Toxicology in Vitro 28 (2014) 875–884

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Detection of key enzymes, free radical reaction products and activated signaling molecules as biomarkers of cell damage induced by benzo[a]pyrene in human keratinocytes

Birgit Bölck [a,*,1], Marwa Ibrahim [b,1], Dirk Steinritz [c,e], Christian Morguet [a], Sandra Dühr [a], Frank Suhr [a], Juxian Lu-Hesselmann [d], Wilhelm Bloch [a]

[a] Department of Molecular and Cellular Sport Medicine, German Sport University Cologne, Germany
[b] Department of Histology, Faculty of Medicine, Tanta University, Tanta, Egypt
[c] Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany
[d] Bundeswehr Institute of Medical Occupational and Environmental Safety, Berlin, Germany
[e] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University, Munich, Germany

ARTICLE INFO

Article history:
Received 2 April 2013
Accepted 17 March 2014
Available online 29 March 2014

Keywords:
Keratinocytes
HaCaT
Benzo[a]pyrene
Apoptosis
Nitrosative stress
Oxidative stress

ABSTRACT

Benzo[a]pyrene (BaP) is a known carcinogenic and cell damaging agent. The underlying cell damaging pathomechanisms have not been totally revealed. Especially BaP-related induction of oxidative and nitrosative stress has not been previously investigated in detail. The presented study investigated these effects in order to elucidate the pathomechanism and as well to identify potential biological markers that may indicate a BaP exposure. Human immortalized keratinocytes (HaCaT cells) were exposed to BaP (1 μM) for either 5 min or 6 h, respectively. BaP-induced cellular damage was evaluated by immunocytochemistry analysis of multiple signaling cascades (e.g. apoptosis, Akt, MAPK, NOS, nitrotyrosine and 8-isoprostane formation), detection of nitrosative stress using diaminofluorescein (DAF-FM) and oxidative stress using 3′ -(p-aminophenyl)fluorescein (APF).

Our results show that BaP exposure significantly enhanced NO and ROS productions in HaCaT cells. BaP led to eNOS-phosphorylation at Ser^{1177}, Thr^{495} and Ser^{116} residues. Using specific inhibitors, we found that the Erk1/2 pathways seemed to have strong impact on eNOS phosphorylation. In addition, BaP-induced apoptosis was observed by caspase-3 activation and PARP cleavage.

Our results suggest that BaP mediates its toxic effect in keratinocytes through oxidative and nitrosative stress which is accompanied by complex changes of eNOS phosphorylation and changes of Akt and MAPK pathways.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds found throughout the environment. They are formed during incomplete combustion of organic matter and are widespread in the atmosphere, food and drinking water. (IARC, 1983; Knize et al., 1999). Some PAH such as benzo[a]pyrene (BaP) are known mutagenic and carcinogenic substances, but underlying pathomechanisms are only partially known. Cell injury due to BaP exposure is not only attributed to the carcinogenic and mutagenic properties, but also to interferences of BaP with cell division, activation of cell death and tissue damage (Burdick et al., 2003; Lidsky and Schneider, 2003; Solhaug et al., 2004; Erb et al., 2005).

BaP was shown to produce quinone derivatives during metabolic processes that in turn are able to generate reactive nitrogen species by redox cycling (Sullvian, 1995). Costa et al. showed that BaP exposure of human skin resulted in increased ROS levels, increased aryl hydrocarbon receptor (AhR) and CYP1A1 expression (Costa et al., 2010). BaP induced free radical formation (reactive oxygen (ROS) and reactive nitrogen species (RNS) and led to DNA and protein damage. In addition, decreased levels of antioxidant

Abbreviations: APF, 3′-(p-aminophenyl) fluorescein; BaP, benzo[a]pyrene; DAF, 4,5-diaminofluorescein; PAH, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; RNS, reactive nitrogen species; O_2^-, superoxide anion; OH, hydroxyl radical; H_2O_2, hydrogen peroxide; NO, nitric oxide; $ONOO^-$, peroxynitrite anion; SOD, superoxide dismutase; 3-NT, 3-nitrotyrosine; 8-isoprostane, 8-epi-prostaglandine; F2 alpha, epi-PGF2a; DU, densitometric units.

* Corresponding author. Address: Department of Molecular and Cellular Sport Medicine, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. Tel.: +49 (0) 221 4982 6116; fax: +49 (0) 221 4982 8370.
E-mail address: Boelck@dshs-koeln.de (B. Bölck).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.tiv.2014.03.010
0887-2333/

scavenger enzymes (superoxide dismutase (SOD) and catalase) are discussed to be involved in the BaP induced carcinogenesis (Kim and Lee, 1997; Miller and Ramos, 2001; Valko et al., 2005).

ROS (superoxide anion (O_2^-), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2)) are able to react with various molecular targets including DNA, proteins and lipids thus leading to cell damage. But more important, ROS have been found to act as signaling molecules in various cellular signaling pathways and redox regulation (Droge, 2002).

Nitric oxide synthases (NOSs) convert the amino acid L-arginine into nitric oxide (NO). NO can react with O_2^- leading to the formation of reactive peroxynitrite ($ONOO^-$) (Szabo, 2003). $ONOO^-$ rapidly hydrolyzes into $^{\cdot}OH$ and nitrogen dioxide (NO_2) (Kroncke et al., 1997). Hydroxyl radicals (e.g. formed by reactive nitrogen species (RNS) pathways) can damage cellular macromolecules by chemical oxidation, while NO_2 can lead to DNA and protein nitration (particularly at tyrosine residues). Nitrotyrosine residues can distort the protein structure and alter its function, leading to potentially pathological results (Amirmansour et al., 1999). NOS-mediated generation of peroxynitrite may be enhanced by O_2^- from ROS pathways. As ROS and RNS pathways interact at various levels, both are discussed to contribute to carcinogenicity and teratogenicity of BaP (Kasapinovic et al., 2004; Ding et al., 2005).

Two NOS isoforms are responsible for the formation of NO in the epidermis or in keratinocytes, namely the inducible NOS (iNOS) and the endothelial NOS (eNOS). Both isoforms play an important role in the physiology and pathophysiology of the skin (Stallmeyer et al., 2002).

Cell damage caused by BaP is mediated and modulated by various intracellular signaling pathways. For example, it was shown that BaP-induced apoptosis depends on different signaling molecules, such as MAPK signaling pathways, including jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (p38), extracellular signal-regulated kinase (Erk) 1/2 as well as the protein kinase Akt pathway (Lidsky and Schneider, 2003; Solhaug et al., 2004; Stern, 2004). Besides the influence of BaP on apoptosis, BaP interacts with hormone receptors leading to the activation of intercellular signaling pathways (Tsai et al., 2004).

Moreover, the combination of various pollutants can enhance cell damage compared to a single pollutant. For example, co-exposure to UVA and UVB rays in BaP pre-treated human keratinocytes amplified ultraviolet damaging effects (Crallan et al., 2005). On the other hand, cadmium was able to alleviate the negative effect of BaP in mouse epidermal JB6 Cl41 cells (Mukherjee et al., 2008). The previous aspects suggest that the detection of ROS or RNS formation, the evaluation of radical reaction products as well as BaP-induced changes of significant signaling cascades could indicate the degree of cell damage and contribute to elucidate the cellular mechanisms occurring upon exposure to BaP. This applies especially to skin and mucous membranes, which are directly exposed to environment pollutants such as BaP. But up till now, specific effects of BaP on keratinocytes have not been thoroughly investigated. Therefore, we have explored the BaP-induced alterations of ROS- and RNS-levels, influences of BaP on MAPK and Akt-Kinase pathways, changes of eNOS-phosphorylation and iNOS expression as well as BaP-induced apoptosis. Our results will pave the way to identify potential novel biomarkers related to BaP exposure and to provide a deeper insight into the pathomechanism of BaP in keratinocytes.

2. Materials and methods

2.1. Cell culture

Spontaneously immortalized HaCaT cells were obtained from the German Cancer Research Center (Heidelberg, Germany; Boukamp et al., 1988). Cells were grown in DMEM culture medium supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 U/ml). The cells were kept at 37 °C in a humidified atmosphere of 5% CO_2. Cell cultures were split and sub-cultured as described for keratinocytes. HaCaTs were seeded for immunocytochemistry onto 0.1% gelatine pre-coated cover slips in 24-multiwell plate. The HaCaT cells were treated with 1 μM BaP (Fluka, Germany) in culture medium for 5 min or 6 h, and compared with control (sham-treated with DMSO as vehicle control).

The effect of different BaP concentrations (0.5, 1, 2 and 3 μM) have been initially tested (using p-Akt, p-Erk and p-eNOS1177 as markers). All tested concentrations resulted a comparable cellular response with the most pronounced effect at 1 μM BaP, which was therefore chosen for further experiments (Data see Table 1).

2.2. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 25 min and rinsed several times with 0.1 M phosphate-buffered saline (PBS). For blocking unspecific binding sites, 5% BSA in Tris-buffered saline (TBS) was used (1 h at room temperature). Prior to each step, cells were rinsed in TBS buffer for three times. Incubation with the primary antibody was performed in a TBS-based solution of 0.8% BSA overnight at 4 °C. The following antibodies were applied (for details see Section 2.5): polyclonal rabbit anti-eNOS (1:400), anti-p-eNOS at Ser^{116} (1:500), anti-p-eNOS at Thr^{495} (1:400), anti-p-eNOS at Ser^{1177} (1:500), anti-AKT1/PKB (1:500), anti-p-Akt1/PKB (Thr^{308}) (1:500), activated anti-Caspase-3 antibody (1:500), anti-c-PARP (1:250), anti-iNOS (1:1500), anti-p-JNK (1:400), anti-3-nitrotyrosine (1:500) or 8-isoprostane (8-epi-prostaglandine F2 alpha (8-epi-PGF2a) 1:1500). After rinsing with TBS, cells were incubated with the corresponding secondary biotinylated goat anti-rabbit IgG (1:400) and biotinylated goat anti-mouse IgG (1:400), followed by a streptavidin horseadish complex (1:150) for 1 h. Finally, the antibody-staining was visualized by 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris–HCl buffer and 0.1% H_2O_2 for 3–5 min. Incubations without the primary antibodies were carried out as negative controls. To objectify the results, we measured the gray values of 100 exposed non-confluent cells from 3 independent experiments. The evaluations of the results were performed blinded. To achieve a total cell number of 100 cells per group a necessary number of randomly chosen visual fields per cover slip were recorded. To ensure a high degree of comparability of all recorded visual fields, background gray values were adjusted. Therefore, the individual background value of each individual cover slip was assessed by defining three rectangular region of interest (ROI) in a cell free area. The exposure setting of the camera was adjusted to achieve a brightness value of 220 ± 5 in order to avoid an unintended over-exposure (sensor saturation = white = 255), for each picture which has been taken. For staining intensity detection, a Leica microscope (Wetzlar, Germany) coupled to a 3-chip CCD-camera (DXC-1850P, Sony, Germany) was used. Magnification for all images was 400-fold. Image analysis was performed using the software "ImageJ" (National Institutes of Health, Bethesda, Maryland, USA). The intensity of the immunocytochemistry stainings was reported as the mean of measured HaCaT cell gray value minus mean of background gray value (referred as densitometric units [DU]). The background gray values were also here measured at three cell free areas from each picture. Magnification for all images was 400-fold.

2.3. Detection of NO radical formation in HaCaT cells using DAF-FM DA

The formation of NO in HaCaT cells was assayed using 4,5-diaminofluorescein diacetate (DAF-FM diacetate), which is a cell membrane permeable form of the NO indicator. Once inside the