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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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**Running title:** Effects of B[a]P and omega-3 fatty acids on plasma membrane

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## **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, polycyclic aromatic hydrocarbon; SFA, saturated fatty acid

## 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 alkalization by affecting Na<sup>+</sup>/H<sup>+</sup> 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.

**Keywords:** lipid rafts; apoptosis; benzo[a]pyrene; eicosapentaenoic acid; docosahexaenoic acid; Na<sup>+</sup>/H<sup>+</sup> exchanger 1

## 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 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<sup>+</sup>/H<sup>+</sup> 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. MATERIAL 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- $\beta$ -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 [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<sub>2</sub> atmosphere, and treated 24 h following seeding as previously described [22-24]. Cells were pre-treated with DHA (140  $\mu$ M), EPA (200  $\mu$ M), DHA:EPA (70:100  $\mu$ M), or DPA (50 and 80  $\mu$ M) for one hour, 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 sets. 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  $\beta$ -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  $\mu$ g 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  $\beta$ -actin (25 ng/mL) antibodies. Regarding raft and soluble fractions, 5  $\mu$ g and 100  $\mu$ g 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). Regarding raft and soluble fractions, 5  $\mu$ g and 100  $\mu$ g respectively were separated on 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were then probed with mouse monoclonal anti-NHE1, mouse monoclonal anti-flotillin or -CD71 antibodies. Examples of full gel western blots images can be seen in Supplementary Figure 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 cholera toxin B (Vybrant® FITC Lipid Raft Labeling Kit; Invitrogen) (0.5  $\mu$ g/mL) for 1 h. DAPI was used to stain nuclei. Fluorescent-labelled cells were captured with a DMRXA Leica microscope and a COHU high performance CCD camera using Metavue software. Data are representative of at least three independent experiments.

## **2.6. DNA adduct analysis by <sup>32</sup>P-postlabeling**

DNA adducts were measured in each DNA sample (4  $\mu$ g) using the nuclease P1 enrichment version of the <sup>32</sup>P-postlabeling method as described previously [28,29]. Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose (Macherey-Nagel, Düren, Germany) were: D1, 1.0 M sodium phosphate, pH 6.0; D3, 4 M lithium formate and 7 M urea, pH 3.5 and D4, 0.8 M lithium chloride, 0.5 M Tris and 8.5 M urea, pH 8.0. After chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers



Grove, IL), and DNA adduct levels (relative adduct labeling [RAL]) were calculated from adduct c.p.m., the specific activity of [ $\gamma$ - $^{32}$ P]ATP and the amount of DNA (pmol of DNA-P) used.

## **2.7. Comet assay**

DNA strand breaks and alkali-labile lesions were detected by the comet assay, and the lesion-specific repair enzyme formamidopyrimidine DNA-glycosylase (FPG) was employed to characterize oxidative damage to DNA as described previously [30]. Nuclei were stained with ethidium bromide and DNA damage scored (50 nuclei per sample) using the Comet IV capture system (version 4.11; Perceptive Instruments, UK). The tail intensity (% tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was measured for each nucleus scored. All samples were measured blind. Mean median % tail DNA was compared by *t*-test analysis.

## **2.8. High-performance liquid chromatography (HPLC) of B[a]P metabolites**

For the analysis of B[a]P metabolites, culture medium (2 mL) from exposed cells was collected and stored at -20°C until further processing. The medium was diluted to 10 mL in H<sub>2</sub>O, applied to a preconditioned (5 mL methanol and 10 ml water) Sep-Pak C<sub>18</sub> cartridge (Millipore Corporation, Milford, MA, USA), followed by a wash with H<sub>2</sub>O (10 mL), and eluted with 100% methanol (5 mL). The methanol eluate was evaporated to dryness at 45°C under a nitrogen stream and suspended in 100  $\mu$ L of 100% methanol. HPLC separation of B[a]P metabolites was performed on a Nova-Pak C<sub>18</sub> 3.9x150 mm<sup>2</sup> column (Waters, Milford, MA, USA) with a Waters 625 LC System, equipped with a LC 240 fluorescence detector (Perkin-Elmer, Beaconsfield, UK). The B[a]P metabolites were separated by a linear gradient of 30-100% methanol in H<sub>2</sub>O for 40 min. For the quantitative determination of B[a]P

metabolites, the following fluorescence conditions were used: 0 min, excitation 380/emission 431; 0.5 min, excitation 341/emission 381; 20 min, excitation 253/emission 410; 27 min, excitation 380/emission 431. The concentrations of B[a]P metabolite standards were determined by ultraviolet absorbance of the compounds dissolved in ethanol and using extinction coefficients from the NIH Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO, USA).

## **2.9. Lipid raft isolation**

Fractionation of lipid rafts was adapted from a previously described method [31]. Briefly, F258 cells ( $2 \times 10^8$ ) were washed in ice-cold PBS and scraped in 3 mL of PBS. Pellets were lysed for 30 min at 4°C in 1 mL 2-(*N*-morpholino)ethanesulfonic acid)-buffered saline (MBS), supplemented with protease inhibitors, containing 1% cold Triton X-100 before homogenization with a tight-fitting dounce homogenizer. Lysates were diluted in 2 mL MBS containing 80% sucrose (w/v) and placed at the bottom of a linear sucrose gradient consisting of 8 mL 5-40% sucrose (w/v) in MBS. Samples were centrifuged at 200,000 g for 20 h at 4°C, and 11 fractions of 1 mL each were collected from the top of the gradient. As previously characterized in F258 cells [22], fractions 1 to 7 correspond to lipid rafts, and fractions 8 to 11 correspond to the non-raft fractions. Protein amounts were measured on each fraction according to the Lowry method (Biorad, Marnes la Coquette, France). Fatty acid and cholesterol analyses were then carried out on each fraction and the calculated amounts were then summed according to DRM [Detergent Resistant Membranes; 1 to 7] and S [Soluble; 8 to 11] fraction pools.

## **2.10. Fatty acid analysis**

After fractionation of lipid rafts, lipids were extracted using Folch method [32]. After saponification, fatty acids were methylated with boron trifluoride (14% in methanol) at 70°C

for 15 min. Fatty acid methyl esters were then extracted with pentane and analyzed by gas chromatography using an Agilent gas chromatography Technologies 6890N (Bios Analytique, Toulouse, France). Identification of fatty acid methyl ester peaks was based on retention times obtained for methyl esters prepared from fatty acid standards.

### **2.11. Cholesterol analysis in lipid raft fractions**

The cholesterol content in DRM (raft) and S (non raft) fractions of each gradient fraction was spectrophotometrically evaluated by a cholesterol oxidase/peroxidase assay using the Infinity Cholesterol kit (Thermo Electron Corp., Eragny sur Oise, France) after lipid extraction by a 2:1 chloroform/methanol mixture (v/v).

### **2.12. Measurement of pH<sub>i</sub>**

pH<sub>i</sub> was monitored using the pH-sensitive fluorescent probe carboxy-SNARF-1-AM (carboxy-seminaphtorhodafluor acetoxy methyl ester; InVitrogen), in HEPES-buffered solution, as previously described [24]. The emission ratio 640/590 nm detected from intracellular SNARF was calculated and converted to a linear pH scale using *in situ* calibration.

### **2.13. Caspase activity measurements**

Adherent and floating cells were lysed in the caspase activity buffer (50 mM HEPES pH=7,5, 150 mM NaCl, 1mM EGTA, 0.1% Tween 20, 10% Glycerol, protease inhibitors). 40 µg of crude cell lysate were incubated with 80 µM DEVD-AMC for 2 h at 37 °C. Caspase-mediated cleavage of DEVD-AMC was measured by spectrofluorimetry (Spectramax Gemini, Molecular Devices, Sunnyvale, CA, USA) at the excitation/emission wavelength pair of

380/440 mm. Three independent experiments, performed in triplicate, were carried out for each experimental condition.

### **2.13. Statistical analysis**

All data are presented as mean  $\pm$  standard error along with the number of observations, n, corresponding to independent cultures used. Analysis of variance of means followed by Newman-Keuls test was performed using Statistica software. Differences were considered significant at the level of  $P < 0.05$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

## **3. RESULTS**

### **3.1. DHA and EPA inhibit B[a]P-induced apoptosis in F258 cells**

In order to study the effects of DHA and EPA on B[a]P-induced apoptosis, F258 cells were pre-treated for 1 h with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), and then co-treated with B[a]P (50 nM) for up to 72 h. Determination of apoptotic cells using Hoechst 33342 staining showed that both DHA and EPA reduced the number of cells exhibiting apoptotic nuclei by 50% (Fig. 1A and 1B). No changes were detected in the amount of B[a]P-induced necrotic cells as determined by propidium iodide staining (data not shown). Measurement of caspase 3/7 activity revealed that DHA decreased B[a]P-induced caspase activation by ~60%, while EPA had less marked effects (Fig. 1C and 1D). Interestingly, co-treating cells with B[a]P and a mixture of the two n-3 PUFAs (*i.e.* DHA 70  $\mu$ M + EPA 100  $\mu$ M) elicited a higher protective effect compared to single n-3 PUFA pre-treatment at their optimal concentration (Fig. 1E). Light microscopy observations also showed that both DHA or EPA resulted in some protection of the cells against B[a]P-induced loss of cell viability. Indeed the cell density was higher in B[a]P / n-3 PUFA co-treated cells compared with cells treated with B[a]P alone (Supplementary Fig. 1).

### 3.2. Effects of DHA and EPA on B[a]P metabolism

It has been reported that n-3 PUFAs can interfere with the metabolism of xenobiotics by reducing the activity of different cytochrome P450 (P450) enzymes [33]. As both DHA and EPA reduced B[a]P-induced apoptosis, we tested the effects of DHA and EPA on the mRNA expression of *CYP1A1* and *CYP1B1* (as both P450 isoenzymes are involved in B[a]P activation). We found that EPA and DHA significantly increased B[a]P-induced *CYP1A1* and *CYP1B1* mRNA levels (Fig. 2A and 2B), although changes were not that marked. At the protein level, P450 1A1 was undetectable as previously reported [34]; no significant effects were observed when considering P450 1B1 protein expression (Fig. 2C and 2D). In order to get further insight into the effects of omega-3 fatty acids on B[a]P metabolism, we analyzed the B[a]P metabolites in the cell culture medium by HPLC. As shown in Supplementary Fig. 2, DHA somewhat changed B[a]P metabolism, as a 2-fold increase in the quantities of B[a]P-r-7,t-8,c-9,t-10-tetrahydroretrol and 9-hydroxyB[a]P was found upon co-exposure with B[a]P and DHA compared to B[a]P alone, which was concomitant with a decrease in B[a]P-trans-9,10-dihydrodiol. In contrast, EPA treatment did not markedly alter the quantities of B[a]P metabolites formed.

### 3.3. Effects of DHA and EPA on B[a]P-induced DNA damage

Previous studies have reported that n-3 PUFA-enriched diets can prevent DNA damage induced by B[a]P or DMBA *in vivo* [3,19]. Here, we tested the effects of DHA or EPA on B[a]P-induced DNA damage measured by the comet assay and <sup>32</sup>P-postlabeling.

The results of the comet assay showed that DHA and EPA had no effects on B[a]P-induced oxidative or non-oxidative DNA damage after a 24 h (Fig. 3A) or a 48 h (data not shown) B[a]P exposure. As a positive control, we found an increase in the quantity of oxidative DNA damage elicited by a 5 μM B[a]P exposure during 48 h (Supplementary Fig. 3). <sup>32</sup>P-

Postlabeling analysis revealed that DHA increased by 54% the levels of B[a]P-DNA adducts (*i.e.* 10-(deoxyguanosine- $N^2$ -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [dG- $N^2$ -BPDE]) after a 48 h co-exposure with B[a]P, whereas EPA had no effect (Fig. 3B). As a change in DNA damage could result in changes in the activation of the p53-related apoptotic pathway, we then decided to look at this pathway. As shown in Fig. 4, the phosphorylation and expression of p53 remained unchanged whatever the fatty acid tested. As no change in the activation by B[a]P of the p53-dependent apoptotic pathway could be observed upon DHA or EPA exposure, we next focused on the other apoptotic pathway involved in our cell model, that is the membrane remodeling-related apoptotic pathway triggered by B[a]P [22].

### **3.4. Effects of DHA and EPA on the lipid composition of plasma membrane microstructures**

As stated above, we have recently found that B[a]P-induced plasma membrane remodeling plays a key role in apoptosis *via* the regulation of intracellular pH [22,24,35]. Based on the fact that DHA and EPA can change membrane microstructure and composition [36], we investigated possible effects of DHA and EPA on plasma membrane.

Gas chromatography was used to measure the percentage of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) in purified lipid rafts (DRM) and in non-raft (S) fractions. We found that DHA and EPA were well incorporated in both domains, with a higher affinity for the non-raft (S) fraction (Fig. 5). DHA had a better penetration in whole plasma membrane compared to EPA. B[a]P did not affect the incorporation of the two n-3 PUFAs in the plasma membrane (Fig. 5). Interestingly, when categorizing the fatty acid detected by gas chromatography in different classes SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) or PUFA and looking at the effect of DHA and EPA, we found that they modified the fatty acid content of the different sub-membrane

fractions (DRM or S). Increased levels of PUFA and SFA were observed in the non-rafts (S) upon DHA or EPA exposure while a decrease was seen in the quantity of MUFA in both raft and non-raft fractions (Fig. 6A-6D). The same results were found in presence of B[a]P (data not shown).

Other effects of DHA and EPA on the plasma membrane content of single fatty acids can be found in Supplementary Fig. 4. After EPA or DHA exposure, the membrane content in oleic acid (C18:1 n-9) decreased concomitantly with an increase in the stearic acid content (C18:0). A marked increase in docosapentaenoic acid (DPA, C22:5 n-3) in both rafts (DRM) and non-rafts (S) fractions was also observed especially after EPA treatment, but this increase was unrelated with EPA protective effects (Supplementary Fig. 5). Cholesterol is an important determinant of raft formation and it was shown previously that B[a]P significantly decreased the level of cholesterol in lipid rafts via an effect on the 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase expression, thereby leading to a remodeling of rafts [22]. This result was confirmed in the present study and we found that DHA or EPA treatment also significantly decreased the amount of cholesterol in lipid rafts; however, no further decrease was observed when cells were co-exposed with B[a]P (Fig. 7).

To examine if the changes in cholesterol-membrane content induced by DHA or EPA affected the plasma membrane integrity, we next tested the effects of these two lipids on raft distribution in cells. The GM1 distribution (a specific marker of lipid rafts) was analyzed by fluorescence microscopy using cholera toxin fragment B coupled to a fluorophore. As expected from our previous study, B[a]P disturbed the organization of cholesterol-enriched microdomains (rafts) as exemplified by the diffuse homogenous GM1 staining compared to control cells (Fig. 8) [22]. None of the PUFAs changed the GM1 distribution alone, but they both prevented the effects of B[a]P (Fig. 8).

### **3.5. Effects of DHA and EPA on the B[a]P-activated NHE1 pathway**

The most important effect of B[a]P-induced plasma membrane remodeling in F258 cells is the activation of a pH-dependent apoptotic pathway [22], which is triggered by a change in NHE1 sub-localization in plasma membrane, thus leading to its activation (with a resulting alkalization) [35,37]. Fig. 9 confirms that B[a]P partially induced a relocation of NHE1 from raft (DRM) to non-raft (S) fractions. Regarding the effects of DHA and EPA, we found that both PUFAs used alone led to a strong decrease of NHE1 protein level in raft microdomains, and no additional effects of B[a]P on NHE1 sub-plasma membrane localization were detected, as further stressed when performing western blotting on pooled fractions (Supplementary Fig. 6A). In order to rule out any possible effect of PUFAs on NHE1 expression that might have explained such an effect on protein level, analysis of NHE1 mRNA expression was performed by RT-qPCR and no significant change was detected whatever the condition tested (Supplementary Fig. 6B).

Regarding NHE1 function, we found that both DHA and EPA alone inhibited NHE1 activation following an intracellular acid load in PS120 cells over-expressing NHE1 (Supplementary Fig. 7). We then tested the effects of PUFAs on the B[a]P-induced, NHE1-related intracellular alkalization in F258 cells [24]. As shown in Fig. 10, both PUFAs prevented the B[a]P-induced intracellular alkalization.

Collectively, these results show that DHA and EPA inhibit NHE1 activation, which interferes with NHE1 activation by B[a]P, thereby protecting cells from apoptosis.

## **4. DISCUSSION**

In vivo studies have shown that n-3 PUFAs can inhibit PAHs-related carcinogenesis [3,19]. In the present study, we have shown that both DHA and EPA inhibit B[a]P-induced



apoptosis in rat hepatic epithelial F258 cells, mostly by interfering with the effects that B[a]P exerts on H<sup>+</sup> homeostasis (Fig. 11A). More specifically EPA and DHA inhibit the NHE1-dependent intracellular alkalization previously shown to be induced in the B[a]P-related apoptosis [24].

We found that DHA and EPA reduce B[a]P-induced apoptosis (50% reduction at optimal concentration). However, DHA appeared to be more effective in inhibiting caspase activation than EPA. Such a differential action was further emphasized by the fact that co-treating cells with B[a]P and a mixture of DHA/EPA nearly completely inhibited apoptosis. Knowing that B[a]P induced both caspase-dependent and independent apoptotic pathways in F258 cells [22,24,38,39], our results indicate that the intracellular mechanisms underlying the protective effects of these lipids might, at least partially, differ.

Recent studies have shown that DHA, but not EPA, dramatically alters cardiac mitochondrial fatty acid composition and prevents mitochondrial dysfunction [40,41]. Thus, an explanation for the differential effects of DHA and EPA on caspase activation might be that DHA, unlike EPA, may act on mitochondrial dysfunction, an event known to be necessary for caspase activation in our model [39]. As recently reviewed, other differential effects between DHA and EPA might also contribute to the different action of these PUFAs on caspase activation [42].

The protective action of PUFAs might have involved inhibition of B[a]P metabolism, since we have previously shown it as of importance in our cell model [23]. Analysis of the rate of B[a]P removal and the total amount of B[a]P metabolites formed suggested some slight modifications of B[a]P metabolism upon DHA but not upon EPA (Figure 11A). There was an increase in the expression of *CYP1A1* and *CYP1B1*, but with apparently no major changes in the levels of P450 enzymes, although a minor increase in P450 enzymes levels cannot be completely ruled out. In contrast, *in vitro* assays using different human P450s have

shown that PUFAs can be metabolized by these enzymes [43], and thus function as competitive inhibitors [33]. The overall result may be some minor changes in B[a]P metabolism and in the balance between activation and detoxification of the molecule. HPLC analysis of B[a]P metabolism suggested some slight effect of DHA on the quantity of some of the metabolites produced. Interestingly, an increase in the amount of B[a]P-r-7,t-8,c-9,t-10-tetrahydrotetrol, a further metabolite of one of the major DNA reactive B[a]P metabolites, was observed. The DHA-related increase in B[a]P tetrol may have significant effects on the DNA damage induced by B[a]P. In fact, it could be related to the increase in the formation of B[a]P-DNA adducts detected by <sup>32</sup>P-postlabeling with DHA. Nevertheless, the cell response to such an increase in DNA damage as measured by p53 phosphorylation remained unchanged (Figure 11A). Since the protective effects of EPA and DHA towards B[a]P-induced apoptosis appeared to be independent of p53 activation, which in our cell model is responsible for the genotoxic-related apoptotic pathway [39], we looked at the membrane remodeling-related apoptotic pathway.

Several studies have shown that supplementation with n-3 PUFAs leads to modifications of the physical properties of biological membranes [44]. Indeed, dietary fatty acids have been found to affect lipid raft composition and organization, with consequences on cell function [45,46].

The most represented SFA and MUFA in F258 plasma membrane are stearic acid (C18:0) and oleic acid (C18:1), respectively. We observed that DHA and EPA increased the membrane content of stearic acid while they decreased that of oleic acid. This could be possibly attributed to an inhibitory effect on  $\Delta$ 9-desaturase (SCD1) expression (Dendelé *et al.*, unpublished data), and lead to an improper ratio (SFA / MUFA) in membranes with consequences on membrane fluidity, protein activities and cell functions [47].

Here we found that both DHA and EPA depleted cholesterol in raft microdomains, with no further effect of B[a]P. This finding is in accordance with previous studies on DHA and EPA using membrane models, suggesting that the reduction was due to the incompatibility between the highly flexible structure of PUFAs and the rigid moiety of cholesterol [48]. We previously found that B[a]P reduced the cholesterol content of lipid rafts *via* the inhibition of the expression of HMG CoA reductase [22]. EPA and DHA may also regulate this cholesterol synthesis rate limiting enzyme [49].

Despite a significant depletion of cholesterol in lipid rafts, it seemed that EPA and DHA did not affect the cellular repartition of lipid rafts as no changes in GM1 staining were observed following EPA or DHA exposure compared to control conditions. However, co-exposure with either n-3 PUFA and B[a]P protected from B[a]P-induced GM1 redistribution. It can be hypothesized that DHA and EPA might replace cholesterol in rafts and consequently form another type of membrane microdomain, which still contains GM1 but less cholesterol (Figure 11B). Thus, the protein content of these newly formed plasma membrane microdomains may also be different, as shown here by the marked decrease of the NHE1 protein level in detergent resistant membranes. As previously suggested, the presence of DHA or EPA in rafts might actually lead to the formation of PUFA domains within membrane lipid rafts, thereby leading to changes in protein lateral organization [20].

Such an effect of n-3 PUFAs on the proteins present in lipid rafts has already been reported, *e.g.* for the epidermal growth factor receptor whose level is decreased in lipid rafts upon DHA exposure [50,51]. In this context, knowing that NHE1 activity is mechano-sensitive [37,52], one might suppose that a decrease in NHE1 protein levels in cholesterol-rich microdomains after PUFA treatment may interfere with the NHE1-dependent apoptotic pathway induced by B[a]P. Indeed, due to this decrease, B[a]P would then be unable to exert its effect on the NHE1 plasma membrane relocation, and hence on its activation and intracellular

alkalinization [35]. In support of this hypothesis we found that B[a]P-induced, NHE1-related intracellular alkalinization was prevented by both DHA and EPA. Regarding the effects of the n-3 PUFA on NHE1 activity, it is important to note that DHA and EPA alone have been reported previously to inhibit  $\text{Na}^+/\text{H}^+$  exchange in cardiac cells [53], and similar effects were observed when we used NHE1-overexpressing PS120 cells (Supplementary Fig. 6). The increase in fluidity previously described for n-3 PUFAs might explain such an inhibition of NHE1, since this transporter is known to be sensitive to membrane fluidity [54]. Regarding the inhibition of the B[a]P-induced NHE1 activation by n-3 PUFA, different possible mechanisms might be put forward: (1) a change in NHE1 expression; (2) alterations in membrane fluidity; (3) the prevention of B[a]P-elicited intracellular signals necessary for NHE1 activation. Regarding the first hypothesis, no significant change in NHE1 mRNA expression was presently observed. However, one might also suppose a change in NHE1 protein level upon PUFA due e.g. to activation of proteasome pathway; this remains to be tested. Considering the second hypothesis, we have found that not only the raft composition changed upon PUFA treatment, but also the composition of non-raft microdomains, notably with an increase in DHA or EPA. As a consequence, the remaining NHE1 protein upon PUFA, mostly detected in non-raft microdomains, would then be surrounded by different lipids (Figure 11B) compared to the B[a]P alone situation, which would then lead to the inhibition of its activity, notably, as suggested above, due to a change in fluidity. Finally, as recently published [35] and presently confirmed, the decrease in NHE1 protein levels in lipid rafts upon B[a]P alone (along with an increase in S fractions under those conditions of treatment, in contrast to the PUFA alone situation) was associated with the activation of the transporter. This translocation from raft to non-raft microdomains was then necessary for calmodulin binding and NHE1 activation by B[a]P [35]. So it seems that the mere exit of NHE1 from raft is not sufficient to activate the transporter, but also requires B[a]P-elicited

intracellular signals. In this context, membrane remodeling due to n-3 PUFA might interfere with the activation of the intracellular signaling pathways, such as calmodulin or the Jun kinase pathway also involved in B[a]P-induced NHE1 activation [35]. Deciphering the exact mechanism involved in the PUFA inhibition of the B[a]P-induced NHE1 activation would require future investigation.

In conclusion, the present study indicates that n-3 PUFAs interfere with B[a]P-induced toxic effects through alterations of H<sup>+</sup> homeostasis and inhibition of the NHE1 pathway. Such effects of nutritional lipids might underlie differential susceptibilities towards the development of PAH-related pathologies.

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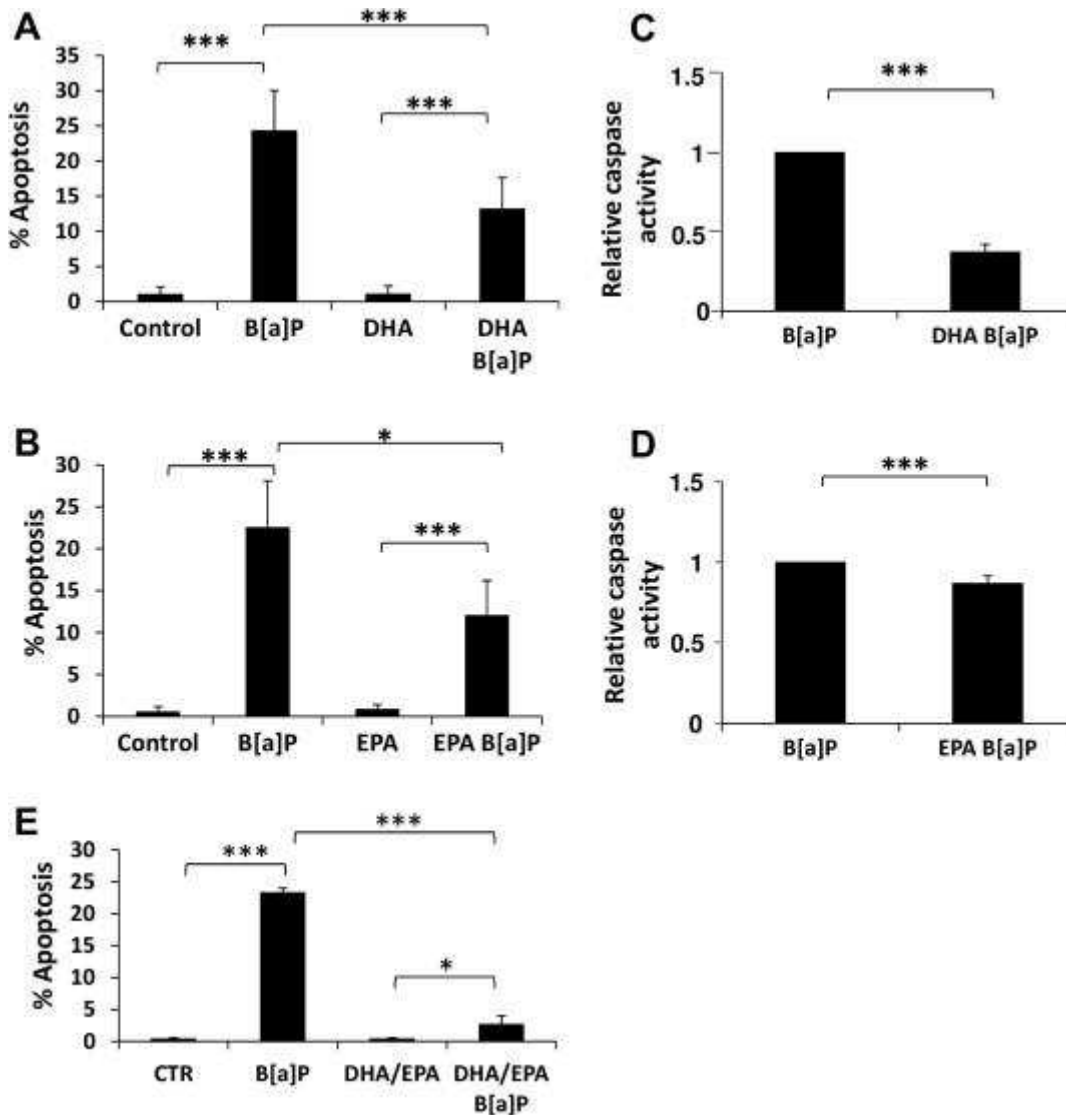


Fig. 1.

Effects of DHA and EPA on B[a]P-induced apoptosis. F258 cells were pre-treated or not 1 h with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P was added and incubation continued for a further 72 h. (A and B) Apoptotic nuclei were analyzed by fluorescence microscopy after staining with Hoechst 33342 ( $n = 12$  independent experiments). (C and D) DEVDase activities, which reflect caspases 3/7 activities, were measured by spectrofluorimetry, averaged from 6 independent experiments and expressed as relative fluorescence unit [RFU]/ $\mu$ g proteins normalized by the RFU of control cells. (E) F258 cells were pre-treated or not 1 h with DHA (70  $\mu$ M) + EPA (100  $\mu$ M) (DHA/EPA) and then co-

treated or not with 50 nM B[a]P for 72 h. Apoptotic nuclei were analyzed by Hoechst 33342 staining.  $N = 4$  independent experiments.

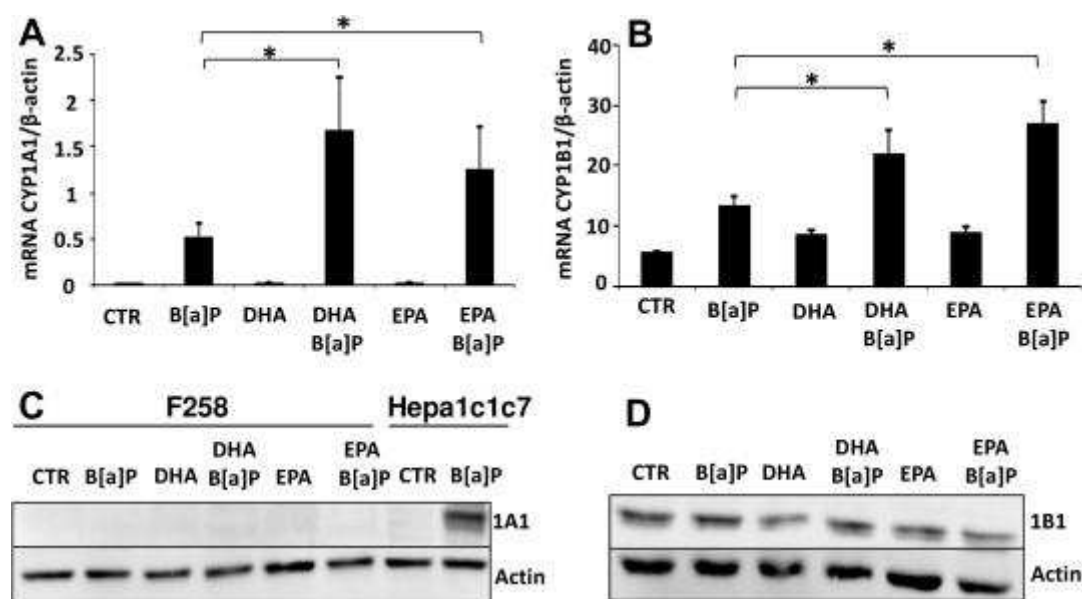


Fig. 2.

Effects of DHA and EPA on expression of P450 1A1 and P450 1B1. F258 cells were pre-treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P was added and incubation continued for a further 48 h. (A and B) Quantitative analysis of *CYP1A1* and *CYP1B1* mRNA levels ( $n = 3$  independent experiments). (C and D) 40  $\mu$ g of whole cell lysate were separated on 10% SDS–polyacrylamide gel electrophoresis. Immunoblots were probed with rabbit polyclonal anti-P450 1B1, -P450 1A1 or mouse monoclonal anti- $\beta$ -actin antibodies. Data are representative of 3 independent experiments. Protein lysates from Hepa1c1c7 cells were used as positive control.

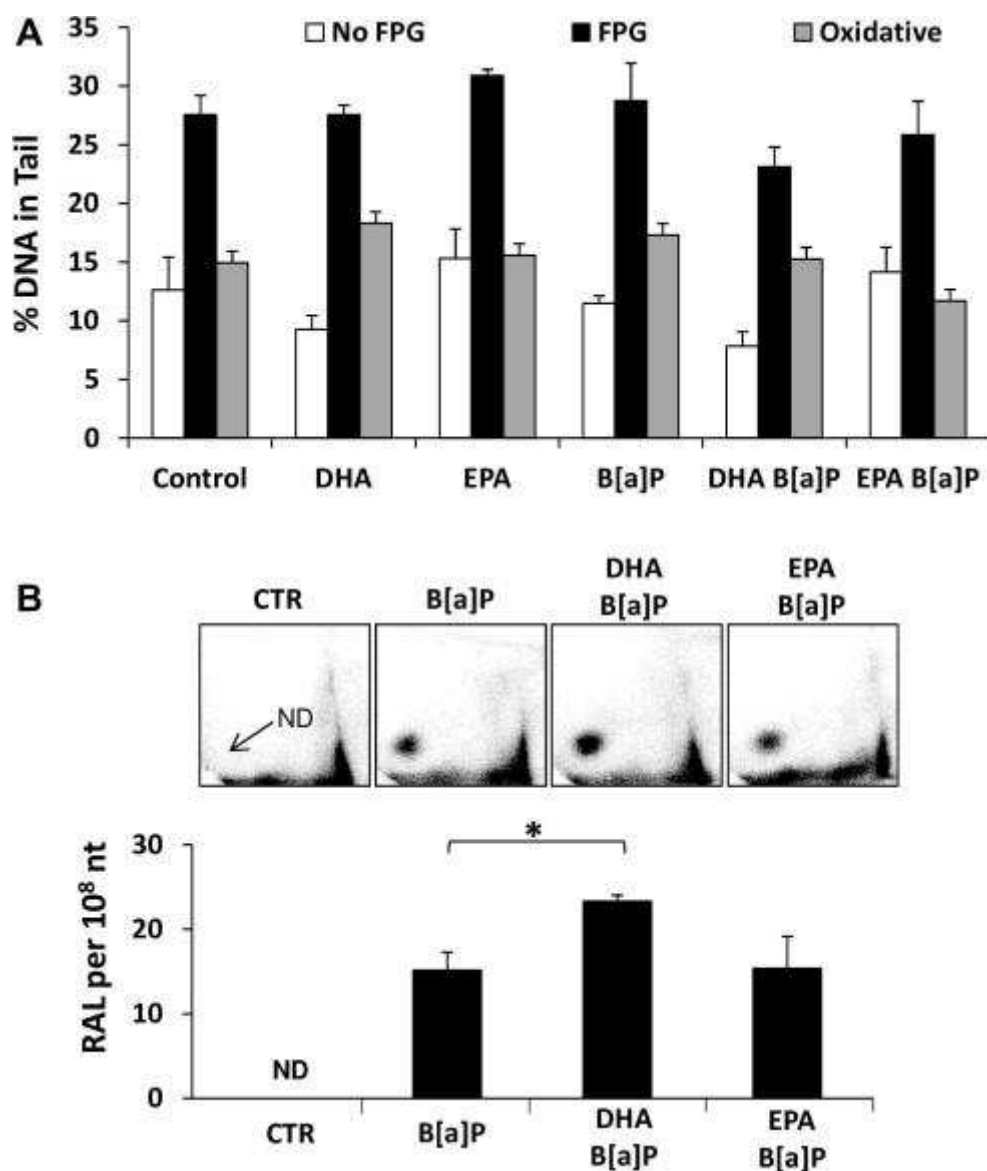


Fig. 3.

Effect of DHA and EPA on B[a]P-induced DNA damage. F258 cells were treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P was added and incubation continued for a further 24 h. (A) The comet assay was used to detect alkali-labile lesions. Formamidopyrimidine glycosylase (FPG) which detects oxidative damage to DNA including 8-oxo-deoxyguanosine (8-oxo-dG) was added in additional experiments ( $n = 3$  independent experiments). (B) Autoradiographic profiles of DNA adducts obtained by  $^{32}$ P-postlabeling. RAL (relative adduct labeling) of total DNA adducts following a 48 h exposure to B[a]P: B[a]P:  $15.16 \pm 3.01$ ; DHA/B[a]P:  $23.33 \pm 1.94$ ; EPA/B[a]P:  $15.37 \pm 4.67$  adducts/ $10^8$  nucleotides ( $n = 3$  independent experiments). ND, not detected.

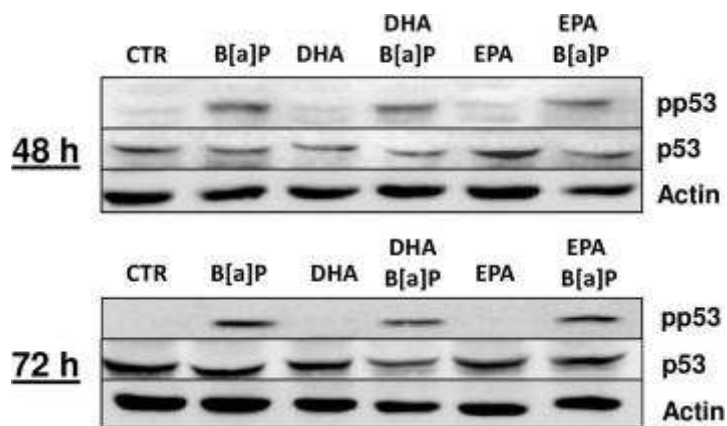


Fig. 4.

Effect of DHA and EPA on p53 activation. F258 cells were pre-treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P was added and incubation continued for a further 48 h or 72 h. 40  $\mu$ g of whole cell lysate were separated on 12% SDS–polyacrylamide gel electrophoresis. Immunoblots were probed with rabbit polyclonal anti-p53, -phospho Ser15 p53 (pp53),-or mouse monoclonal anti- $\beta$ -actin antibodies. Data are representative of at least 3 independent experiments.



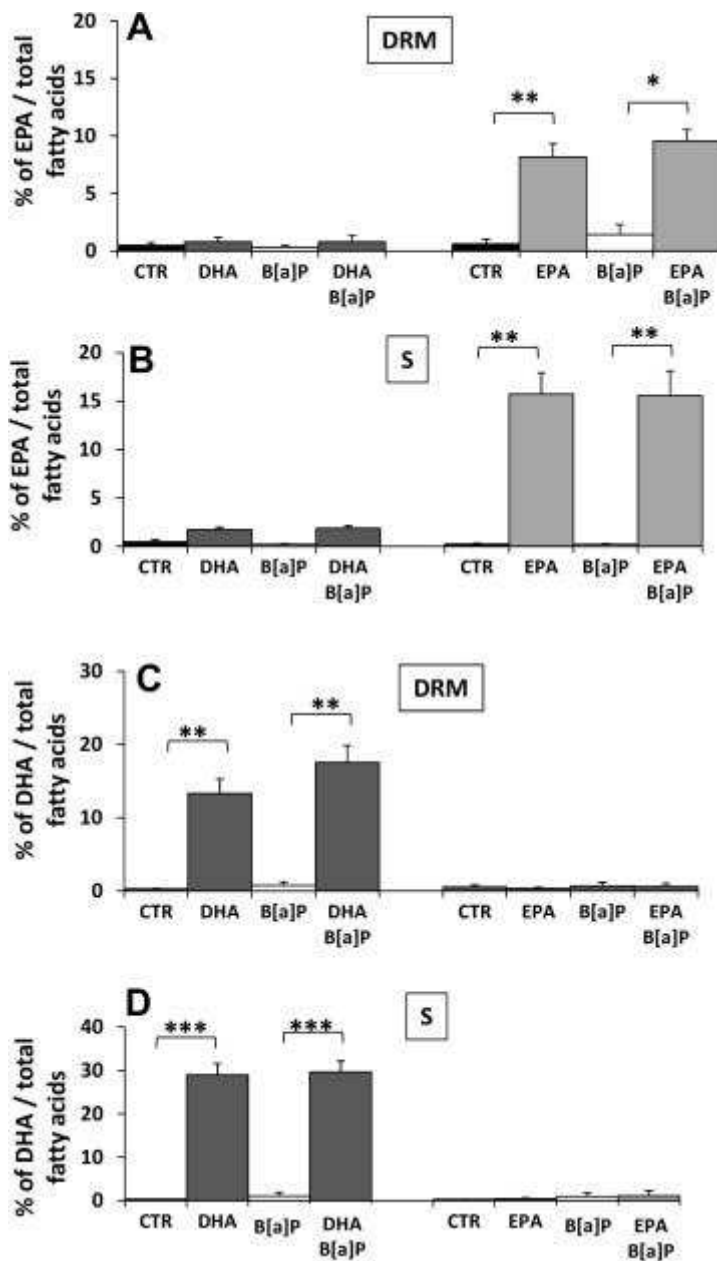


Fig. 5.

EPA (A,B) and DHA (C,D) incorporation in plasma membrane. Gas chromatography analysis of fatty acids from F258 cells pre-treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P was added and incubation continued for a further 48 h. The percentages of each EPA and DHA in raft (DRM; A,C) or non-raft (S; B,D) fractions were averaged from 4 independent experiments.

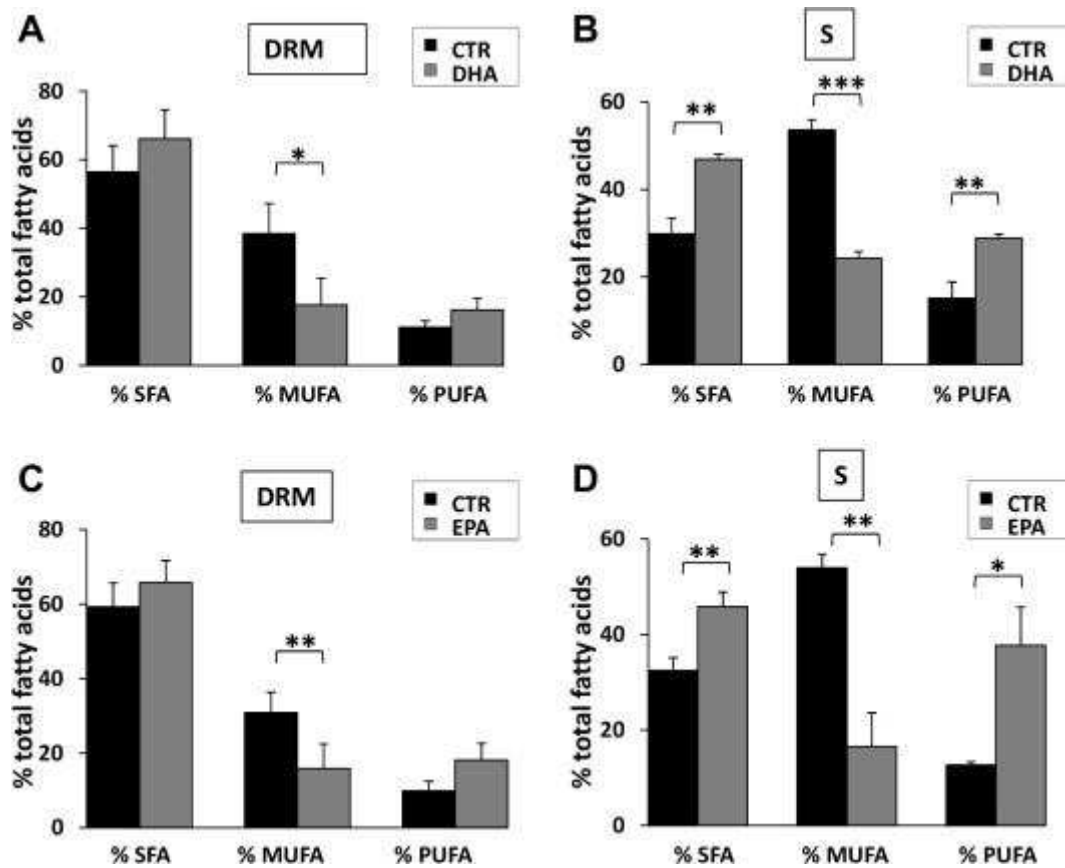


Fig. 6.

DHA (A,B) and EPA (C,D) change plasma membrane fatty acid composition. Gas chromatography analysis of fatty acids from F258 cells pre-treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P or not was added and incubation continued for a further 48 h. The percentages of saturated (SFA), polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids in raft (DRM, A,C) or non-raft (*S*; B,D) fractions were averaged from 5 independent experiments.

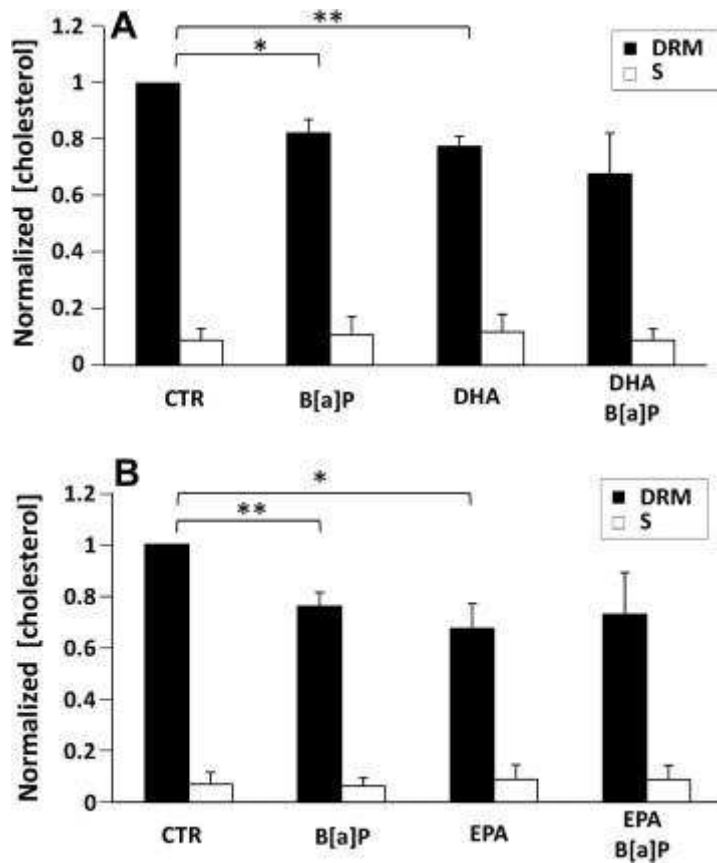


Fig. 7.

DHA (A) and EPA (B) decrease membrane cholesterol. F258 cells were pre-treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P or not was added and incubation continued for a further 48 h. After fractionation of lipid rafts, lipids were extracted by a 2:1 chloroform/methanol (v/v) mixture. The amount of cholesterol was determined by spectrophotometry in raft (DRM) and non-raft (S) fractions and results averaged from 4 independent experiments.

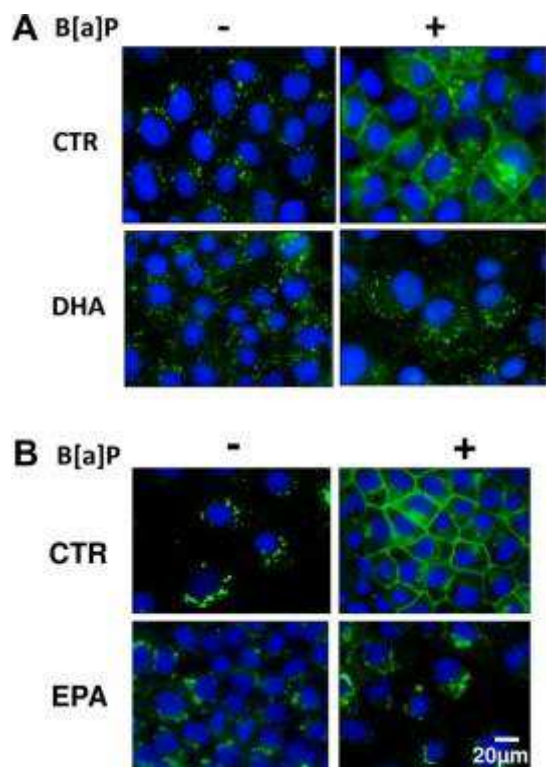


Fig. 8.

DHA (A) and EPA (B) inhibit B[a]P-induced reorganization of cholesterol-rich microdomains. F258 cells were pre-treated or not with DHA (140 μM) or EPA (200 μM), after which 50 nM B[a]P or not was added and incubation continued for a further 72 h. After fixation, staining of cells was performed with FITC-conjugated-cholera toxin subunit B (to visualize ganglioside GM1; green). DAPI (blue) stained the nuclei. Cells were viewed by fluorescence microscopy (magnification  $\times 400$ ). The experiments were repeated at least 3 times, with similar results. Scale bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

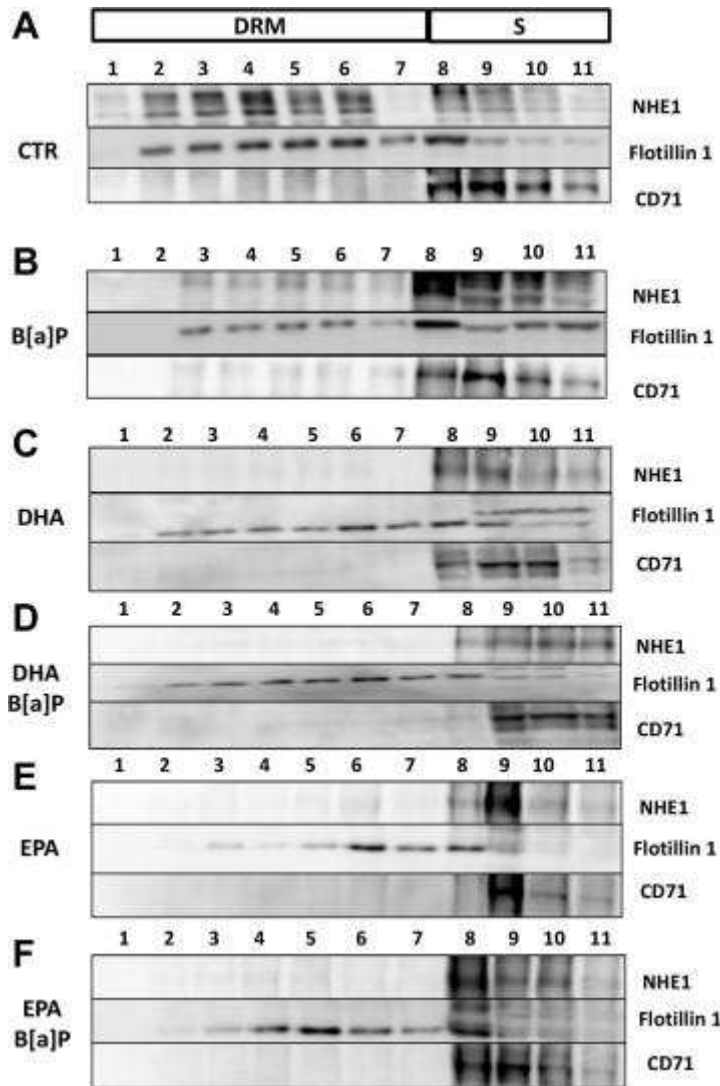


Fig. 9.

Effect of DHA and EPA on NHE1 localization. F258 cells were pre-treated or not (A,B) with DHA (140  $\mu$ M; C,D) or EPA (200  $\mu$ M; E,F), after which 50 nM B[a]P or not was added and incubation continued for a further 48 h. Raft (DRM) and non-raft (S) fractions were isolated on sucrose gradient and ultracentrifugation. Fractions 1–7 correspond to lipid rafts, and fractions 8–11 correspond to the non-raft fractions. 5  $\mu$ g of raft fractions and 100  $\mu$ g of soluble fractions were separated on 10% SDS–polyacrylamide gel electrophoresis. Immunoblots were probed with mouse monoclonal anti-NHE1, mouse monoclonal anti-flotillin, or -CD71 antibodies. Data are representative of at least 3 independent experiments.

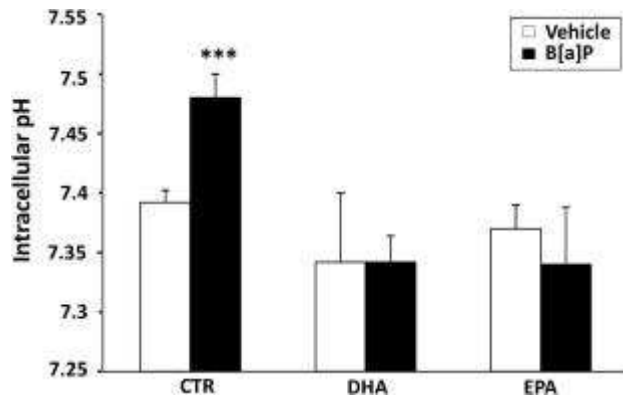


Fig. 10.

Effect of DHA and EPA on intracellular pH. F258 cells were pre-treated or not with DHA (140  $\mu$ M) or EPA (200  $\mu$ M), after which 50 nM B[a]P or not was added and incubation continued for a further 48 h. Resting pH<sub>i</sub> measurements were performed by microspectrofluorimetry using the pH-sensitive probe, carboxy-SNARF-1-AM.

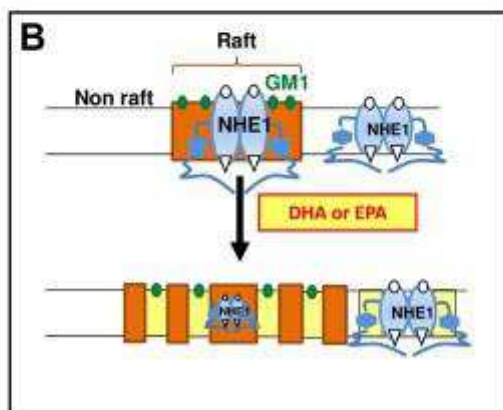
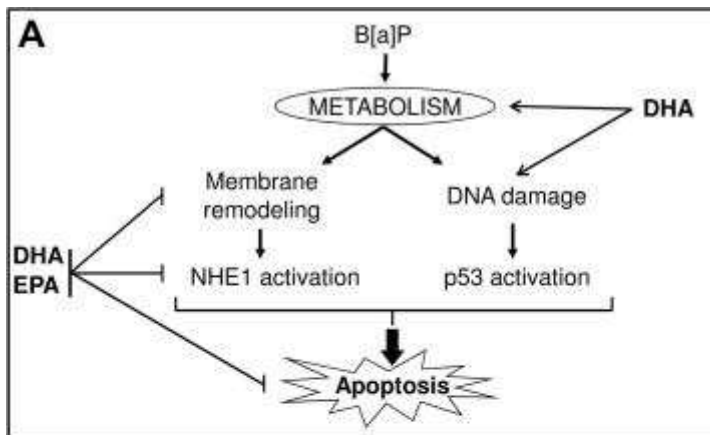


Fig. 11.

Schematic representation of the effects of DHA and EPA. (A) Effects of DHA and EPA on previously proposed intracellular signaling pathways involved in the apoptosis induced by B[a]P in F258 cells, and (B) on the microstructure and sub-membrane localization of NHE1 when used alone or in presence of B[a]P; the size of NHE1 in (B) refers to the amount of protein presently detected in the two types of microdomains.