Estrogen-Like Response to p-Nonylphenol in Human First Trimester Placenta and BeWo Choriocarcinoma Cells

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p-Nonylphenol (p-NP) is a metabolite of alkylphenol ethoxylates used as surfactants in the manufacturing industry. Although it is reported to have estrogenic activity and to be transferred from the mother to the embryo, no data are available on its effects on the development of the human placenta. In the present study, we investigated estrogen receptors’ (ERs) expression in the first trimester human placenta. Using an in vitro model of choric villous explants, we then compared the effects of p-NP and 17β-estradiol (17β-E2). Finally, a trophoblast-derived choriocarcinoma cell line, BeWo, was used as a model of trophoblast cell differentiation. Our results showed that the first trimester placenta expresses three ER-α isoforms of 67, 46, and 39 kDa and one ER-β isoform of 55 kDa. Immunohistochemistry revealed the expression of ER-α in the villous cytotrophoblast, whereas ER-β was mainly expressed by the syncytiotrophoblast. Treatment of explant cultures with p-NP (10^-7M) and 17β-E2 (10^-9M) significantly increased β-hCG secretion and cell apoptosis but did not modify ER expression. After 72 h of exposure, hormone release was significantly higher in p-NP- than 17β-E2-treated explant cultures. By this time, cleavage of caspase-3 was evident in cultures treated with 17β-E2 and p-NP. In BeWo cells, a caspase-3 band of 20–16 kDa was evident after 1 h of treatment with p-NP and after 24 h of treatment with 17β-E2 or forskolin. These findings suggest that the human trophoblast may be highly responsive to p-NP and raise concern about maternal exposure in early gestation.

Key Words: p-nonylphenol; endocrine disruptors; estrogen receptors; human placenta; human choriocarcinoma; trophoblast apoptosis.

Increasing evidence suggests that exposure to certain man-made chemicals present in the environment may interfere with endocrine function in humans and wildlife (Safe, 1995; Weiss, 1998). Alkylphenol ethoxylates are a class of nonionic surfactants introduced in the 1940’s and used in detergents, paints, pesticides, personal care products, and plastics (Soto et al., 1991; White et al., 1994). The most commonly used alkylphenol ethoxylates in consumer products have nine carbon atoms with branched alkyl chains and are thus known as nonylphenol ethoxylates. These chemicals are discharged into aquatic environments with urban and industrial wastewater. Here they are broken down microbially into nonylphenol ethoxylate by-products and the final degradation intermediate nonylphenol (Rudel et al., 2003; White et al., 1994). These metabolites are also used as intermediates in the chemical industry (Müller and Schlatter, 1998).

Human exposure to p-nonylphenol (p-NP) may occur by cutaneous absorption, ingestion of contaminated food or water, and inhalation (Guenther et al., 2003; Monteiro-Riviere et al., 2000). Great concern has been expressed about this compound, after Soto et al. (1991) accidentally found that p-NP, released by polystyrene, induced progesterone receptor and cell proliferation in estrogen-dependent breast cancer cells (MCF-7). Estrogenic activity of p-NP has been then investigated in a number of in vitro and in vivo studies. By binding to estrogen receptors (ERs), p-NP may disrupt normal endocrine function, promoting reproductive failure and carcinogenesis in estrogen-sensitive tissues (Blair et al., 2000; Sonnenschein and Soto, 1998). p-NP acts directly via the ERs, displacing 17β-estradiol (17β-E2) from human cell line ERs (Soto et al., 1995; White et al., 1994) and specifically inhibiting binding of 17β-E2 to ERs (Kwack et al., 2002).

Interestingly, in vivo studies have shown that maternal exposure to p-NP resulted in an increase in uterine calbindin-D 9k (CaBP-9k) expression in maternal and neonatal rat uteri (Hong et al., 2004; Kwack et al., 2002). These and other studies suggest that p-NP can cross the placenta and cause reproductive and developmental toxicity. Reports on several endocrine disruptor chemicals (bromodichloromethane and organochlorine compounds) describe the effect of these substances on human trophoblast (Chen et al., 2003, 2004; Hamel et al., 2003), while no functional studies regarding the effects of p-NP on the placenta have yet been reported.

Previous studies show that the placenta is an estrogen-target tissue and that estrogens have important physiological roles in...
regulating functional differentiation of the placental villous trophoblast (Bukovsky et al., 2003a; Cronier et al., 1999; Rama et al., 2004). Expression of ER-α and -β protein was recently demonstrated in the human placenta at term (Bukovsky et al., 2003b). Expression of ER-α, -β, and -γ mRNAs was also recently reported to increase in the placenta throughout gestation (Fujimoto et al., 2005).

The aim of the present study was as follows: (1) to investigate protein expression of ERs in first trimester human placentas, (2) to compare the effects of p-NP and 17β-E2 on trophoblast differentiation-apoptosis by an in vitro model of chorionic villous explants, and (3) to verify the role of p-NP in a trophoblast-derived choriocarcinoma cell line, BeWo.

We demonstrate that first trimester placenta, like the placenta at term, expresses ER proteins α and β and that p-NP exerts estrogen-like activity on trophoblast, inducing hCG secretion and caspase-3 activity, two main markers of trophoblast differentiation and apoptosis (Straszewski-Chavez et al., 2005). Unexpectedly, at equimolar concentrations of 10⁻⁸M, the effect of p-NP was significantly greater and longer lasting than that of 17β-E2.

**MATERIALS AND METHODS**

**Tissue collection.** First trimester placentas (n = 25) were obtained after elective terminations of pregnancy at weeks 7–12 of gestation, with the consent of patients and approval of the hospital Ethics Committee (Siena, May 2004). A portion of each was immediately snap frozen and stored at −80°C for protein analysis; another portion was fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis. The rest was rinsed in cold phosphate-buffered saline (PBS) to remove excess blood and processed for explant cultures within 2 h. Human term placental tissues (n = 5) were snap frozen immediately after collection and stored at −80°C for western blot analysis.

**Isolation and treatment of chorionic villous explants.** Chorionic villous explant cultures were established from 7–9 week human placentas. Villous explants were dissected as described by Caniggia et al. (1997). Briefly, small fragments of villous tips (15–20 mg wet weight) were placed on Millicell CM culture dish inserts (Millipore Corp, Bedford, MA), previously coated with 180 μl undiluted matrigel (Collaborative Research, Inc., Bedford, MA), and inserted in 24-well plates. Explants were cultured in serum-free Dulbecco’s modified Eagle’s medium/F12 without phenol red (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma Chemical Co., St Louis, MO). Explant cultures were incubated overnight at 37°C in 5% CO₂ for attachment to the matrigel. The next day, the culture medium was replaced with medium supplemented with 17β-E2 (10⁻⁸M) (Sigma Chemical Co.) or p-NP (10⁻⁸M), both dissolved in 0.1% ethanol or with medium plus ethanol (controls). This concentration was selected far below the levels found in human samples (Inoue et al., 2004; Tan and Nohd, 2003).

At predetermined incubation times (4, 24, 48, and 72 h), explants were removed from matrigel and washed in PBS. Part of each was frozen and stored at −80°C until processing for western blot analysis for ERs and caspase-3 expression. Culture medium was stored at −80°C until assay for β-hCG.

**BeWo cell culture and treatment.** BeWo cells (Istituto Zooprofilattico Sperimentale, Brescia, Italy) were cultured in Ham’s F-10 without phenol red (Sigma Chemical Co.) supplemented with 10% FBS (Biochrom, Berlin, Germany), 100 U/ml penicillin/streptomycin, and 2 mM glutamine (Sigma Chemical Co.) in 75-cm² flasks (Becton Dickinson Labware, Franklin Lakes, NJ) in a humidified atmosphere of 20% air and 5% CO₂ at 37°C until 70–80% confluence. After overnight storage in serum-free conditions, BeWo cells were exposed to 17β-E2 (10⁻⁸M) or p-NP (10⁻⁸M). Cell differentiation was induced by addition of 10 μM forskolin (Sigma Chemical Co.). Control cells were cultured in serum-free medium containing vehicle (0.1% ethanol). Cells were harvested at 1, 6, and 24 h and processed for western blot analysis of caspase-3 expression.

**Western blot.** Placental tissue, villous explants, and BeWo cells were homogenized at predetermined times of incubation in ice-cold lysing buffer (Tris-HCl 50 mM, NaCl 50 mM, Triton X-100 1%, sodium deoxycholate 1%, and SDS 0.1%) containing sodium orthovanadate 100 mM and a protease inhibitor cocktail containing 4-(2-aminoethyl benzene)sulfonflouride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma Chemical Co.). Protein lysates were clarified by centrifuging at 13,000 × g for 15 min at 4°C. Protein concentration was determined by Quick Start Bradford Protein Assay (BioRad Laboratories, Hercules, CA). Proteins (50 μg for placenta tissue, 75 μg for villous explants, and 100 μg for BeWo cells) were separated on 10 and 12% polyacrylamide gel for ERs (ER-α and ER-β) and caspase-3, respectively, in the presence of SDS and β-mercaptoethanol. After electrophoresis, the gel was removed and equilibrated in transfer buffer (20 mM Tris, 190 mM glycine, and 20% [v/v] methanol pH 8.3) for 5 min at room temperature. Proteins were transferred to nitrocellulose filters (Hybond-C, Amersham International, Little Chalfont, UK) for 1.5 h. The blot was incubated in blocking solution (5% [w/v] powdered milk in 10 mM PBS, 0.1% Tween 20) for 1 h and exposed to rabbit anti-human ER-α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000, rabbit anti-human ER-β antibody (Affinity Bioreagent, Golden, CO) diluted 1:1000, and goat anti-human caspase-3 antibody (R&D Systems, Abingdon, UK) diluted 1:1000 with overnight incubation at 4°C. The nitrocellulose filter was washed three times with PBS (0.1% Tween 20 in PBS 10 mM) and exposed for 1 h to the swine anti-rabbit antibody for ERs (dilution 1:3000) or rabbit anti-goat antibody for caspase-3 (dilution 1:1000), respectively, labeled with peroxidase (BioRad Laboratories) at room temperature. The blot was washed three times with PBST and visualized with a chemiluminescence kit (Immuno-Star Chemiluminescent Kit) (BioRad Laboratories) according to the manufacturer’s instructions. Equal loading of the proteins was confirmed by staining the blots with a 10% (v/v) Ponceau S solution (2% Ponceau S in 30% trichloroacetic acid/50% sulfosalicylic acid, Sigma Chemical Co.).

**Immunohistochemistry.** Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded first trimester placental tissue. Tissue sections (4 μm) were processed by the streptavidin-biotin method with specific antibodies for ER-α and ER-β: rabbit anti-human ER-α (Santa Cruz Biotechnology) and rabbit anti-human ER-β (Affinity Bioreagent) diluted 1:200 and 1:20, respectively. Sections were dewaxed, rehydrated, and washed in TBS (Tris buffer saline, 20 mM Tris-HCl, and 150 mM NaCl pH 7.6). Antigen retrieval was carried out by incubating the sections in sodium citrate buffer (10 mM pH 6.0) in a microwave oven at 750 W for 15 min. Slides were preincubated with normal swine serum (Dako, Copenhagen, Denmark) and incubated overnight at 4°C with the anti-ER-α and -ER-β primary antibodies. After washing with TBS for 5 min, the slides were incubated with a swine anti-rabbit antibody (Dako) labeled with biotin (dilution 1:500). The reaction was revealed using streptavidin-biotin complex (Dako). Sections were not counterstained. Slides were mounted with aqueous mounting (Merck, Darmstadt, Germany) and examined by light microscope. For each stain, a negative control was carried out, replacing the specific antibody with nonimmune serum immunoglobulins at the same concentration as the primary antibody.

**β-hCG assay.** The concentration of β-hCG in the explant culture medium was assessed by a commercial immunoenzymometric assay (Radim SpA, Pomezia, Italy) and expressed in relation to protein concentration, determined from explant lysates by Quick Start Bradford Protein Assay (BioRad Laboratories) at 4, 24, 48, and 72 h of incubation.
Statistical analysis. Values are means ± SEM. Data were compared by ANOVA followed by Fisher’s least significant difference post hoc test as appropriate. All p values ≤ 0.05 were considered statistically significant.

RESULTS

Expression of ERs in Human Placental Tissue

Western blot analysis performed in first trimester (7–12 weeks) and term placental tissues revealed expression of ER-α corresponding to the three known isoforms of 67, 46, and 39 kDa (Denger et al., 2001) (Fig. 1A). Analysis using antibody against ER-β showed one band of 55 kDa (Fig. 1B). Immunohistochemistry in first trimester placenta sections showed strong expression of ER-α in most chorionic villi, predominantly in the villous cytotrophoblast layer (Fig. 2A). Weak staining for ER-α was also observed in some stromal cells of the villous core while no staining was observed in the syncytiotrophoblast (Fig. 2A). ER-β was localized in the syncytiotrophoblast layer and in only few cells of the cytotrophoblast; no staining was observed in the stromal region (Fig. 2B).

Effects of p-NP and 17β-E2 on First Trimester Villous Explant Cultures

ER expression. Treatment of first trimester villous explant cultures with p-NP or 17β-E2 did not produce significant differences compared to untreated cultures (controls) in the expression of ERs at 24, 48, and 72 h of incubation (Fig. 3A). Western blot analysis showed expression of three bands for ER-α at 67, 46, and 39 kDa and a single band of 55 kDa for ER-β (Fig. 3B).

β-hCG concentration. β-hCG concentrations increased throughout incubation in treated (17β-E2 or p-NP) and untreated cultures (controls) (Fig. 4). A significant increase in β-hCG production with respect to control cultures was observed at 48 h in 17β-E2– (p ≤ 0.05) and in p-NP (p ≤ 0.05)–treated cultures. Although a reduced effect for 17β-E2 was observed at 72 h, a massive response was observed in p-NP–treated cultures at the same time (p ≤ 0.001, p-NP–treated vs. control cultures and p ≤ 0.05, p-NP–treated vs. 17β-E2–treated cultures).

Trophoblast apoptosis. The increased levels of β-hCG associated with p-NP treatment suggested differentiation of cytotrophoblast into syncytiotrophoblast with an increase in apoptosis (Huppertz et al., 2002). As cleaved caspase-3 expression has been documented as a marker of trophoblast apoptosis, we assessed its expression by western blot analysis in explants treated with p-NP or 17β-E2 or medium alone (control cultures) after 24, 48, and 72 h of incubation. The three bands of the cleaved form of caspase-3 (20, 18, and 16 kDa) were detected in p-NP– and 17β-E2–treated explant cultures after 72 h of incubation (Fig. 5). None of these bands were detected in control cultures (Fig. 5).

Apoptosis in BeWo Cells

The cleaved forms of caspase-3 were found in p-NP–treated cultures after 1 h of treatment, whereas the caspase pathway only started to be activated in forskolin and 17β-E2 cultures after 24 h of incubation (Fig. 6). A sharp decrease in the procaspase form was also observed in p-NP–treated cultures after 6 h. This 32-kDa band disappeared at 24 h, suggesting complete cleavage of the protein (Fig. 6).

DISCUSSION

Placental development is a key event for the success of pregnancy (Cross et al., 1994). Abnormalities in this event may lead to embryo developmental defects and death (Chaddha et al., 2004). It is therefore important to identify factors that may interfere with the processes characterizing the first phases of pregnancy.

In our study, we investigated whether p-NP, a metabolite of alkylphenol ethoxylates, may interfere with placental development. We demonstrated that the first trimester human placenta...
is an estrogen-target tissue expressing ER-α and ER-β proteins. Using an in vitro model of chorionic villous explants, we then compared the effects of p-NP and 17β-E2 on first trimester human trophoblast. Unlike cultures of isolated cells, this model has the advantage of preserving the topology of chorionic villi and maintaining the paracrine relations between the different cell components, i.e., cytotrophoblast and syncytiotrophoblast, trophoblast, and stromal cells (Genbacev et al., 1992). This is why it appears to be a good model for studying substances interfering with the homeostasis of placentation (Miller et al., 2005). Using explant cultures from first trimester placentas, we demonstrated that p-NP mimics the effects of estrogens, increasing hCG secretion and cell apoptosis. Surprisingly, at equimolar concentrations of 10^{-9} M, the effect of p-NP on hCG secretion was significantly greater than that of 17β-E2. Moreover, hCG secretion induced by p-NP increased up to 72 h, whereas that induced by 17β-E2 peaked at 48 h and was much lower by 72 h. Evidence of cell apoptosis, shown by caspase-3 fragmentation, was detected at 72 h in 17β-E2– and p-NP–treated tissues. Since the trophoblast is not the only cell component in villous explant cultures, we used a trophoblast-derived choriocarcinoma cell line, BeWo, to verify the effects of p-NP on cell differentiation, syncytialization, and apoptosis. Previous reports showed that 17β-E2 treatment of BeWo cells...
E2– and incubation. Note that caspase-3 fragments of about 20–16 kDa were obtained in E2, E2 or kDa was drastically reduced at 6 h in shows equal protein loading.

induced differentiation by formation of multinucleate syncytial structures (Rama et al., 2004). In this study, we demonstrated that like 17β-E2 and forskolin, an inducer of cell differentiation, p-NP induces apoptosis in BeWo cells by cleavage of caspase-3. This effect was observed after 1 h of treatment with p-NP but not until 24 h of treatment with 17β-E2 or forskolin. The apoptotic effect of p-NP by the caspase-3 pathway was recently demonstrated by Kudo et al. (2004) in neural stem cells.

Taken together, the present findings indicate that p-NP exerts estrogen-like effects on first trimester placentas, affecting trophoblast differentiation and apoptosis. It is noteworthy that p-NP activity was greater and longer lasting than that of 17β-E2, suggesting that metabolism of p-NP in the trophoblast produces more stable and possibly more active metabolites.

Reports on in vitro systems, e.g., recombinant yeast (Gaido et al., 1997), primary trout hepatocytes (Flouriot et al., 1995), MCF-7 cell line (Blom et al., 1998; Nagel et al., 1997), and transfected avian cells (White et al., 1994), have shown that p-NP is about 1000–10,000 times less potent than 17β-E2. These studies also showed that the lowest effective concentrations of p-NP ranged from 100nM to 1 μM. Our findings show that p-NP is more potent than 17β-E2 at 10⁻⁹M, a concentration 100–1000 times less than that reported in other in vitro systems and at least 1000 times lower than the levels found in humans (Inoue et al., 2000; Kawaguchi et al., 2004; Tan and Nohd, 2003). In fact, concentration of 10⁻⁹M used in the present study is about 220.36 ng/l, while the levels of p-NP detected in human samples vary from 0.3 to 221.7 ng/ml in plasma and blood samples (Inoue et al., 2000; Kawaguchi et al., 2004) and 15.17 ng/ml in human cord blood samples (Tan and Nohd, 2003), suggesting that the trophoblast is extremely sensitive to this chemical.

In a recent paper, Bukovsky et al. (2003b) showed that cell nuclei distant from the syncytiotrophoblast exhibited many ER-α, while cell nuclei associated with the syncytiotrophoblast showed fewer ER-α and some ER-β. The same authors also showed that estrogens may play a role, via ER-α, in the stimulation of cytotoxic trophoblast differentiation into syncytiotrophoblast (Bukovsky et al., 2003a). In line with Bukovsky et al. (2003b), we showed here that expression of ER-α is mainly localized in the cytotrophoblast whereas that of ER-β is predominantly distributed in the syncytiotrophoblast of the chorionic villi. Further studies are necessary to investigate the exact role of each isoform and the mechanism of action of estrogen-like compounds.

Differentiation of trophoblast into syncytiotrophoblast is a physiological event in placental development: the internal cytotrophoblast cells aggregate and their plasma membranes fuse (Potgens et al., 2002). The syncytiotrophoblast, the external layer of villi, is an endocrine tissue producing hCG and placentl lactogen. It also exchanges nutrients and gases between the mother and fetus. The physiological turnover of placental epithelium produces syncyial knots which are shed apotopically into the maternal bloodstream (Huppertz et al., 2002; Straszewski-Chavez et al., 2005). Abnormal apoptosis, leading to faulty placentaion, is involved in several gestational disorders including preeclampsia (Allaire et al., 2000; Crocker et al., 2004).

Our finding of increased hCG secretion and cell apoptosis after exposure to the endocrine disruptor p-NP suggest that maternal exposure to this chemical may lead to aberrant or adaptive placental cell turnover in the uterus. This could cause early termination of pregnancy, gestational pathologies, and fetal growth defects.

In conclusion, our data show that p-NP induces an estrogen-like response in first trimester human placenta by increasing trophoblast differentiation and apoptosis. The power of p-NP on early placenta even at low concentrations raises considerable concern about the implications of exposure to this chemical for the fetus and pregnancy.

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