Toxicology Letters 206 (2011) 289–299

Contents lists available at ScienceDirect

Toxicology Letters

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

Mechanisms involved in lipid accumulation and apoptosis induced by 1-nitropyrene in Hepa1c1c7 cells

N. Podechard[a], X. Tekpli[a], D. Catheline[c], J.A. Holme[d], V. Rioux[c], P. Legrand[c], M. Rialland[e], O. Fardel[a,b], D. Lagadic-Gossmann[a], V. Lecureur[a,*]

[a] EA4427-SeRAIC, Team "Toxicity of Polycyclic Aromatic Hydrocarbons", IFR140, Université de Rennes 1, Faculté des Sciences Pharmaceutiques et Biologiques, IRSET, France
[b] Département HITC, Centre Hospitalier Universitaire, Rennes, France
[c] Laboratoire de Biochimie, INRA-Agrocampus, 35042 Rennes, France
[d] Division of Environmental Medecine, Norwegian Institute of Public Health, Oslo, Norway
[e] Centre de recherche INSERM «Lipides, Nutrition, Cancer», UMR866, Université de Bourgogne, Dijon, France

ARTICLE INFO

Article history:
Received 30 July 2010
Received in revised form 20 July 2011
Accepted 21 July 2011
Available online 26 August 2011

Keywords:
Apoptosis
Cholesterol
Fatty acids
Hepatoma cells
1-Nitropyrene
Stearoyl-CoA desaturase 1

ABSTRACT

1-Nitropyrene (1-NP) is a nitro-polycyclic aromatic hydrocarbon (nitro-PAH) present in diesel exhaust and bound to particular matter in urban air. We show that 1-NP and the referent PAH benzo(a)pyrene (BP) induce apoptosis and a lipid accumulation dependent on cytochrome P450 1A1-metabolites in mouse hepatoma cells, whereas 1-amino-pyrene had no effect. The caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-fmk), inhibits 1-NP-induced apoptosis, but failed to alter 1-NP-triggered lipid accumulation determined by Nile red staining. We further show that cholesterol and fatty acid contents are modified after nitro-PAH exposure and that 1-NP-induced cholesterol level is partially involved in related apoptosis. In parallel, the activity of the stearoyl-CoA desaturase 1 (SCD1), determined by fatty acid analysis, and its expression are reduced by 1-NP. The role of SCD1 in 1-NP-induced apoptosis is demonstrated in cells down-expressing SCD1, in which an increased apoptosis is observed, whereas the SCD1 overexpression elicits the opposite effects. In contrast, changes in SCD1 gene expression have no effect on the induced lipid accumulation. Moreover, 1-NP increases the activity of the AMP-dependent protein kinase (AMPK) leading to a caspase-independent apoptosis. Overall, our study demonstrates that the 1-NP-induced apoptosis is caspase- and AMPK-dependent, and is associated to a decrease of SCD1 expression which results in an alteration of lipid homeostasis.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs are a ubiquitous group of environmental pollutants. They are often produced by incomplete combustion of organic material including gasoline and diesel. In urban air, PAHs are not only often adsorbed on particular matter, but also found in soil and water. Humans are commonly exposed to such compounds not only via air, but also through diet. PAHs may exert a wide range of toxic effects, and some of them have been identified as carcinogenic to humans. Benzo[a]pyrene (BP) is an important carcinogenic PAH, whereas 1-nitropyrene (1-NP) is the most abundant nitro-PAH in diesel exhaust particles.

The deleterious effects of these environmental chemicals have partly been linked to the formation of reactive electrophilic metabolites. In the mouse hepatoma cell line Hepa1c1c7, these compounds have been found to increase cytochrome P-450 (CYP)1A1 expression (Landvik et al., 2007). The binding of BP, a well known ligand of the aryl hydrocarbon-receptor (AhR), to this receptor leads to its nuclear translocation and activation of AhR-dependent gene transcription such as CYP1A1. Nitro-PAHs require metabolic activation by nitroreduction or ring oxidation to react with DNA; but CYP enzymes are also reported to reduce nitro-PAHs (H.J. Kim et al., 2005).

Several reports demonstrate that BP causes apoptotic and necrotic cell death (Holme et al., 2007), while 1-NP was found to cause apoptosis as well as a non-apoptotic programmed cell death with "paraptotic" characteristics (Landvik et al., 2007; Asare et al., 2008). Recently, we also observed that 1-NP exposure resulted in a very marked increase of lipid droplets in Hepa1c1c7 cells (Asare et al., 2009); however the mechanisms involved are still unknown.

Liver plays a major role in lipid homeostasis, but hepatocytes are not a physiological site of lipid storage, and development of

* Corresponding author at: EA4427-SeRAIC, Faculté des Sciences Pharmaceutiques et Biologiques, 2 Avenue du Pr L. Bernard, 35043 Rennes cedex, France. Tel.: +33 02 23 23 47 88; fax: +33 02 23 23 47 94.
E-mail address: valerie.lecureur@univ-rennes1.fr (V. Lecureur).

0378-4274/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2011.07.024

steatosis is usually associated with cellular dysfunction and apoptosis. Nevertheless, it is well known that several liver toxic xenobiotics may alter lipid metabolism by leading to accumulation of lipid droplets that can cause a fatty liver (Prince et al., 1993). An effect of the strong AhR ligand, TCDD, as well as some of the PAHs on lipid accumulation has previously been observed in human macrophages (Vogel et al., 2004; Podechard et al., 2009). Similarly, hepatic lipid accumulation was observed in mice exposed to TCDD (Shen et al., 1991; Matsumura et al., 1997). Interestingly, some recent findings have also suggested lipid droplet accumulation to be more directly involved in cell death processes (Schaffer, 2003). So, the link between lipid homeostasis and apoptosis induced by aromatic hydrocarbon (AH) pollutants remains to be characterized.

In liver cells, a prolonged activation of the AMP-dependent protein kinase (AMPK), an enzyme involved in lipid homeostasis, can trigger apoptosis and activation of caspase-3 (Meisse et al., 2002). AMPK can be activated by an inhibition of the stearoyl CoA desaturase (SCD1), a key enzyme of lipid metabolism catalyzing Δ9-desaturation of the saturated fatty acids (SFA) palmitate and stearate to the monounsaturated fatty acids (MUFA) palmitoleate and oleate, respectively (Scaglia et al., 2009). Furthermore, the ratio between SFA and MUFA seems to be involved in the regulation of cell growth, as loss of SCD1 has been reported to be associated with a reduced proliferation as well as an increased apoptosis (Scaglia and Igal, 2005, 2008; Minville-Walz et al., 2010).

In the present study, we have extended our previous observations showing that 1-NP induces lipid accumulation (Asare et al., 2009) and apoptosis (Solhaug et al., 2004; Landvik et al., 2007). We have sought to understand the relationship between lipid accumulation and apoptosis induced by PAHs. Therefore, we have compared the effects of 1-NP to those observed after exposure to its non-toxic amine metabolite 1-amino-pyrene (1-AP) and BP and, we have characterized the changes in lipid composition. Furthermore, the role of AMPK and SCD1 in the perturbation of lipid homeostasis and apoptosis induced by 1-NP was examined.

2. Materials and methods

2.1. Chemicals

BP, 1-NP, 1-AP, Oil red O (ORO), N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-fmk), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and methyl-β-cyclodextrin D (MβCD) were provided by Sigma–Aldrich (St Louis, MO). TCDD was obtained from Cambridge Isotope Laboratories (Cambridge, MA). 5-Aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR), compound C (CpC) and cylic-pifithrin-α (PFT) were provided by Calbiochem (La Jolla, CA). Chemicals were commonly prepared as stock solution in DMSO. Final concentration of DMSO did not exceed 0.2% (v/v) and control cultures received DMSO only.

2.2. Culture and treatments

The mouse hepatoma cell line Hepa1c1c7 (purchased from the European Collection of Cell Culture) was maintained in MEMα medium with L-glutamine without ribonucleosides and deoxyribonucleosides (Gibco, Cergy Pontoise, France) and supplemented with 10% fetal calf serum and 0.1 mg/ml gentamycin. Cells were routinely kept in logarithmic growth phase at 1–9 × 10^6 cells/75 cm^2 flasks by splitting twice a week. Cells were seeded near confluence (90 × 10^3 cells/cm^2) a day before treatment and the medium changed before exposures. Inhibitors were added for 1 h before the treatment, for the indicated time points.

2.3. Cellular lipid accumulation

Cellular lipid accumulation was assessed by staining lipid droplets by ORO and cellular lipids by Nile red dye as previously described (Podechard et al., 2009). Cells stained by ORO were observed and counted on a ZEISS axiolab microscope, whereas the fluorescence emitted by cells stained by Nile red was measured at 584 ± 20 nm by flow cytometry (FACScalibur, Becton Dickinson, Le-Pont-de-Claix, France), analyzed with CellQuest software and expressed as mean of fluorescence intensity (MFI).

2.4. Cell viability assays

PAH effect on cell viability was evaluated using the MTT assay as previously described (Fardel et al., 1997). Fluorescence detection of apoptotic and necrotic cells was performed using a LEICA DMRXA microscope after Hoechst 33342 (5 μg/ml) and propidium iodide (PI) (10 μg/ml) staining of floating and adherent cells collected after treatment. Cells with condensed and/or fragmented nuclei were counted as apoptotic and PI-stained cells were counted as necrotic cells. Results were expressed as percentage of the total number of cells.

2.5. Ethoxyresorufin O-deethylase activity assay

EROD activity, corresponding to the *O*-deethylation of ethoxyresorufin, and mainly supported by CYP1A1 enzyme, was measured as described previously (Sparfel et al., 2006). In brief, cells were incubated in PBS at pH 7.4 containing 50 μM ethoxyresorufin and 1.5 mM salicylamide (Sigma–Aldrich), and kinetic reading was performed at 37 °C with a SpectraMax Gemini XS spectrofluorimeter (Molecular Devices, Sunnyvale, CA) over a 45 min period.

2.6. Caspase activity assays

Cell lysates (50 μg) obtained in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 0.1% Tween 20, 10% glycerol, 100 μM phenylmethylsulfonyl fluoride, 10 mM DTT supplemented with an EDTA-free cocktail protease inhibitor (Roche Diagnostic, Meylan, France)) were incubated for 30 min at 37 °C in a caspase assay buffer containing DEVD-AMC for caspase-3 or VDVAD-*p*NA for caspase-2 activities (Calbiochem). Caspase-3 activity was measured at 37 °C by monitoring fluorescence at 0, 1 and 2 h with a SpectraMax Gemini XS spectrofluorimeter using excitation/emission wavelengths 380/440 nm. Caspase-2 activity was measured at 37 °C by monitoring absorbance at 0, 1 and 2 h with a SpectraMax spectrophotometer at 400 nm. Caspase activities were determined as fluorescence or absorbance intensity/h/mg protein and expressed relatively to the control.

2.7. RNA isolation and reverse transcription-real time quantitative PCR (RT-qPCR) analysis

RNA expression was analyzed through RT-qPCR assays. Briefly, total RNA was isolated from cells using TRIzol (Invitrogen). Total RNA (1 μg) was then reverse transcribed using the RT Applied Biosystem kit (Courtaboeuf, France). qPCR assays were next performed using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystem). Gene-specific primers were used as follows: SCD1 sense: 5′-GCGATACACTCTGGTGCTCA-3′; SCD1 antisense: 5′-CCCAGGGAAACCAGGATATT-3′. Amplification curves of the PCR products were analyzed with the ABI Prism SDS software using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to an 18S RNA endogenous reference.

2.8. Western blot analysis

Total cellular protein preparation and Western blots were performed exactly as previously described (Lecureur et al., 2005) using anti-phospho-AMPKα (thr172), anti-Phospho-Acetyl-CoA-Carboxylase (Ser79) and anti-AMPKα antibodies obtained from Cell Signaling Technology Inc. (Danvers, MA) or using anti-GAPDH (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) antibody. Moreover, the polyclonal rabbit anti-SCD1 antibody was obtained from Ozols (1997). After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h, bands were visualized by autoradiography using chemiluminescence.

2.9. Transfection of siRNAs and plasmid

The sequences of iRNA targeting SCD1 mRNA: iSCD1v4 and iSCD1v5 were respectively: 5′-UGCCACAAGACAUUAUAUATT-3′ and 5′-GUAGAUUGUCUCGAGAGAATT-3′ and for control experiment, the sequence of iRNA control (iCT) was: 5′-AGGUAGUGUAAUCGCCUUGTT-3′. pSCD1 corresponds to a human SCD1 expression plasmid cloned in pcDNA3. Transfection of pcDNA3, pSCD1 (1.6 μg/ml) or siRNA (0.1 μM) into Hepa1c1c7 cells was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The analysis of SCD1 mRNA expression was realized 24 h after the beginning of transfection, whereas the analysis of SCD1 protein expression was performed 48 h post-transfection. Twenty-four hours after the beginning of transfection, iSCD1-transfected cells were exposed to 1-NP during 24 h.

2.10. Fatty acid extraction and analysis

At the end of the incubation, cells were washed twice with ice-cold PBS solution and harvested in 1 ml PBS. Cell suspensions were then centrifuged at 800 × *g* for 4 min. The supernatant was discarded and the cell pellet kept for lipid extraction. Lipids were extracted twice with a mixture of hexane/isopropanol (3:2, v/v), after acidification with 1 ml HCl 3 M (Rioux et al., 2005), and dissolved in 1 ml of