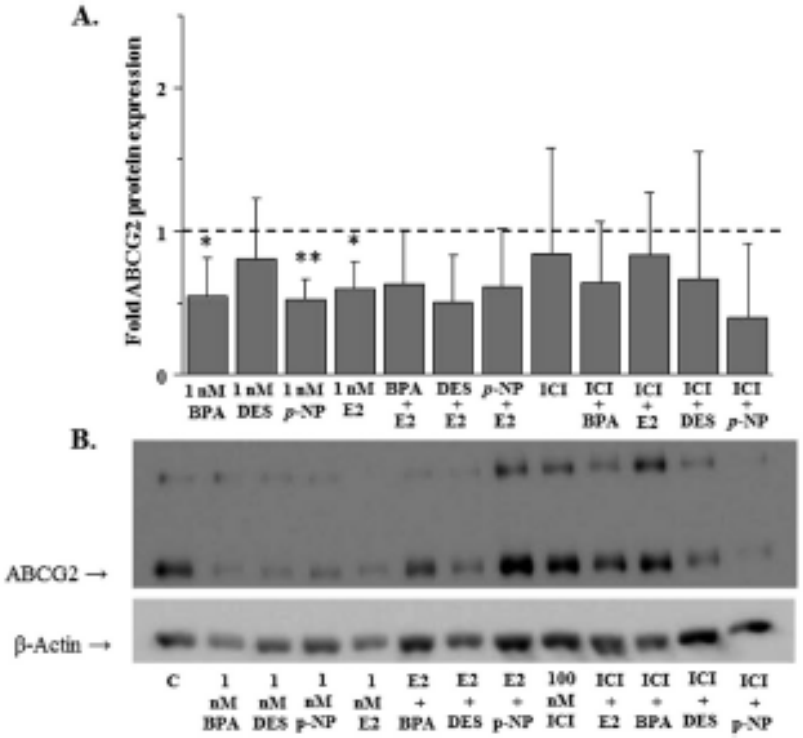


Fig. 5. The effect of the estrogen receptor antagonist fulvestrant on the responses by the chemicals on the expression of ABCG2 protein in term chorionic villous placental explants after exposure for 48 h. (A) Densitometry of immunoblots after exposure to vehicle (C = control, 0.1% EtOH in all samples), bisphenol A (BPA, 1 nM), diethylstilbestrol (DES, 1 nM), *para*-nonylphenol (*p*-NP, 1 nM), estradiol (E2, 1 nM), the combination of BPA, DES or *p*-NP with E2 (both 1 nM), or to fulvestrant alone (ICI 182780, 100 nM) or in combination with each chemical (1 nM BPA, DES, *p*-NP or E2). Values represent mean ± SD (statistical analysis by Student's *t*-test using SPSS). *$P < 0.05$, **$P < 0.01$. In all cases $n = 4$ except for samples where ICI is present ($n = 3$). (B) Representative immunoblot showing the effects of the chemicals on the expression of the ABCG2 protein (~75 Da). β-actin was used as a loading control.

Table III
The effects of estrogens and xenoestrogens on ABCG2 expression are contradictory.

Transporter	Action	Chemical	Species/cell line	References
ABCG2	Induction of mRNA	Estradiol (estrone, estriol)	human breast cancer cell line	Ee et al., 2004, Li et al., 2013, Pradhan et al., 2010, Zhang et al., 2006
			human placental choriocarcinoma cells	Ervesenko et al., 2007, Pitouillac et al., 2009, Wang et al., 2008, Yasuda et al., 2006
	Induction of protein	Estradiol (estrone)	human breast cancer cells	Li et al., 2013, Pradhan et al., 2010, Zhang et al., 2006
			human placental choriocarcinoma cells	Ervesenko et al., 2007, Pitouillac et al., 2009, Wang et al., 2008, Yasuda et al., 2006
	Reduction of mRNA	Estradiol	human breast cancer cells	Honorat et al., 2008, Wu et al., 2012
			human placental choriocarcinoma cells	Wang et al., 2006, Wang et al. 2008
	reduction of protein	estradiol (estrone)	human breast cancer cells	Honorat et al., 2008, Imai et al., 2005, Wu et al., 2012
			human placental choriocarcinoma cells	Wang et al., 2006, Wang et al. 2008
		DES	human breast cancer cells	Imai et al., 2005

case of biological material is the high interindividual variation seen also in our results. For instance, genetic polymorphisms of ABC transporters increase the variation of molecular effects of compounds among individuals (Hutson et al., 2010; Ni and Mao, 2011).

5. Conclusions

Our study confirms the earlier findings by other groups that human term placenta expresses less ABCG2 protein than first trimester placenta. The environmental xenoestrogens, BPA and *p*-NP, downregulated the protein expression of the human placental ABCG2 protein in term placenta, while they did not have any effect on first trimester placenta. The mechanisms of action of these two chemicals may differ. However, ERs appear to be involved in the effects of both, BPA and p-NP. The information provided by the study is important because nothing was known in the literature up to now about the role of xenoestrogens on the expression of human placental ABCG2 which is important for transport of endogenous compounds, like nutrients, and protection from xenobiotics, such as drugs and carcinogens.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.03.034.

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