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Induction of PUMA-α and down-regulation of PUMA-β expression is associated with benzo(a)pyrene-induced apoptosis in MCF-7 cells

Marjo Tampio[a,*], Piia Markkanen[b], Katja A. Puttonen[a], Eveliina Hagelberg[a], Hannu Heikkinen[a], Kati Huhtinen[a], Jarkko Loikkanen[a], Maija-Riitta Hirvonen[b,c], Kirsi H. Vähäkangas[a]

[a] University of Kuopio, Department of Pharmacology and Toxicology, P.O. Box 1627 (Yliopistonranta 1C), FI-70211 Kuopio, Finland
[b] National Institute for Health and Welfare, Environmental Toxicology Unit, P.O. Box 95, FI-70701 Kuopio, Finland
[c] University of Kuopio, Department of Environmental Science, P.O. Box 1627, FI-70211 Kuopio, Finland

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ABSTRACT

Benzo(a)pyrene (BP) forms benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)-DNA adducts in human breast adenocarcinoma MCF-7 cells, leading to p53 protein induction and phosphorylation. Although BP-induced apoptosis in rodent cells is known, it is still unclear in human cells. Here we have analyzed the effects of BP on p53 related apoptotic proteins, cell cycle and cell death in MCF-7 cells. PUMA-protein (p53 up-regulated modulator of apoptosis) levels were changed after BP exposure so that PUMA-α protein was statistically significantly increased whereas PUMA-β protein was statistically significantly decreased. PUMA-protein levels were also investigated in ZR-75-1 cells, where PUMA-α protein was statistically significantly increased. Cytochrome *c*, which is released from mitochondria during apoptosis to form the apoptosome, was increased in cytoplasmic fraction after BP exposure in MCF-7 cells. Increased apoptosis was also seen after 48 and 72 h BP exposure (2.5 and 5 μM). In addition, BP decreased dose dependently cell viability (2.5 and 5 μM) and increased ROS formation (1 and 10 μM). Our results suggest that PUMA-α protein is involved in BP-induced cell death most likely through a p53 dependent apoptotic pathway.

1. Introduction

p53 tumor suppressor protein has a central role in cell function. It is involved in the regulation of cell cycle, DNA repair and apoptosis (Hainaut and Vähäkangas, 1997; Sengupta and Harris, 2005). In nucleus, p53 protein is a transcription factor that increases the expression of several pro-apoptotic proteins. One of these important apoptotic proteins is PUMA (p53 up-regulated modulator of apoptosis) which is essential to apoptosis induced by DNA damage (Vousden, 2005; Wang et al., 2007). PUMA gene codes for four different isoforms of the protein of which α and β, containing the BH3 domain, are the functional ones (Nakano and Vousden, 2001). Once induced, PUMA protein operates in mitochondrial membrane where it targets the same proteins as p53. PUMA binds to anti-apoptotic Bcl-2 and Bcl-$_{XL}$ proteins preventing their effects (Nakano and Vousden, 2001; Yu et al., 2001; Yee and Vousden, 2008) and it can activate pro-apoptotic Bax (Kim et al., 2006; Yee and Vousden, 2008; for review see Chipuk and Green, 2008). Bax protein is one of the Bcl-2 family molecules that have the capacity to permeabilize the outer mitochondrial membrane leading to cytochrome *c* release to cytosol (Antonsson et al., 2001; Chipuk and Green, 2008). Although PUMA protein is known to be involved in apoptosis triggered by chemical exposures, like cytostatics (Jiang et al., 2006; Middelburg et al., 2005) and methylamine (Solé et al., 2008), its role in benzo(a)pyrene (BP)-induced apoptosis is unknown. In addition, the role and function of different PUMA isoforms during apoptosis is not very clearly described in the literature.

BP, a polycyclic aromatic hydrocarbon (PAH) is a model compound in studies of carcinogenic and mutagenic effects of PAHs. The ultimate mutagenic and carcinogenic metabolite of BP is BP-7,8-dihydrodiol-9,10-epoxide (BPDE) which can bind to DNA, form BPDE-DNA adducts (Bjelogrlic et al., 1994; Rämet et al., 1995; Tapiainen et al., 1996) and may lead to programmed apoptotic cell death (Solhaug et al., 2004, 2005). In addition, reactive oxygen species (ROS) can also be formed during BP metabolism through ortho-quinone pathway. BP-induced ROS production may enhance the carcinogenicity of BP (for a review, see Xue and Warshawsky, 2005) since increased ROS levels can damage the DNA (e.g. DNA strand breaks) (Flowers et al., 1997) or induce cell proliferation (Burdick et al., 2003). DNA damage caused by BP (Rämet et al., 1995; Plíšková et al., 2005; Myllynen et al., 2007) and ROS (Chandel et al., 2000; Achanta and Huang, 2004) is associated with p53 induction. p53-Associated BP-induced apoptosis has been shown in rodent cells (Ko et al., 2004; Solhaug et al., 2004, 2005; Andrysík et al., 2006; Holme et al., 2007; Topinka et al., 2008) and in human breast carcinoma MCF-7 cells (Plíšková et al., 2005; Sadikovic and

* Corresponding author. Tel.: +358 40 355 2407; fax: +358 17 16 2424.
E-mail address: Marjo.Tampio@uku.fi (M. Tampio).

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Rodenhiser, 2006; Tampio et al., 2008). However, apoptosis related proteins in the p53 pathway in human cells after BP-induced cell damage are so far still poorly characterized.

Human MCF-7 breast cancer cells are capable of metabolizing BP to BPDE (Rämet et al., 1995) which is a prerequisite for a useful cell model in BP research. Although MCF-7 cells have a functional deletion in CASP-3 gene, it has been shown that chemicals or medicines can induce apoptosis in these cells (Jänicke et al., 1998; Plíšková et al., 2005; Sadikovic and Rodenhiser, 2006; Tampio et al., 2008). BP has been shown to cause apoptosis and loss of cell proliferation in human breast cancer cell lines (MCF-7 and T47-D) (Sadikovic and Rodenhiser, 2006) and expression of apoptosis related genes in MCF-7 cells (Hockley et al., 2007). Our earlier studies on MCF-7 breast cancer cells with a wild type p53 gene have shown that formed BPDE causes p53 protein induction (Rämet et al., 1995) and phosphorylation (Tampio et al., 2008) confirming the activation of p53. To our knowledge, there are no earlier published studies on the effect of BP on PUMA expression. Here we found that in MCF-7 cells BP induces PUMA-α and down-regulates PUMA-β expression in association with apoptotic cell death.

2. Materials and methods

2.1. Cell culture

MCF-7 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose (BioWhittaker, Belgium) and supplemented with 4 μg/ml insulin (Gibco, UK), 10 μg/ml gentamicin (Gibco, UK), 10 nM estradiol (Sigma, USA) and 9% heat inactivated foetal bovine serum (FBS, Gibco, UK) at 37 °C in a cell culture incubator with a humidified atmosphere containing 95% air and 5% CO_2. ZR-75-1 breast cancer cells were obtained to compare the apoptotic effects of BP. ZR-75-1 cells were cultured in RPMI 1640 containing 0.3 g/l L-glutamine (BioWhittaker, Belgium) and supplemented with 10 mM HEPES (BioWhittaker, Belgium), 1 mM Na-pyruvate (BioWhittaker, Belgium), 10 μg/ml gentamicin (Gibco, UK) and 9% heat inactivated foetal bovine serum (FBS, Gibco, UK) at the same atmosphere as MCF-7 cells. Cells were exposed to 1, 2.5, 5 or 10 μM BP (Sigma, dissolved in dimethyl sulfoxide, DMSO, Sigma). Control cells were exposed to 0.1% DMSO, the solvent of BP. Cells were exposed on petri dishes or 48-well plate and all the experiments with different time points and concentrations were repeated at least three times.

2.2. Preparation of total cell fraction for immunoblotting

After exposure, cells were washed with ice cold PBS and scraped from the plates in 150 μl of EMSA B buffer (20 mM HEPES pH 7.6; 20% glycerol; 500 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA pH 7.6; 0.1% NP 40) supplemented with protease inhibitors (Complete Mini Protease Inhibitor cocktail tablets, Roche Diagnostics GmbH, Mannheim, Germany; used according to manufacturer's instruction). Cell suspension was incubated on ice for 30 min and centrifuged (15 min at 13,000 × *g*, 4 °C). Supernatants were collected and protein concentrations were measured with Bradford method using protein assay reagent (Bio-Rad Laboratories). Samples were stored at −80 °C until analyzed by immunoblotting. All experiments were carried out in cell culture plates. One experiment comprised of one plate (no parallels) per exposure done in different days with newly made BP-solution. The experiments were repeated three times ($n = 3$).

2.3. Preparation of cytosolic and mitochondrial fractions for immunoblotting

After exposure, the medium was transferred from the plates to 15 ml tubes. Cells were washed with PBS and detached with 0.05% trypsin–0.02% EDTA and suspended to cell growth medium. Cells from the medium and from the plate were combined and collected with centrifugation. Thereafter, cells were fractioned according to ApoAlert® Cell Fractionation Kit (Clontech). 27 G needle was used for the homogenization (45 passes). After fractionation protein concentrations were measured [as above, with Bradford method using protein assay reagent (Bio-Rad Laboratories)]. Samples were stored at −80 °C until analyzed by immunoblotting. To control the purity of mitochondrial and cytosolic fractions, mitochondrial-specific COX4 was immunoblotted from selected samples. The absence of COX4 in cytosolic fraction showed that the fractionation had been successful (data now shown). All experiments were carried out in cell culture plates. One experiment comprised of one plate (no parallels) per exposure done in different days with newly made BP-solution. The experiments were repeated five times ($n = 5$).

2.4. Immunoblotting

Proteins (10 or 20 μg) were separated electrophoretically at 200 V (BioRad Power Pac 200) in 15% polyacrylamide gel containing SDS. Protein transfer to PVDF membrane, blocking in 5% non-fat cow milk–TBS–0.1% Tween, and primary and secondary antibody incubations, as well as washing steps were carried out as previously (Tampio et al., 2008). Primary antibodies used were mouse monoclonal Anti-cytochrome c (Pharmingen, dilution 1:2000), mouse monoclonal Anti-COX4 (ApoAlert® Cell Fractionation Kit, Clontech, dilution 1:500), rabbit polyclonal Anti-Puma (Cell Signaling, dilution 1:1000) and mouse monoclonal Anti-Bax (Santa Cruz Biotechnology, dilution 1:1000). Secondary antibodies used were Anti-mouse-IgG HRP-labeled (Amersham NA9310 for cytochrome *c* and Bax, dilution 1:2000), Anti-mouse-IgG HRP-labeled (Chemicon AP124P for COX4, dilution 1:2000) or Anti-rabbit-IgG HRP-labeled (Cell Signaling for PUMA, dilution 1:1000). Protein bands were visualized with ECL+ Plus system (Immunoblotting detection system, Amersham BioSciences) according to the manufacturer's instructions with ImageQuant™ RT ECL™ imaging system (GE Healthcare, USA). Page Ruler Plus Prestained Protein Ladder (Fermentas, Canada) was used to compare the molecular weights of the bands and mouse monoclonal Anti-β-actin-IgG (Sigma, USA, dilution 1:2 million) was used as a loading control. Densitometric analysis was carried out using QuantityOne®-program (1-D Analysis Software, version 4.6.3, Bio-Rad Laboratories Inc., USA).

2.5. Immunostained cytochrome c visualized by confocal microscopy

Glass coverslips (#1.5) were moved to the cell culture dishes (Ø9 cm, Greiner Bio-One) and coated with 0.1% rat tail collagen (type 1; BD Biosciences, Bedford, MA, USA) in 0.1% acetic acid. After careful washing, 2×10^5 MCF-7 cells were seeded and grown for 24 h before BP exposure. All experiments were carried out in cell culture plates. One experiment comprised of one plate (no parallels) per exposure done in different days with newly made BP-solution. The experiments were repeated three times ($n = 3$). Cytochrome *c* release was visualized by confocal microscopy using an immunofluorescent method with slight modifications described by Hietakangas et al. (2003). Briefly, the cells were fixed in ice cold 3% paraformaldehyde for 20 min after which they were permeabilized with 0.1% Triton X-100 in PBS at room temperature (RT) for 10 min. After a wash with PBS, the samples were incubated with blocking solution, 5% goat serum (GS; Gibco, Paisley, UK) in PBS, in a humidified chamber at RT for 1 h. Then the samples were incubated with dilution of 1:150 Anti-cytochrome *c* (Pharmingen, San Diego, CA, USA) in 0.01% Triton X-100–1.5% GS–PBS at 37 °C for 2 h. The samples were washed well and moved onto the 1:150 diluted Alexa Fluor 488 goat Anti-mouse-IgG (Molecular Probes, Eugene, Oregon, USA) antibody drops in the chamber, and incubated at RT for 45 min. After careful washings, the samples were incubated with 2.5 μM propidium iodide (Molecular Probes), to stain nuclei, for 5 min. The samples were washed once and mounted on the objective slides with Vectashield (Vector Laboratories, Burlingame, CA, USA) sealed with clear nail polish. The slides were protected from light and stored at 4 °C until visualization with UltraView confocal image system (PerkinElmer, UK) attached to Nikon Eclipse TE300 microscope (Nikon corp., Japan). Images were taken with 100× oil immersion objective (Plan Fluor, Nikon) with a cooled digital charge-coupled device (CCD) camera (PerkinElmer) and further processed with Adobe Photoshop CS.

2.6. Detachment of the cells for MTT test and flow cytometric analysis

The exposed cells were collected as in chapter "Preparation of cytosolic and mitochondrial fractions for immunoblotting". After centrifugation, supernatant was discarded and the cell pellets were suspended in 1 ml of PBS. Suspension (200 μl) was diluted to 1:5 in PBS for the MTT test. For the DNA content analysis (200 μl) and for the PI exclusion test (200 μl) the suspension was centrifuged and resuspended in PBS. All experiments for the MTT test and flow cytometric analysis were carried out in cell culture plates. One experiment comprised of one plate (no parallels) per exposure done in different days with newly made BP-solution. The experiments were repeated three times ($n = 3$).

2.7. The MTT test

Cell viability was measured by the MTT test where exogenously administrated MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is converted to the colored formazan by the functional mitochondria of the cells (Mosmann, 1983). Briefly, the diluted cell suspension and MTT-solution (Sigma–Aldrich Corp., St. Louis, MO, USA) were added in duplicate to the 96-well plate. Sodium dodecyl sulfate (SDS) buffer was added after 2 h of incubation at +37 °C. Incubation was continued overnight and absorbance was measured with a microplate reader (Victor^3, PerkinElmer, Finland) at the wavelength of 570 nm and analyzed by WorkOut software version 1.5 (Dazdaq Ltd., Brighton, UK). The proportion of viable cells in exposed samples was compared to a control sample (Penttinen et al., 2007).

2.8. DNA content analysis

DNA content was analyzed by propidium iodide (PI) staining of permeabilized cells, where apoptotic cells can be identified as the cells containing fragmented DNA (sub-G1 fraction) (Darzynkiewicz et al., 1992). This method also provides infor-