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7,12-Dimethylbenzanthracene induces apoptosis in RL95-2 human endometrial cancer cells: Ligand-selective activation of cytochrome P450 1B1

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ABSTRACT

7,12-Dimethylbenzanthracene (DMBA), a polycyclic aromatic hydrocarbon, exhibits mutagenic, carcinogenic, immunosuppressive, and apoptogenic properties in various cell types. To achieve these functions effectively, DMBA is modified to its active form by cytochrome P450 1 (CYP1). Exposure to DMBA causes cytotoxicity-mediated apoptosis in bone marrow B cells and ovarian cells. Although uterine endometrium constitutively expresses CYP1A1 and CYP1B1, their apoptotic role after exposure to DMBA remains to be elucidated. Therefore, we chose RL95-2 endometrial cancer cells as a model system for studying DMBA-induced cytotoxicity and cell death and hypothesized that exposure to DMBA causes apoptosis in this cell type following CYP1A1 and/or CYP1B1 activation. We showed that DMBA-induced apoptosis in RL95-2 cells is associated with activation of caspases. In addition, mitochondrial changes, including decrease in mitochondrial potential and release of mitochondrial cytochrome *c* into the cytosol, support the hypothesis that a mitochondrial pathway is involved in DMBA-induced apoptosis. Exposure to DMBA upregulated the expression of AhR, Arnt, CYP1A1, and CYP1B1 significantly; this may be necessary for the conversion of DMBA to DMBA-3,4-diol-1,2-epoxide (DMBA-DE). Although both CYP1A1 and CYP1B1 were significantly upregulated by DMBA, only CYP1B1 exhibited activity. Moreover, knockdown of CYP1B1 abolished DMBA-induced apoptosis in RL95-2 cells. Our data show that RL95-2 cells are susceptible to apoptosis by exposure to DMBA and that CYP1B1 plays a pivotal role in DMBA-induced apoptosis in this system.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and toxic environmental contaminants that are produced during incomplete combustion of organic materials. Major sources of human exposure are cigarette smoke, charbroiled food, and automobile exhaust. Among PAHs, 7,12-dimethylbenzanthracene (DMBA) is one of the most potent of these mutagenic, teratogenic, and carcinogenic compounds; it is associated with the induction of tumors in breast tissue and skin (Cavalieri et al., 1991; Chidambaram and Baradarajan, 1996; Detmar et al., 2006). It is probably the best-studied PAH, along with benzo[a]pyrene (B[a]P). In addition, DMBA is immunosuppressive and has been shown to cause a severe loss of bone marrow cellularity (Ward et al., 1984). It has been suggested that DMBA-induced bone marrow cytotoxicity is a consequence of apoptosis in developing leukocytes (Hardin et al., 1992; Yamaguchi et al., 1997).

DMBA, like other PAHs, requires transformation by cytochrome P450 (CYP) and microsomal epoxide hydrolase (mEH) in order to express its carcinogenicity; its 3,4-diol derivative is thought to be a procarcinogen that is further modified by CYP to produce the ultimate carcinogen, 1,2-epoxide-3,4-diol-DMBA (DMBA-DE), which binds to DNA (Cheng et al., 1988a). Thus, induction of CYP gene expression, specifically of the CYP1 family, is a prerequisite for the transformation of PAHs, which is also modulated by the aryl hydrocarbon receptor (AhR), and the AhR nuclear translocator (Arnt) (Alexander et al., 1997; Kleiner et al., 2004). The CYP1 family includes CYP1A1,

Abbreviations: α-NF, α-Naphthoflavone; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; B[a]P, benzo[a]pyrene; CHO, cyclohexene oxide; CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1; $DiOC_3(6)$, 3′,3′-dipropyloxacarbocyanine; DMBA, 7,12-dimethylbenzanthracene; DMBA-DE, DMBA-3,4-diol-1,2-epoxide; EH, epoxide hydrolase; Ellipticine, 5,11-Dimethyl-6H-pyrido[4,3-b]carbazole; EROD, ethoxyresorufin O-deethylase; PAHs, polycyclic aromatic hydrocarbons; TMS, 2,3′,4,5′-tetramethoxystilbene; XREs, xenobiotic-response elements; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone.

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CYP1A2, and CYP1B1 and is involved in the oxidation of PAHs (Larsen et al., 1998; Heidel et al., 1998). The involvement of CYP1A1 and CYP1B1 has been identified in PAH-induced immunotoxicity and carcinogenicity, as well as cytotoxicity (Heidel et al., 2000; Kleiner et al., 2002; Buters et al., 2003). However, their role in DMBA processing has been observed mostly in lymphocytes (Galván et al., 2003; Mann et al., 1999).

In humans, CYP1A1 is expressed in the liver, pancreas, thymus, prostate, mammary, uterus, and small intestine, whereas CYP1B1 expression is abundant in the kidney, prostate, breast, and uterus (Shimada et al., 1996). The CYP1B1 amino acid sequence is highly conserved across human, rat, and mouse species (80% identity), and the similarity between CYP1B1 and CYP1A1 is also high (40% identity) (Walker et al., 1995). The CYP1B1 enzyme has been shown to catalyze the regioselective modification of DMBA (Pottenger and Jefcoate, 1990; Otto et al., 1992). Because CYP1B1 is expressed in various tissues and is inducible by exposure to dioxins and PAHs, it is important to elucidate the role of CYP1B1 in the oxidation and activation of environmental carcinogens. Although the cytotoxicity of DMBA has been demonstrated in many cell types and tissues such as MCF-7 breast cancer cells (Ciolino et al., 2002), MA-10 Leydig cells (Mandal et al., 2001), ovarian follicles (Tsai-Turton et al., 2007; Rajapaksa et al., 2007), and the adrenal gland (Lindhe et al., 2002), the precise cellular and biochemical mechanism(s) underlying cytotoxicity-induced apoptosis require further investigation.

DMBA-induced apoptosis has been well demonstrated in bone marrow B cells (Yamaguchi et al., 1997; Mann et al., 1999; Ryu et al., 2005) and ovarian cells (Tsai-Turton et al., 2007; Matikainen et al., 2001; Matikainen et al., 2002). Although the uterine endometrium constitutively expresses CYP1A1 and CYP1B1 (Shimada et al., 1996; Hakkola et al., 1997; Vadlamuri et al., 1998), the precise toxicological mechanisms of these enzymes following exposure to DMBA remain to be determined. Previously, we have demonstrated that B[a]P-induced apoptosis in RL95-2 endometrial cancer cells is selectively mediated by CYP1A1 (Kim et al., 2007) and that CYP1B1 expression is regulated by B[a]P. Consequently, we chose RL95-2 endometrial cancer cells as a model system for studying DMBA-induced cytotoxicity and cell death; we hypothesized that exposure to DMBA causes apoptosis in this cell type by activation of CYP1A1 or CYP1B1. Here, we demonstrate that human endometrial RL95-2 cells are susceptible to apoptosis by exposure to DMBA, that DMBA-induced apoptosis may be evoked ultimately by the metabolite(s) that is (are) converted from DMBA via CYP1B1 activation.

Materials and methods

Reagents and antibodies. 7,12-Dimethylbenzanthracene (DMBA), benzo-[a]-pyrene (B[a]P), α-naphthoflavone, dimethyl sulfoxide (DMSO), Hoechst 33258, glutaaldehyde, phenylmethyl-sulfonyl fluoride (PMSF), paraformaldehyde, propidium iodide (PI), RNase A, Hoechst33258, 3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide; thiazolyl blue (MTT), pyrene, 5,11-dimethyl-6H-pyrido[4,3-b]carbazole (ellipticine), cyclohexene oxide (CHO), 2,3′,4,5′-tetramethoxystilbene (TMS), CYP1A1 and CYP1B1 isozymes, and anti-actin, were purchased from Sigma (St. Louis, MO, USA). Anti-AhR, AIF, cytochorome *c*, Hsp90, and CYP1A1 were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-cleaved caspase-3, caspase-7, caspase-8, and caspase-9 came from Cell Signaling Tech (Beverly, MA, USA). Anti-PARP and zVAD-fmk were purchased from Calbiochem (San Diego, CA, USA). AnnexinV-FITC was from BD pharmingen (San Diego, CA, USA). Anti-CYP1B1 was obtained from Gentest (San Jose, CA). Anti-rabbit and mouse Ig-conjugated with horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and CMXRos were purchased from Invitrogen Corporation (Carlsbad, CA, USA). FITC-conjugated anti-rabbit IgG and anti-mouse IgG was from Vector Laboratory (Burlingame, CA, USA).

Cell culture. RL95-2 cells (human endometrial adenocarcinoma cell line; American Type Culture Collection, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 HAM (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1.2 g/l sodium bicarbonate supplemented with 10 μg/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated in a humidified incubator at 37 °C with 5% CO_2 and were exposed to DMBA when the confluency reached 20%.

MTT cell viability assay. Cells were seeded in 12-well plates at a density of 5×10^5 cells per well. After treatment at an appropriate time, the culture medium was removed and replaced with a medium containing 0.5 mg of MTT dissolved in PBS (pH, 7.2), After 4 h, the formed crystals were dissolved with 200 μl of DMSO. The intensity of the color in each well was measured at a wavelength of 490 nm using a microplate reader (BIOTEK EL-312e, VT, USA).

Flowcytometric cell death assay. The cells were harvested, fixed with 95% ethanol for 24 h, incubated with 0.05 mg/ml PI and 1 μg/ml RNase A at 37 °C for 30 min, and analyzed by flowcytometry, using an Epics XL and analysis software (EXPO32™; Beckman Coulter, MI, USA). The cells belonging to the sub-G1 population were considered to be apoptotic cells; the percentage of each phase of the cell cycle was determined.

Annexin V cell death assay. The cells were stained using the AnnexinV-FITC Apoptosis Detection kit (BD Biosciences, NJ, USA) according to the manufacturer's protocol. Stained cells were analyzed by flowcytometry.

Hoechst 33258 staining. The cells were stained in Hoechst 33258 (4 μg/ml) for 30 min at 37 °C, fixed for 10 min in 4% paraformaldehyde (PFA), and then observed under an Axiophot microscope (Zeiss, Germany).

Western blot analysis. Whole-cell lysates were prepared by incubating cell pellets in lysis buffer [30 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), 1 mM Na_3VO_4, 25 mM NaF, 10 mM $Na_4P_2O_7$] for 30 min on ice. After the insoluble fractions were removed by centrifugation at 20,800×*g* at 4 °C for 30 min, the supernatants were collected and protein concentration was determined with a BCA protein assay kit (Pierce Biotechnology, Woburn, MA, USA). The same amounts of proteins (~30 μg) in SDS-gel sample buffer [2% (w/v) SDS, 100 mM DTT, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 60 mM Tris–HCl, pH 6.8] were boiled at 95 °C for 10 min and resolved by 7–15% gradient SDS-PAGE. For Western blots, the gels were subsequently equilibrated in transfer buffer [26 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3], and the separated proteins were transferred to a 0.2-μm nitrocellulose membrane in a Tris/glycine transfer buffer containing 10% methanol by using a Mini Trans-Blot Cell Western blotting apparatus (Bio-Rad, Hercules, CA, USA) at 100 V for 1 h at 4 °C. Nonspecific binding sites were blocked with 5% (w/v) nonfat dry milk in TBS-T (25 mM Tris, 137 mM NaCl, 3 mM KCl, and 0.05% Tween-20). The blots were incubated for 1 h each at 37 °C in primary and appropriate HRP-conjugated secondary antibodies diluted in TBS-T according to the manufacturer's instructions. The membrane was then washed in TBS-T, followed by washing in TBS alone. The signals were detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in the LAS-3000 detector (Fujifilm, Japan). Immunoblotting for β-actin was performed in every experiment as an internal control.

Immunocytochemistry. Harvested cells were attached on the slide glass by cytospin centrifugation. The cells were fixed with 4% PFA, washed with PBS, and incubated with 0.2% Triton X-100. Then, the cells were incubated with the appropriate primary antibody in 1% bovine serum albumin at RT. For secondary antibody reaction, the cells

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