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Protective action of *n*-3 fatty acids on benzo[a]pyrene-induced apoptosis through the plasma membrane remodeling-dependent NHE1 pathway

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ABSTRACT

Plasma membrane is an early target of polycyclic aromatic hydrocarbons (PAH). We previously showed that the PAH prototype, benzo[a]pyrene (B[a]P), triggers apoptosis *via* DNA damage-induced p53 activation (genotoxic pathway) and *via* remodeling of the membrane cholesterol-rich microdomains called lipid rafts, leading to changes in pH homeostasis (non-genotoxic pathway). As omega-3 (*n*-3) fatty acids can affect membrane composition and function or hamper *in vivo* PAH genotoxicity, we hypothesized that addition of physiologically relevant levels of polyunsaturated *n*-3 fatty acids (PUFAs) might interfere with B[a]P-induced toxicity. The effects of two major PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were tested on B[a]P cytotoxicity in the liver epithelial cell line F258. Both PUFAs reduced B[a]P-induced apoptosis. Surprisingly, pre-treatment with DHA increased the formation of reactive B[a]P metabolites, resulting in higher levels of B[a]P-DNA adducts. EPA had no apparent effect on B[a]P metabolism or related DNA damage. EPA and DHA prevented B[a]P-induced apoptotic alkalinization by affecting Na^+/H^+ exchanger 1 activity. Thus, the inhibitory effects of omega-3 fatty acids on B[a]P-induced apoptosis involve a non-genotoxic pathway associated with plasma membrane remodeling. Our results suggest that dietary omega-3 fatty acids may have marked effects on the biological consequences of PAH exposure.

1. Introduction

Environmental variables influence the incidence and progression of disease. Dietary fat is an environmental variable that has been associated experimentally and epidemiologically with diverse physiopathological states (e.g. inflammation, diabetes, cardiovascular disease, and cancer). However, the impact of fat on the incidence of disease appears to depend on the type of fatty acids present in diet. With regard to tumorigenesis, *in vivo* studies in rodents have indicated that omega-3 (*n*-3) fatty acid-enriched diets would protect against chemical-induced carcinogenesis whereas omega-6-enriched diets would favor it [1–3].

Several health benefits of dietary consumption of *n*-3 polyunsaturated fatty acids (PUFAs) have been described, among them, a decrease in the risk of cardiovascular diseases [4,5], reduction of the risk of developing colorectal cancer [6], inhibition of the growth of tumor cells [7–9], and enhancement of healthy cell survival [10]. *n*-3 PUFAs are not synthesized *de novo* by human cells. The most effective way to increase the *n*-3 PUFA content in plasma

Abbreviations: B[a]P, benzo[a]pyrene; P450, cytochrome P450; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PAH, polycylic aromatic hydrocarbon; SFA, saturated fatty acid.

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or tissues is *via* dietary supplementation [11]. Usual dietary intake comes mainly from oily fish (salmon, mackerel, sardine, tuna), but also from some vegetable oils and seaweeds. However these products, especially fish and seafood, may also be contaminated with toxic compounds. Indeed, most fishes are at least to some degree contaminated with diverse environmental chemicals, such as persistent organic pollutants including dioxins or polycyclic aromatic hydrocarbons (PAHs) [12–14]. Nonetheless, several studies have indicated that the risk of exposure to chemical contaminants *via* these products could be partially offset by the fatty acid-associated health benefits [15,16]. In fact, *in vivo* experiments in rodents showed that *n*-3 PUFA supplementation could prevent the adverse effects of PAHs [17,18]. However, the underlying mechanisms for such protective actions remain to be determined.

The *n*-3 PUFAs have been suggested to inhibit *in vivo* the genotoxic effects of benzo[a]pyrene (B[a]P) in mice or 7,12-dimethylbenz[a]anthracene in rat [3,19]. The *n*-3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are known to be rapidly incorporated into plasma membrane and to increase membrane fluidity as well as to decrease the cholesterol content of lipid raft microdomains [20]. Such an impact on membrane properties is important to consider since we have recently found that B[a]P-induced plasma membrane remodeling, more particularly changes in lipid raft biochemical characteristics, plays a key role in apoptosis *via* the regulation of intracellular pH. Additionally, several other compounds might also partly exert their toxic effects through plasma membrane dysfunction [21,22].

We thus hypothesized that addition of *n*-3 fatty acids might interfere with B[a]P-induced toxicity. More specifically we examined if DHA and EPA could modulate B[a]P-induced apoptosis by affecting the levels of DNA damage or the plasma membrane microstructure in rat liver epithelial F258 cells. This cell model was chosen since we previously showed the existence of two pathways involved in B[a]P-induced apoptosis: a p53-dependent pathway triggered by DNA damage and most interestingly, a pH-dependent apoptotic pathway related to the activation of the Na^+/H^+ exchanger 1 (NHE-1) by membrane remodeling [21,22]. The present study shows that DHA and EPA protect F258 cells against B[a]P-induced apoptosis, most likely by inhibiting the B[a]P effects at the plasma membrane level, and hence the NHE-1-dependent pathway.

2. Materials and methods

2.1. Chemicals

B[a]P, DEVD-AMC (Asp-Glu-Asp-7-amino-4-methylcoumarin), DHA and EPA were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hoechst 33342 was purchased from Invitrogen (Invitrogen, France). DPA (docosapentaenoic acid) was purchased from Cayman Chemical (Ann Harbor, USA). Rabbit polyclonal anti-p53 phospho Serine 15, mouse monoclonal anti-β-actin, anti-CD71, and anti-CYP1A1 antibodies were from Santa Cruz Biotechnology (Tebu-bio SA, Le Perray en Yvelynes, France). Mouse monoclonal anti-NHE1 and anti-flotillin were purchased from Becton Dickinson (BD Biosciences, San Jose, CA). Rabbit polyclonal antibody against CYP1B1 was from Alpha Diagnostics (San Antonio, USA).

2.2. Cell culture and apoptosis measurement

The F258 rat liver epithelial cell line originated from the liver of 10 day-old fisher rats and was kindly provided by Pr. Olivier Fardel and co-workers [23]. Cells were cultured in Williams'E medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 IU/mL penicillin, and 0.5 mg/mL streptomycin, at 37 °C under a 5% CO_2 atmosphere, and treated 24 h following seeding as previously described [22–24]. Cells were pre-treated with DHA (140 μM), EPA (200 μM), DHA:EPA (70:100 μM), or DPA (50 and 80 μM) for 1 h, after which B[a]P (50 nM) was added and incubation continued for a further 24, 48 or 72 h. The DHA concentration used was lower than with EPA due to cytotoxicity. These concentrations were chosen in order to be as close as possible to those detected in plasma after dietary supplementation (0.5–0.7 mM) [25]. All PUFAs were prepared in methanol and final concentration of this vehicle in culture medium did not exceed 0.12% (v/v); for all treatments without PUFAs, the same concentration of vehicle as for PUFAs-treated cells was included. B[a]P stock solutions were prepared in dimethyl sulfoxide; final concentration of this vehicle in culture medium was <0.05% (v/v), and control cultures received the same concentration of vehicle as B[a]P-treated cultures. Microscopic detection of apoptosis was performed in both floating and adherent cells, using Hoechst 33342 labeling, as previously described [24]. The caspase activity assay using the fluorescent substrate DEVD-AMC was performed as previously described [22].

2.3. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen cultured cells by the Izol-RNA Lysis Reagent (5 PRIME) and reverse transcribed using qScript cDNA Synthesis kit (Quanta Biosciences). Quantitative analysis of the specific expression of various genes was performed by real-time PCR on an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA), as previously described [26]. After isolation, RNA concentration was measured and only the RNA with ratio of absorbance 260/280 > 1.8 were used for experiments. To assess the successful amplification of the target genes, a standard curve was made for each primer set. In addition, for each primer set a dissociation curve was analyzed in order to ensure that there were no contaminating products in the reaction; the product of PCR reaction was also analyzed in a 2% agarose gel. The expression levels of target genes were normalized relative to the expression of the β-actin gene.

2.4. Western blotting

After treatment, cells were harvested, centrifuged, washed with PBS and lysed for 10 min on ice in Cytobuster lysis buffer (Invitrogen, Paisley, UK). The lysis buffer was complemented with protease inhibitor (complete, Mini; Protease Inhibitor Cocktail, Roche Applied Science). DNA and cell debris were removed by centrifugation. Western blot analysis was performed not only in whole cell lysates but also in the detergent resistant membrane (DRM; raft) and soluble (*S*; non-raft) fractions (see below for raft isolation), as previously described [22,27]. In short, 40 μg of whole cell lysate were separated on 12% SDS–polyacrylamide gel electrophoresis. Immunoblots were then probed with rabbit polyclonal anti-p53 (100 ng/mL), -phospho Ser15-p53 (200 ng/mL), or mouse monoclonal anti-CYP1A1 (100 ng/mL), -CYP1B1 (100 ng/mL) or β-actin (25 ng/mL) antibodies. Regarding raft and soluble fractions, 5 and 100 μg respectively were separated on 10% SDS–polyacrylamide gel electrophoresis. Immunoblots were then probed with mouse monoclonal anti-NHE1 (100 ng/mL), mouse monoclonal anti-flotillin (50 ng/mL) or -CD71 antibodies (200 ng/mL). Examples of full gel Western blots images can be seen in Supplementary Fig. 8.

2.5. Immunofluorescence assay for detecting GM1

Cells were fixed 30 min at 4 °C with 4% paraformaldehyde in PBS; after washing, cells were incubated for 1 h with a blocking solution (2% BSA in PBS) and subsequently, with FITC-coupled