Endocrine-Disrupting Potential of Bisphenol A, Bisphenol A Dimethacrylate, 4-n-Nonylphenol, and 4-n-Octylphenol in Vitro: New Data and a Brief Review

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Endocrine disruptors (EDs) are exogenous compounds that have the potential to interfere with hormonal regulations and the normal endocrine system and consequently cause health effects in animals and humans (U.S. Environmental Protection Agency 2000). EDs include environmental persistent organohalogens, pesticides, and industrial chemicals such as some plasticizers and surfactants. The involvement of EDs in disruption of development, reproduction, the immune system, and the neural system has been supported in a wide range of fish and animal species, whereas for human beings the ED hypothesis is still controversial (Bonefeld-Jørgensen 2004; Bonefeld-Jørgensen and Ayotte 2003; Owens and Koeter 2003; vom Saal and Hughes 2005). Most EDs are synthetic compounds, some of which were designed to act as estrogens (e.g., oral contraceptives), whereas many were designed for other purposes and accidentally possessed estrogenic activity, such as plasticizers (Krishnan et al. 1993; Soto et al. 1991). Naturally occurring xenoestrogens in the environment include phytosterogens produced by plants reported to have pleiotropic effects, including antioxidative and apoptotic activity, inhibitors of kinases, and suggested anticancer actions on prostate and breast carcinomas (Basly and Lavier 2005; Mueller 2002; Sirtori et al. 2005). Other EDs such as the pesticides vinclozolin, procymidone, and p,p′-dichlorodiphenyl-dichloroethene (DDE) possess in vitro and in vivo estrogenic actions and can affect male reproduction in animals (Gray 1998). Bisphenol A (BPA) and BPA dimethacrylate (BPA-DM) are monomers used largely in polycarbonate plastic and polystyrene resins and as dental sealants. Halogenated derivatives of BPA, such as tetrabromobisphenol A (TBBPA), are widely used as flame-retardants for building materials, paints, plastic products including epoxy resin, electronic circuit boards, and other electronic equipments. Depolymerization of these products results in BPA and its derivatives, which leach into foods (Brotorns et al. 1995), into infant formula from plastic bottles (Biles et al. 1999), into saliva of patients treated with dental sealants (Olea et al. 1996; Pulgar et al. 2000), and in fresh food at the microgram to milligram per kilogram level (Vivacqua et al. 2003). BPA and TBBPA have been detected in the concentration range of 0.1–10 ppb in human blood, urine, and fetal tissues, and related BPA levels in blood and fat tissues have also been reported (Ikekuzi et al. 2002; Schonfelder et al. 2002; Thomsen et al. 2001; vom Saal and Hughes 2005).

Alkylphenol ethoxylates (APEs) are widely used surfactants and detergents in domestic and industrial products and are commonly found in wastewater. In sewage treatment plant effluents, APEs are degraded to the more resistant alkylphenols such as 4-n-nonylphenol (nNP) and 4-n-octylphenol (nOP). Data from studies across many regions of the world have shown significant levels in samples of every environmental compartment examined, including fish muscle tissue (Ying et al. 2002), and they are generally ubiquitous in food as well (Guenther et al. 2002).

The sex steroid receptors such as the estrogen receptors (ER) α and ERβ and the androgen receptor (AR) belong to the nuclear receptor family and are ligand-dependent transcription factors (Bjornstrom and Sjoberg 2005; Schwabe and Teichmann 2004; Verrijdt et al. 2003). The genomic-mediated pathway of EDs via ER and AR includes steps such as binding of ligand to receptor, translocation into nucleus, and binding of the receptor–ligand complex to a specific DNA response element causing gene expression.

Several years ago BPA, BPA-DM, nNP, and nOP were reported to elicit estrogenic activity (Andersen et al. 1999; Krishnan et al. 1993; Soto et al. 1991; Steinmetz et al. 1998; White et al. 1994). Since then, numerous studies have been carried out to assess the endocrine disruption potentials of these industrial compounds in vitro and in vivo (Alonso-Magdalena et al. 2006; Choi and
to advance the onset of pubertal spermatogenesis in rats (Atanassova et al. 2000). The androgen:estrogen ratio is among other things determined by aromatase (CYP19) activity that is responsible for the irreversible estrogen biosynthesis from androgens (Jones et al. 2006; Seralini and Moslemi 2001; Simpson et al. 2002). Depressed ovarian aromatase activity in the red mullet was suggested to be caused by nNP and nOP (Martin-Skilton et al. 2006), whereas increased CYP19 gene expression was reported in nNP-exposed zebrafish (Kazeto et al. 2004), and nNP- or BPA-exposed medaka fish liver (Min et al. 2003). In rats, a decreased serum 17β-estradiol (E2) and aromatase mRNA level in Leydig cells was interpreted to play a role in inhibited testicular steroidogenesis by BPA (Akingbemi et al. 2004). Interestingly, although no effect of BPA was observed on CYP19 mRNA levels in human placental JEG-3 cells, a time- and concentration-dependent modulation of the aromatase activity was reported suggesting an interaction between the enzyme and BPA (Nativelle-Serpentini et al. 2003). In summary, effects on aromatase activity caused by the alkylphenols and BPA have been reported (Nishizawa et al. 2005a). In mice Hepa-1c1c7 cells were exposed to BPA and nNP and it was shown that AhR mRNA expression in nNN-exposed mice embryos, disrupt the expression of AhR and related factors and xenobiotic metabolizing enzymes (Nishizawa et al. 2005a). In mice Hepa-1c1c7 cells, nNP suppressed CYP1A1 expression by antagonizing the dioxin-responsive element (DRE) binding of nuclear AhR (Jeong et al. 2001). In parallel with E2, an estrogenic effect of nNP was observed in marine fish, Gobius niger, and the P4501A1 inhibition by nNP was mediated through activation of the AhRR (Maradonna et al. 2004). Also, in Atlantic salmon nNP was suggested to have an impact on the metabolism of endogenous and exogenous substrates by modulation of hepatic CYP1A1 via AhR (Meucci and Arukwe 2006). In summary, BPA and nNP are both able to affect AhR action in cell cultures, in murine fetuses, and/or in fish.

The aim of the present study was to analyze the relative ED potencies of BPA, BPA-DM, nNP, and nOP (Figure 1) in four key in vitro bioassays for ED effects including ER, AR, and AhR transactivation and aromatase activity.

**Materials and Methods**

**Materials.** BPA was obtained from Sigma-Aldrich Co. (Birmingham, UK). BPA-DM was purchased from Aldrich Chemical Co. (St. Louis, MO, USA). nNP and nOP were purchased from Lancaster Synthesis Ltd. (Birmingham, UK) and Aldrich Chemical Co., respectively. The four chemicals (purity ≥ 98%), and TCDD, 98% (Cambridge Isotopes Laboratories Inc., Andover, MA, USA) were dissolved in dimethyl sulfoxide (DMSO) (BDH Laboratory Supplies, Pool, UK). The E2 (Sigma, St. Louis, MO, USA) was dissolved in 96% ethanol (extra pure; Merck, Darmstadt Germany). Luciferin and fluorescamine were from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and Molecular Probes (Eugene, OR, USA).
respectively, and bovine serum albumin from Promega (Madison, WI, USA); [1β-3H]Androst-4-ene-3,17-dione was from PerkinElmer (Boston, MA, USA), and both 4-androstene-3,17-dione (4-AD) and 4-androsten-4-ol-3,17-dione (4-AOD) were from Sigma Aldrich (Milwaukee, WI, USA). Methyltrienolone (R1881) was purchased from Mikromol Gmbh (Luckenwalde, Germany). The structures of the test chemicals are shown in Figure 1.

**ER-activated luciferase expression assay.** We used the stable transfected MVLN cell line, derived from the human breast adenocarcinoma MCF-7 cell line (Pons et al. 1990), to assess effects on ER–luciferase transactivation as described by Bonefeld-Jorgensen et al. (2005). The luciferase data were corrected to cell protein, and the results are given as relative light unit (RLU)/microgram protein. Each compound was tested in triplicate in at least three independent assays alone and as co-treatment with 25 µM E2 [40% of the effect concentration (EC50) of E2]. An E2 dose–response control (0.05–500 pM E2) was performed in parallel each analysis day as described (Bonefeld-Jorgensen et al. 2005). The maximal (EC100) and half maximal (EC50) effective concentrations of E2 were 150 pM and 33 pM, respectively.

**Aromatase activity.** We performed the assay protocol as described by Drenth et al. (1998), with minor modifications. Human JEG-3 choriocarcinoma cells (no. HTB-36; ATCC, Manassas, VA, USA) were maintained in minimum essential medium (Invitrogen, Life Technologies, Glasgow, UK) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 64 mg/L garamycin, and 1 mM sodium pyruvate (Gibco BRL, Life Technology, Gaithersburg, MD, USA). Cells were seeded in 24-well culture plates (Nunc) at 4 × 10^4 cells/well in 1 mL culture medium for 2 days (∼50% confluence). Serum-free medium ± test compound and/or control were added (maximum 0.1% DMSO) and incubated (37°C, 5% CO2/95% O2) for 18 hr (∼90% confluence), and then medium was removed, cells were washed with phosphate-buffered saline (PBS), and 0.5 mL serum-free medium containing 0.2 µCi [1β-3H]Androst-4-ene-3,17-dione and 10 nM unlabeled 4-AD, corresponding to the Kav value of the enzyme, was added. After 2 hr of incubation, the aromatization was terminated by placing the 24-well plates on ice. We extracted 200 µL of the water phase with 4 mL hexane (Packard BioScience, Groningen, the Netherlands) in a 6-mL vial for scintillation (Packard BioScience) and assayed for radioactivity (Packard Liquid Scintillation Analyzer, model Tri-carb 2200; Packard Instrument, Meriden, CT, USA).

**AR-reporter gene assay.** We tested AR transactivation in a luciferase reporter assay. Chinese hamster ovary (CHO) cells were transiently transfected with the expression vector pSVAR0 (human AR) and the MMTV-LUC reporter plasmid as described by Vinggaard et al. (2002), except that most pipetting procedures were performed using a Biomek2000 laboratory robot (Beckman Coulter, Fullerton, CA, USA) and that 0.1 nM R1881 was used as the AR agonist. Each test compound was tested at concentrations of 0.15, 0.3, 1.3, 2.5, 5, 10, 20, and 40 × 10^{-6} M in three independent assays as triplicates, and within each assay all data were related to 0.1 nM R1881, which was set to 100%. Using the SigmaStat program, we performed analysis of variance, and if data were statistically significant followed with a Dunnett’s test (p < 0.05). Dose–response analysis was performed in Sigma Plot fitting the curves to the “four parameter logistic curve” fit, and IC50 (half maximal inhibitory concentration) was calculated for each test compound. We determined cytotoxicity in parallel by transfecting cells with a plasmid (pSVAR13) encoding for a constitutively active AR, which lacks the ligand-binding domain (Vinggaard et al. 2002). These experiments were designed exactly as was the AR reporter gene assay except that the ratio between pSVAR13 and MMTV-LUC was 2:100.

**Results**

**Effects on ER transactivity.** BPA and BPA-DM elicited ER-mediated dose–response luciferase activity in the concentration range of 10^{-8} to 5 × 10^{-7} M, with the maximum response being approximately 75% of the natural ligand E2 induced maximum response. The EC50 values were 3.9 µM for BPA and 4.8 µM for BPA-DM. (Figure 2, Table 1). Co-treatment of MVLN cells with 25 pM E2 (EC50) and each of the two compounds showed at 5 µM and 10 µM a further increased estrogenic effect above the E2 EC50 control, whereas the significant decreased response at 50 µM might be the beginning of cell cytotoxicity (Figure 2B). Although lower maximum induction was observed, the alkylphenols nNP and nOP alone also elicited a clearly significant estrogenic response in the concentration range of 10^{-8} M to 10^{-6} M and 10^{-5} M, respectively, with EC50 values of 8.9 µM and 4.9 µM, respectively (Figure 2, Table 1). We observed further increase of the 25 pM E2 induced activity level for nNP and nOP at 5 × 10^{-6} and 5 × 10^{-5}–10^{-4} M, respectively (Figure 2B); cytotoxicity was observed at 25 and 50 µM, respectively (Table 1).

**Effects on AR transactivity.** BPA, BPA-DM, nNP, and nOP elicited a significantly antiandrogenic effect on the R1881-induced...

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AR activity in the range of 0.60–20 µM, with IC$_{50}$ values of 1.0, 2.3, 14.1, and 1.1 µM, respectively, and a maximum inhibition (MI) of 90, 89, 56, and 92%, respectively. We determined cytotoxicity for BPA, BPA-DM, and nNP at concentrations > 40 µM and for nOP at concentrations > 20 µM (Figure 3 and Table 1).

**Aromatase activity.** The inhibitor control 4-AOD caused an aromatase inhibition in the concentration range of 10$^{-9}$ M to 10$^{-5}$ M, with an IC$_{50}$ of 6 × 10$^{-5}$ M and 100% inhibition at 10$^{-5}$ M (Table 1). BPA and BPA-DM decreased the aromatase activity significantly at 10$^{-4}$ M, with MI of 59% and 40%, respectively. nNP and nOP decreased the aromatase activity in the range 10$^{-5}$–10$^{-5}$ M and 10$^{-2}$–10$^{-5}$ M, respectively, with MI at 10$^{-2}$ M of 71% and 47%, respectively. Cytotoxicity was observed at 10$^{-4}$ M for both nNP and nOP (Table 1).

**Effects on AhR transactivation.** Effects on AhR transactivation data are shown in Table 1 and Table 2. Decreased AhR activity was observed for BPA alone and on co-treatment with TCDD-EC$_{50}$ at 5 × 10$^{-5}$ to 10$^{-4}$ M and 10$^{-4}$ M, respectively. BPA-DM alone weakly but significantly increased the CALUX activity at 10$^{-5}$ M. Although weak compared with TCDD, nNP alone elicited significant (~4-fold) dose–response AhR-CALUX activity in the range of 5 × 10$^{-8}$ M to 10$^{-4}$ M, with an EC$_{50}$ of 24 µM. Moreover, nNP further increased and decreased the TCDD-induced AhR activity at 2.5–5 × 10$^{-5}$ M and 10$^{-4}$ M, respectively. For nOP alone, weak increased AhR-CALUX activity was observed at 10$^{-5}$–2.5 × 10$^{-4}$ M, whereas a decrease was observed at 5 × 10$^{-5}$ M and 10$^{-4}$ M. At 10$^{-4}$ M, nOP decreased TCDD-induced activity by 60%. None of the compounds elicited any toxicity in Hepa1.1 2cR cells at the tested concentration range. Rather, each of the compounds caused a significantly increased cell proliferation at 10$^{-4}$ M. However, we cannot exclude that the decreased CALUX activity observed at 10$^{-4}$ M for all compounds, except nNP alone, might be a response due to beginning cytotoxicity.

**Discussion**

In the present study we demonstrated that BPA, BPA-DM, nNP, and nOP elicited an impact on most of the selected end points: ER, AR, AhR, and aromatase activity. Estrogenicity was observed for the four compounds where BPA and nNP showed the highest relative potency compared with E$_2$, supporting previously reported in vitro and in vivo data. The four chemicals also antagonized AR transactivation in a concentration-dependent manner, with IC$_{50}$ values in the order BPA ≥ nOP ≥ BPA-DM < nNP. In addition, all four chemicals inhibited the aromatase activity in JEG-3 cells. Furthermore, nNP activated AhR in a concentration-dependent manner, as BPA-DM induced AhR activity at 10$^{-3}$ M only, whereas BPA inhibited the AhR activity at 5 × 10$^{-5}$ to 10$^{-4}$ M, and nOP elicited a weakly induced and a decreased AhR activity at lower and higher concentrations, respectively. Thus our in vitro data indicate that the four industrial chemicals have the potential to affect several cellular pathways systems, including gene expressions regulated via the steroid receptors ER and AR, the conversion of testosterone into estrogen by aromatase, and the function of AhR, involved in syntheses of steroids such as estrogens and metabolism of steroids and xenobiotic compounds.

MVLN cells, derived from MCF-7 cells (Pons et al. 1990), express both ERα and ERβ (Gaido et al. 1998; Grunfeld and Bonefeld-Jørgensen 2004; Hofmeyer and Bonefeld-Jørgensen 2004) that can bind the ER response element vitellogenin, *vit-tk*, in...
front of the luciferase gene (Gruber et al. 2004). Thus the estrogenicity demonstrated for the four tested compounds in our analyses is mediated by transactivation of ERs and/or ERα. Moreover, based on previous reports including E-SCREEN, ER transactivation, binding assays to the two ER subtypes, and effects observed with the ER antagonists ICI 182,780 and tamoxifen (Gutendorf and Westendorf 2001; Scippo et al. 2004; Vivaqua et al. 2003; Wilson et al. 2004), we can conclude that the estrogenic potential of these four compounds is accomplished through the ERs. However, we cannot exclude that other cellular mechanisms are involved, such as activation of the transcription factor cAMP-responsive element binding protein via binding to nonclassical membrane ERs (Quesada et al. 2002). In addition, BPA and nNP effects were exerted predominantly by the parent compounds and not by their metabolites (Legler et al. 2002).

We observed an antagonistic effect on R1881-induced AR transactivation of the four tested chemicals. To our knowledge, the AR antagonism of BPA-DM has not previously been reported. As we performed the present study, another study using transiently transfected [human AR (hAR) and MMTV-CAT] African monkey kidney CV-1 cells reported an antagonizing effect on AR by BPA, nNP, and nOP with similar IC50 values, except that nOP had an IC50 approximately 100 times higher (97.1 μM) (Xu et al. 2005) than ours. In contrast, in a recently established stably transfected CHO-AR-LUC cell line, only BPA but not nNP and nOP elicited anti-AR effects (Roy et al. 2004); similarly, BPA but not nNP antagonized AR action in stable transfected (hAR and MMTV-luciferase) PC-3 prostate cells (Paris et al. 2002; Sultan et al. 2001). Our AR antagonistic data for BPA and nNP are consistent with results obtained in yeast (Lee et al. 2003) and transiently transfected (hAR and ARE2-luciferase) NIH3T3 cells (Kitamura et al. 2005). In contrast, BPA showed no effect on AR action in transiently transfected (hAR and MMTV-luciferase) human hepatoma HepG2 cells (Gaido et al. 2000). Whole cell binding assays showed that BPA has the potential of binding to the AR (Paris et al. 2002). We suggest that the anti-AR action observed in the present study for BPA, BPA-DM, nNP, and nOP indicates that the chemicals have the ability to bind the AR and thus compete with endogenous androgens for binding and regulating AR-dependent gene expression. However, indirect interference with other transcriptional factors involved in AR transactivation may also play a role. The discrepancy between the reported AR effects of the compounds might be caused by different sensitivities of the various assays, differences in co-factors in the different cell lines, and/or the use of different AR response elements (Sommer and Haendler 2003; Verrijdt et al. 2003). However, it is important to clarify the cause(s) of the discrepancies in the use of AR-reporter analyses to assess the potential actions of chemicals on androgenic processes in vitro.

Table 1. ER, AR, AhR, and aromatase characteristics of the four test chemicals alone.

| Assay | LOEC (M) | MOEC (M) | Maximum % | REP | EC50/IC50 | Cytotoxicity (M) |
|-------|----------|----------|------------|-----|----------|-----------------|
| ER    | E2       | 1 × 10^{-11} | 1.5 × 10^{-10} | —   | 1        | 3.3 × 10^{-10} | —               |
|       | BPA      | 1 × 10^{-4}  | 1 × 10^{-5}   | —   | 1 × 10^{-4} | 3.9 × 10^{-6}  | —               |
|       | BPA-DM   | 5 × 10^{-4}  | 1 × 10^{-5}   | —   | 2 × 10^{-5} | 3.8 × 10^{-6}  | —               |
|       | nNP      | 1 × 10^{-3}  | 1 × 10^{-4}   | —   | 1 × 10^{-3} | 6.9 × 10^{-6}  | 2.5 × 10^{-5}  |
|       | nOP      | 5 × 10^{-4}  | 1 × 10^{-5}   | —   | 2 × 10^{-6} | 4.9 × 10^{-6}  | 5.0 × 10^{-5}  |
| AhR   | BPA-DM   | 5 × 10^{-6}  | 2 × 10^{-6}   | 90  | ND       | 1.1 × 10^{-6}  | > 10^{-5}      |
|       | nNP      | 2.5 × 10^{-6} | 2 × 10^{-6}   | 56  | ND       | 1.4 × 10^{-6}  | > 10^{-5}      |
|       | nOP      | 0.6 × 10^{-6} | 1 × 10^{-5}   | 92  | ND       | 1.1 × 10^{-6}  | > 2 × 10^{-5}  |
| Aromatase | 4-ADO | 1 × 10^{-3}  | 1 × 10^{-4}   | 100 | 1        | 6.5 × 10^{-12} | —               |
|       | BPA      | 1 × 10^{-4}  | 1 × 10^{-4}   | 59  | 1 × 10^{-5} | —               | —               |
|       | BPA-DM   | 1 × 10^{-4}  | 1 × 10^{-4}   | 40  | 1 × 10^{-5} | —               | —               |
|       | nNP      | 1 × 10^{-4}  | 1 × 10^{-5}   | 71  | 1        | —               | —               |
|       | nOP      | 1 × 10^{-4}  | 1 × 10^{-5}   | 47  | 1 × 10^{-4} | —               | —               |
| AhR   | TCDD     | 2 × 10^{-12} | 1 × 10^{-8}   | —   | 1        | 6.8 × 10^{-12} | —               |
|       | BPA-DM   | 5 × 10^{-6}  | 1 × 10^{-6}   | 54  | ND       | > 10^{-7}      | > 10^{-4}      |
|       | nNP      | 5 × 10^{-6}  | 1 × 10^{-5}   | 44  | ND       | > 10^{-4}      | > 10^{-4}      |
|       | nOP      | 1 × 10^{-4}  | 2.5 × 10^{-5} | 44  | ND       | > 10^{-4}      | > 10^{-4}      |

Abbreviations: —, no data; LOEC, lowest effect concentration in molar (M); MOEC, maximal effect concentration in molar (M); ND, not determined; REP, relative potency.

Figure 3. AR antagonism of the test chemicals on co-exposure with R1881 in transient transfected CHO-K1 cells. Cells were transiently transfected with the AR expression pSVAR0 and the reporter pMMTV-LUC vector.

Endocrine-disruption potential of BPA, BPA-DM, nNP, and nOP

In the synthesis of steroid hormones from cholesterol, the aromatase enzyme is pivotal by its irreversible conversion of androgens to estrogens (Simpson et al. 2002). Aromatase, which is located in the endoplasmic reticulum membrane, is expressed in several tissues and cell types in humans, including adipose tissue, various sites of the brain, and testicular Leydig cells in males (Jones et al. 2006). Aromatase activity and endogenous estrogens (and ERs) are important in male reproduction (Barcellos 2006). In rat Leydig cells, BPA inhibited testicular steroidogenesis by a decrease in 17α-hydroxylase/17-20 lyase and CYP19 expression causing decreased testosterone and E2 synthesis (Akingbemi et al. 2004). After 18-hr exposure of JEG-3 cells to BPA, BPA-DM, nNP, or nOP, we observed a significant inhibition of aromatase activity. To our knowledge, this is the first report showing BPA-DM, nNP, and nOP effects on aromatase activity in mammalian cells. nNP and, to lesser extent nOP, caused a dose-dependent inhibition of aromatase activity in the range of 10^{-9}–10^{-7} M. Our data are supported by an earlier report examining BPA in JEG-3 cells, in which a significant effect was observed on aromatase activity but not on gene expression (Nativelle-Serpentini et al. 2003). Given the observation that short (2 hr) exposure increased and long (18 hr) exposure decreased aromatase activity, and that parallel transfections with CYP19 cDNA elicited similar effects, Nativelle-Serpentini et al. (2003) concluded that the xenobiotics acted at the aromatase protein level. In summary, the tested plasticizers seem to have the potential to affect the aromatase activity and thus the synthesis of estrogens in mammals as well as in fish (Kazeto et al. 2004; Martin-Skilton et al. 2006; Min et al. 2003).

The present study suggests that BPA, BPA-DM, nNP, and nOP have the potential for indirect AhR-mediated actions on xenobiotic metabolism, steroid synthesis, and metabolism. This is the first report, to our knowledge, showing the ability of BPA-DM
and nOP to affect the transactivation of AhR. BPA and BPA-DM elicited in the 10⁻⁴ M range an antagonistic and agonistic AhR effects, respectively. We observed a clear dose-dependent AhR activation for nNP, although it was weak compared with that for TCDD. In previous in vitro studies, BPA and nNP had no effects on their own, but antagonized TCDD-induced CYP1A1 mRNA and 7-ethoxyresorufin-O-deethylase (EROD) activity levels in Hepa-1c1c7 cells via interference with AhR:DRE binding and/or transport into the nucleus and/or the action of co-transcription factors (Jeong et al. 2000, 2001). Similarly to BPA and nNP, estradiol was shown to antagonize AhR:TCDD-induced CYP1A1 expression and EROD activity, and weakly induce EROD activity via AhR not involving ER (Jeong and Lee 1998). In vitro, the endocrine disruption potential of BPA on mice embryonic development was demonstrated at very low doses (0.02 µg/kg/day) (Nishizawa et al. 2005a, 2005b).

We observed weak increased and decreased AhR activity at lower (10⁻⁸ M) and higher (10⁻³ M) nOP concentrations, respectively, and nOP also induced ER activity at ~ 10⁻⁶ M. We wonder whether this estrogenic mimic of nOP can be explained by the fact that the biofocal effects of estradiol on AhR can either induce or antagonize AhR function (Jeong and Lee 1998; Kharat and Saatcioglu 1996). That nOP elicited AhR agonism at lower concentrations and reduced AhR transactivation at higher concentrations might be attributed to inhibitory cross-talk of AhR and ER. However, the specific mechanisms involved must be further studied.

The demonstration in AhR knock-out mice that AhR is an important factor in female reproduction by regulating the expression of ovarian aromatase elucidates the physiological role of AhR and also suggests AhR as a mediator of endocrine disruption (Baba et al. 2005). Antiestrogenic actions mediated via AhR are well described, in which AhR:ligand inhibits ER binding to ERE (Pocar et al. 2005) and increases pro-apoptotic degradation of ER (Wormke et al. 2003). A further perspective of AhR as a factor in ED is given by AhR’s estrogenic potential in absence of estrogen via the agonist-activated AhR:Arnt interaction, with ERα and/or ERβ leading to transcriptional activation of ER-regulated genes (Ohtake et al. 2003). In addition, antiandrogenic actions of TCDD have pointed out two possible mechanisms: blocking of AR-induced gene expression and AhR–AR cross-talk possibly involving competition of co-agonists (Barnes-Ellerbe et al. 2004; Jana et al. 1999).

Hypothetically, AhR might play a role in regulating the cell ratio of androgen estrogens via activation of CYP19, thereby affecting male and female reproduction, affecting estrogenic actions via ERs, inducing cell metabolism via, for example, CYP1A1, and inhibiting AR functions.

In summary, our in vitro data demonstrated that the four tested chemicals have the potential to affect central endocrine pathways through their capacity to affect the function of the nuclear receptors ER, AR, AhR, and aromatase activity. BPA-related compounds and alkylphenols have been found in human fluids in 0.1–10 nM (von Saal and Hughes 2005) and 0.4–13.9 ng/mL urine (Kawaguchi et al. 2004; Kudlenyik et al. 2003), respectively. In this study, the lowest observed effect concentrations for the BPA phenols and the alkylphenols were in the 100–1,000 nM and 10–1,000 nM ranges, respectively. Although the effective concentrations in vitro for the tested compounds were ≥ 1,000 times higher than the level found in humans, their ability to act via more than one mechanism might enhance the biologic effect in the intact organism, because the final response will likely be determined as a sum of the interactions of all pathways implicated. Furthermore, because most humans are exposed to several chemicals simultaneously and some EDs have been shown to act additively in vitro and in vivo (Birkhoj et al. 2004; Nellemann et al. 2003; Rajapakse et al. 2002), potential mixture effects should also be taken into consideration in the risk assessment.

Table 2. AhR agonistic and antagonistic effects of the 4 test chemicals.

| Concentration | BPA | BPA-DM | nNP | nOP |
|---------------|-----|--------|-----|-----|
| 0.1% DMSO     | 1.03 ± 0.04 | 1.00 ± 0.05 | 0.98 ± 0.07 | 0.99 ± 0.04 |
| 60 µM TCDD    | 14.84 ± 6.26* | 14.84 ± 6.26* | 13.89 ± 7.32* | 13.89 ± 7.32* |

**Concentrations up to 10⁻⁴ M were not cytotoxic to the cells, whereas concentrations > 10⁻⁴ M elicited cytotoxicity to the Hepa1.2cR cell.** The results given refer to effects observed at concentrations eliciting no cytotoxicity (n = 3).

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