BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 117–120 (1997)
ARTICLE NO. RC976053

Benzo(*a*)pyrene, but Not 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, Alters Cell Proliferation and c-Myc and Growth Factor Expression in Human Placental Choriocarcinoma JEG-3 Cells

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Received December 10, 1996

This study compared the effects of benzo(*a*)pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), two aryl hydrocarbon receptor (AhR) agonists, on proliferation and gene expression in the human placental choriocarcinoma JEG-3 cell line. BaP significantly inhibited [^{3}H]thymidine incorporation, whereas no effect of TCDD was observed over a 7 day period. TCDD and BaP both showed induction of cytochrome P450 1A1 (CYP1A1), whereas only BaP caused a significant loss of EGFRs. Exposure to 10 μM BaP significantly increased the steady state mRNA level of transforming growth factor (TGF)-β1, while the c-myc mRNA levels were decreased by 61%. In contrast, TCDD showed no changes in mRNA levels for TGF-β1 and c-myc. Thus, although both compounds induce CYP1A1, only BaP inhibits cell proliferation which is correlated with disruption of expression of significant regulators of trophoblast growth.

Reproductive and developmental toxicity has been a focus of recent studies on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. TCDD-like compounds have been implicated to be environmental endocrine disruptors and growth disregulators by altering gene expression through the aryl hydrocabon receptor (AhR) (1). The induction of cytochrome P450 1A1 (CYP1A1) is one of the most sensitive biomarkers of exposure to AhR agonists (1). This laboratory previously reported that benzo(*a*)pyrene (BaP), a major toxicant in cigarette smoke (2), induced CYP1A1 expression in association with a decrease in epidermal growth factor receptors (EGFRs) in human placental choriocarcinoma cells (3). In this regard, exposure to cigarette smoke or polychlorinated biphenyls/dibenzofurans (PCB/PCDF) during pregnancy has been found to be associated with decreased placental EGFRs and low birth weight in infants (4, 5). Altered placental function may be among the most sensitive end points for the assessment of developmental risk from human exposure to TCDD-like compounds, and may underlie a number of the developmental toxicities observed following *in utero* exposure. Little is known, however, regarding the toxicity of TCDD-like compounds in the human placenta.

The present study was undertaken to investigate whether TCDD and BaP altered cell proliferation and expression of EGFR, transforming growth factor (TGF)-β1 and c-myc genes in the human trophoblastic JEG-3 cell line. Coordinated expression of these genes is recognized to be important for the regulation of trophoblast proliferation, differentiation and invasiveness (6, 7). The results indicate that BaP, but not TCDD, exposure is associated with altered cell proliferation and disregulation of EGFR, TGF-β1 and c-myc gene expression, changes which are significant potential mechanisms of fetoplacental toxicity.

MATERIALS AND METHODS

Materials. BaP was obtained from the Sigma Chemical Co. (St. Louis, MO), and TCDD from Midwest Research Institute (Kansas City, MO) through the National Cancer Institute Chemical Carcinogen Reference Repository. Plasmids containing cDNA for TGF-β1 (phTGFB-2), c-myc (pG1-5′-c-myc), *CYP1A1* (phP1-450-3′) and β-actin (no. 65128) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). [α^{32}P]dCTP was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

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Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 1A1; BaP, benzo(*a*)pyrene; EGFR, epidermal growth factor receptor; PCB/PCDF, polychlorinated biphenyls/dibenzofurans; TGF, transforming growth factor.

0006-291X/97 $25.00

Cell culture. The human placental trophoblastic choriocarcinoma cell line JEG-3 (ATCC HTB36) was obtained from ATCC and cultured in minimum essential medium supplemented with 10% FBS, as previously described (3). All experiments were initiated when cells were at approximately 40-60% confluence. Cells were treated with BaP or TCDD in 0.1% DMSO or vehicle alone (0.1% DMSO).

[^{3}H]Thymidine incorporation assay. Cells were cultured at 5.0 × 10^4 cells/well in 24-well plates for 20 hr and treated with 10 nM TCDD and 10 μM BaP for 1, 3, 5 and 7 days. Medium was changed every 24 to 48 hr. Cells were transferred to serum-free medium 20 hr before the addition of tritiated thymidine and pulsed with 1 μCi/ml [^{3}H]-thymidine for the last 3 hr. After trypsinization, cells were harvested onto glass fiber filter strips with a cell harvester, and incorporated radioactivity was determined by liquid scintillation counting.

Western analysis. The immunoreactive proteins were analyzed as previously described (3). Briefly, 100 μg protein of the whole cell lysate in 1 × SDS-sample buffer was electrophoresed, transferred, and incubated sequentially with sheep anti-human EGFR (Upstate Biotechnology, Inc., Lake Placid, NY) or goat anti-rat CYP1A1 (Gentest, Woburn, MA), followed by peroxidase conjugated anti-sheep IgG or anti-goat IgG. The immunoreactive bands were visualized by incubation with 3-amino-9-ethylcarbazole, and quantitated by densitometry and NIH Image software.

Northern analysis. Poly (A)$^+$ RNA was prepared by using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Poly (A)$^+$ RNA (10 μg) was denatured, fractionated in 1% agarose formaldehyde gel and transferred to positively charged nylon membrane (Magna Charge, Micron Separations Inc., Westboro, MA). The probes used were a *CYP1A1* 1.0 kb, TGF-β1 2.1 kb and β-actin 1.0 kb EcoRI fragment, and a c-myc 1.6 kb SstI fragment, respectively. The DNA fragments were labeled with [α^{32}P]dCTP using a random primer labeling kit (Stratagene, La Jolla, CA). Hybridization to radiolabled probes was carried out for 2 to 4 hr at 68°C in ExpressHyb hybridization solution (CLONTECH Laboratories, Inc., Palo Alto, CA). The presence of specific RNA was detected by autoradiography, after which filters were stripped and reprobed sequentially with each of the respective DNA probes. Hybridization signals were quantitated by densitometry and NIH Image software, with each message standardized to the β-actin transcript.

Data analysis. Student's *t* test was used to assses the effect of BaP and TCDD treatment, using the Macintosh StatView512^{+TM} program. Experiments were performed using triplicate cultures at each concentration of BaP and TCDD or time point.

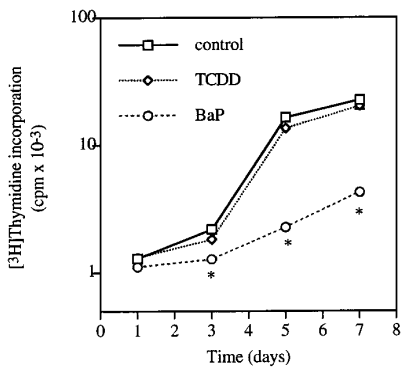

FIG. 1. Time-course of the effects of TCDD and BaP on JEG-3 cell proliferation. Cells were subcultured at 5.0 × 10^4 cells/well in 24-well plates for 20 hr, and treated with 10 nM TCDD, 10 μM BaP or 0.1% DMSO in the presence of serum. [^{3}H]Thymidine incorporation was determined as described in Methods. Cpm values are the mean ± SE of six replicate cultures from separate experiments. The SEs were too small to be shown. * $p < 0.05$ as compared with control.

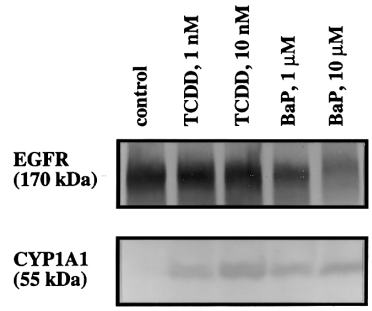

FIG. 2. Western immunoblot analysis of the effects of TCDD and BaP on EGFR and CYP1A1. Cells were exposed to TCDD or BaP for 48 hr. Total cellular protein, 100 μg, was electrophoresed, transferred, and immunostained with anti-EGFR or anti-CYP1A1 as described in Methods.

RESULTS

Effects of TCDD and BaP on JEG-3 cell proliferation. In cells cultured in the presence of serum, exposure to BaP produced a time-dependent inhibition of [^{3}H]-thymidine incorporation over a 7 day period (Fig. 1). The [^{3}H] incorporation was significantly decreased by 42, 86 and 81% at 3, 5 and 7 days, respectively, following 10 μM BaP treatment. In contrast, no alteration in [^{3}H]thymidine incorporation was observed following exposure to 10 nM TCDD for 1 to 7 days. Similar results were obtained in parallel experiments in which cell number was quantitated (data not shown).

Effects of TCDD and BaP on CYP1A1 and EGFR proteins. Both TCDD (1 and 10 nM) and BaP (1 and 10 μM) markedly induced expression of CYP1A1 protein. The level of total cellular EGFR protein was unaltered following exposure to 1 and 10 nM TCDD for 48 hr (Fig. 2), nor were changes observed up to 96 hr following exposure to 10 nM TCDD. In contrast, BaP treatment resulted in a significant concentration-related decrease in the level of EGFRs, being reduced by 40 and 60% at 1 and 10 μM BaP, respectively, as previously described (3). Similar results were obtained in parallel experiments which measured [^{125}I]-EGF binding to whole cells (data not shown). Thus, in contrast to BaP, TCDD-mediated induction of CYP1A1 was not associated with a loss of EGFRs.

Effects of TCDD and BaP on the steady-state mRNA levels for TGF-β1 and c-myc. Northern blot analysis was used to examine the effects of TCDD and BaP on the steady state mRNA levels for TGF-β1 and c-myc. Induction of CYP1A1 by the AhR ligands was used as a positive control. Exposure to 10 nM TCDD for 6 to 120 hr (5 days) had no effect on the steady state mRNA levels for TGF-β1 and c-myc (Fig. 3). In contrast, BaP treatment resulted in a time-dependent change in the steady state mRNA levels for TGF-β1 and c-myc. The TGF-β1 mRNA level was significantly increased at 24

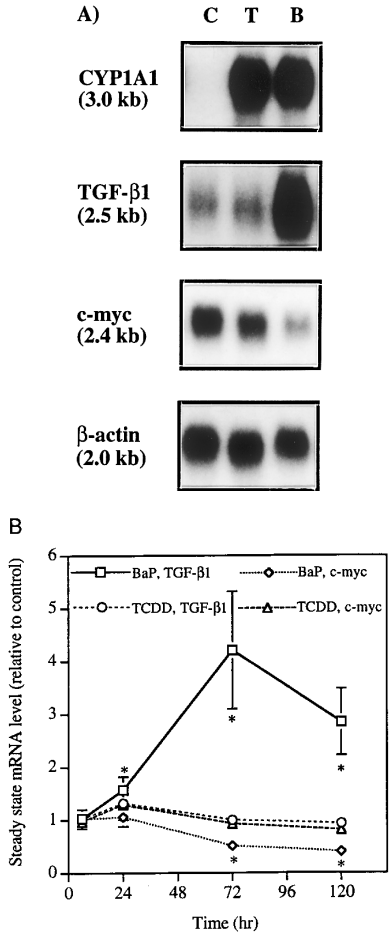

FIG. 3. Effects of TCDD and BaP on the steady state mRNA levels for TGF-β1 and c-myc. Cells were treated with 0.1% DMSO (C), 10 nM TCDD (T), or 10 μM BaP (B). Poly $(A)^+$ RNA was extracted for Northern analysis. A) Representative autoradiograms of the Northern blot following 72 hr treatment; B) Quantitation of the TGF-β1 and c-myc mRNA, with the ratio of each message to β-actin in the control cells being set as 1. Values are the mean $\pm$ SE of three experiments. The points without the SE bars indicate that the individual SEs were too small to be shown. * $p < 0.05$ as compared with control.

hr after the addition of BaP (Fig. 3B), and the level at 72 hr was increased 4-fold over control and remained significantly elevated for 5 days. In contrast, the c-myc mRNA level was not changed at 6 and 24 hr, but was significantly decreased by 50% at 72 hr, and further depressed by 60% at 5 days in cultures treated with 10 μM BaP. In data not shown, the steady state level of EGFR mRNA was not significantly changed by BaP or TCDD, as we have previously reported for BaP (3).

DISCUSSION

This study demonstrates that major differential effects were observed on gene expression in JEG-3 cells with two AhR agonists, BaP which is a polycyclic hydrocarbon toxicant in cigarette smoke, and TCDD which is a reference compound for halogenated aromatics. The present finding that TCDD had no effect on EGFRs in the presence of the induction of CYP1A1 does not support a direct relationship between CYP1A1 induction and EGFR downregulation in JEG-3 cells. This result was unexpected in that TCDD has been found to downregulate EGFR in non-placental cell lines and tissues, including keratinocytes, hepatoma cells, uterus and liver (1). Moreover, the role of the AhR in mediating EGFR downregulation has been supported by structure-activity studies and experiments with Ah-responsive and -nonresponsive congenic mice (1). Our results, however, demonstrate that the induction of CYP1A1 by TCDD is apparently not sufficient to cause the downregulation of the EGFR in JEG-3 cells, since both TCDD and BaP bind to the AhR and induce CYP1A1, but only BaP alters EGFR protein level.

Trophoblast proliferation has been shown to be negatively regulated by TGF-β1 and positively correlated with the level of c-myc and EGFR (6-8). The present study observed inhibition of JEG-3 cell proliferation following BaP treatment which may be mediated by the upregulation of TGF-β1 in association with the downregulation of EGFR and c-myc. During early pregnancy, highly proliferative and invasive trophoblasts are needed to produce the basic structure of the human placenta (7, 8), and the inhibition of trophoblastic cell proliferation by BaP may interfere with normal placental development. TCDD has been shown to inhibit MCF-7 human breast cancer cell proliferation (9), as well as to stimulate proliferation in the mouse embryonic palate and ureter epithelial cells (10, 11). In the present study, no alteration in cell proliferation was observed following TCDD exposure in BeWo cells. Thus modulation of cell proliferation by TCDD appears to be tissue-specific.

The BaP-mediated alterations in TGF-β1 and c-myc gene expression may underlie mechanisms by which xenobiotics such as those found in cigarette smoke lead to fetal intrauterine growth retardation. Studies with human trophoblasts have shown that TGF-β1 upregulates tissue inhibitor of metalloproteinases and extracellular matrix proteins such as oncofetal fibronectin, as well as downregulates the activity of urokinse type-plasminogen activator and collagenase type IV (12-14). Interestingly, c-Myc has been found to repress collagen gene expression (15). Therefore, BaP-mediated upmodulation of TGF-β1 and downmodulation of c-myc may lead to accumulation of extracellular matrix such as collagen in human placenta. In this regard, placentas from women who smoke cigarettes have been shown to exhibit thickening of the basement membrane and increased collagen content of the villous stroma (16). In mice c-myc mutant embryos are small and retarded in development compared with their littermates (17), providing direct evidence that c-myc is necessary for normal embryonic development.

In contrast, this study has shown that TCDD had no effect on the level of TGF-β1 and c-myc mRNA, which is in agreement with the observations in non-placental cell lines (18-20). The observed dissociation between TCDD and BaP effects suggests that the BaP-mediated alterations in cell proliferation and growth factor gene expression may be a consequence of metabolism of BaP to reactive metabolites, which does not occur with TCDD due to its resistance to metabolism. The potential for toxicity caused by BaP metabolites may be biologically important because the inducibility of CYP1A1 has been demonstrated in human placenta as early as the first trimester of pregnancy (21). Evidence has shown that placental CYP1A1 activity was induced by maternal cigarette smoking and PCB/PCDF exposure, and the induced activity was able to activate BaP and related polycyclic hydrocarbons into reactive metabolites (22).

In summary, although both TCDD and BaP induced CYP1A1 mRNA and protein expression, only BaP inhibited JEG-3 cell proliferation, which was correlated with disruption of expression of EGFR, TGF-β1 and c-myc in this cell line.

ACKNOWLEDGMENTS

Portions of this work were presented at the annual meeting of the Society of Toxicology (Baltimore, MD, March 1995 and Anaheim, CA, March 1996). This work was supported by NIH Grant NIEHS 07375.

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