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n-3 Polyunsaturated fatty acids alter benzo[*a*]pyrene metabolism and genotoxicity in human colon epithelial cell models☆

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ABSTRACT

Dietary carcinogens, such as benzo[a]pyrene (BaP), are suspected to contribute to colorectal cancer development. n-3 Polyunsaturated fatty acids (PUFAs) decrease colorectal cancer risk in individuals consuming diets rich in PUFAs. Here, we investigated the impact of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid on metabolism and genotoxicity of BaP in human cell models derived from the colon: HT-29 and HCT-116 cell lines. Both PUFAs reduced levels of excreted BaP metabolites, in particular BaP-tetrols and hydroxylated BaP metabolites, as well as formation of DNA adducts in HT-29 and HCT-116 cells. However, EPA appeared to be a more potent inhibitor of formation of some intracellular BaP metabolites, including BaP-7,8-dihydrodiol. EPA also reduced phosphorylation of histone H2AX (Ser139) in HT-29 cells, which indicated that it may reduce further forms of DNA damage, including DNA double strand breaks. Both PUFAs inhibited induction of CYP1 activity in colon cells determined as 7-ethoxyresorufin-*O*-deethylase (EROD); this was at least partly linked with inhibition of induction of CYP1A1, 1A2 and 1B1 mRNAs. The downregulation and/or inhibition of CYP1 enzymes by PUFAs could thus alter metabolism and reduce genotoxicity of BaP in human colon cells, which might contribute to known chemopreventive effects of PUFAs in colon epithelium.

1. Introduction

Colorectal cancer (CRC) represents one of leading causes of cancer-related death in Western countries, and it is a significant global health concern. The pathogenesis of CRC is a multistep process involving accumulation of mutations in specific oncogenes and tumor suppressor genes, as well as alterations of their expression induced by epigenetic changes, and it is significantly affected by lifestyle and environmental factors, in particular by diet (Ahmed, 2003). Therefore, dietary interventions, using biologically active food components as chemopreventive agents, have received a considerable attention, both in CRC and in other cancer types (Umar et al., 2012). During last decades, a number of experimental and epidemiological studies have supported the idea that the quantitative and qualitative content of essential polyunsaturated fatty acids (PUFAs), in particular the ratio of n-3/n-6 PUFAs in the diet, play a role in the etiology of colon cancer (Blasbalg et al., 2011). The anti-inflammatory and anti-cancer effects of a number of n-3 PUFAs have been widely documented, especially those of docosahexaenoic (DHA, 22:6) and eicosapentaenoic (EPA, 20:5) acids. High levels of DHA and EPA are found especially in fish oil, and both PUFAs are frequently used as dietary supplements. The molecular mechanisms of n-3 PUFA action include changes in membrane structure, alterations of membrane-associated signal transduction, direct interactions with transcription factors, altered eicosanoid biosynthesis or increased lipid peroxidation which causes an irreversible cell damage, in particular in tumor cells (Fuentes et al., 2018). A large number of studies has also documented the efficacy of n-3 PUFAs in supporting cancer therapy, including an improved response of the tumor to drug treatment (Klek, 2016; Morland et al., 2016).

Numerous animal studies have implicated chemical carcinogens present in diet, such as heterocyclic aromatic amines or polycyclic aromatic hydrocarbons (PAHs), to be involved in colorectal carcinogenesis (Gilsing et al., 2012; Wang et al., 2011). PAHs are a large family of toxic compounds generated from the combustion of organic

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materials, diesel exhaust and industrial waste. Exposure to PAHs has been associated with human cancers in various organs, including skin, lung, colon and bladder cancer (IARC, 2010). In humans, diet contributes substantially to exposure to PAHs, in particular in non-smokers, and cooking procedures have been proposed to significantly affect amount of PAHs that is present in consumed food (Domingo, 2017; Ma and Harrad, 2015; Phillips, 1999; Rose et al., 2015). Benzo[*a*]pyrene (BaP) is known human carcinogen, and a widely distributed environmental toxicant, that is found in cigarette smoke, industrial emissions or diet. The intestine is the primary site for numerous ingested xenobiotics, including toxicants, carcinogens, and drugs, and previous studies have implicated BaP as a causative agent in several cancer types, including CRC (Sachse et al., 2002). Mutagenicity and carcinogenicity of BaP strongly depend on its metabolism that is mediated primarily by cytochrome P450 family 1 (CYP1) enzymes. These form, together with microsomal epoxide hydrolase, the ultimate genotoxic metabolite benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), which forms covalent DNA adducts, primarily 10-(deoxyguanosin-*N*2-yl)-7,8,9- trihydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene (dG-*N*2-BPDE) adduct both *in vitro* and *in vivo* (Baird et al., 2005; Phillips and Venitt, 2012; Shimada et al., 2002). Therefore, the activity of CYP1A1 enzyme has been proposed to play a major role in organ-dependent detoxification and/or bioactivation of BaP, which makes it an attractive target for chemoprevention of carcinogenic effects of PAHs (Mescher and Haarmann-Stemmann, 2018; Shi et al., 2010). BaP can also be metabolized to BaP-quinones that may undergo redox cycling and contribute to oxidative stress and further DNA damage (Penning, 2014; Penning et al., 1999). Oxidative DNA damage, as well as formation of stable DNA adducts may both lead to formation of DNA double strand breaks (DSBs), which contribute to chromosomal aberrations and carcinogenesis. Interestingly, BaP has been also shown to stimulate DNA DSB repair *in vitro* and *in vivo*, through induction of expression of various DNA repair pathway genes (Tung et al., 2014).

Previously, dietary fish oil, which is a rich source of n-3 PUFAs, has been shown to play a protective role in PAH-induced carcinogenesis and to significantly reduce levels of DNA adducts, thus supporting the hypothesis that fish oil can be used as an anti-inflammatory, as well as chemopreventive agent (Barhoumi et al., 2014; Zhou et al., 2011). However, until now, only several studies have addressed the effects of individual PUFAs on BaP metabolism and/or genotoxicity (e.g. in liver cells or lung carcinoma model), and their results have indicated that the effects of PUFAs are likely to be cell type- and/or tissue-specific (Barhoumi et al., 2014; Dendelé et al., 2014). Presently, little is known about the impact of PUFAs on CYP1-mediated metabolism and genotoxicity of BaP in human colon cells, a potential target of dietary carcinogens. Therefore, in the present study, we investigated effects of EPA and DHA on BaP metabolism and genotoxicity in human colon cancer cell models.

2. Material and methods

2.1. Cell culture

Human colon cancer cell lines HT-29 and HCT-116 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in McCoy's 5A medium (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with penicillin-streptomycin (50 mg/l; Biosera, Nuaille, France) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco). Cells were routinely passaged twice a week and maintained at 37 °C in 5% CO_2 and 95% humidity.

2.2. Chemicals and experimental design

For the experiments, the cells were counted using a CASY (model TT, Roche, Czech Republic), seeded (3×10^4 cells per cm^2) in cell culture dishes (TPP, Trasadigen, Switzerland) and allowed to attach for 48 h. The medium was exchanged prior to treatment. DHA (Cayman Chemical, Ann Arbor, MI, USA; #90310) and EPA (Cayman Chemical, #90110) were dissolved in 96% ethanol and stock solutions were stored under nitrogen at −80 °C. Both DHA and EPA were diluted in the growth medium directly prior to the experiments and used at 50 or 100 μM final concentration. BaP (CAS No. 50-32-8, Ehrenstorfer, Augsburg, Germany) was dissolved in DMSO, stored in the dark, and used at final concentration of 10 μM or 30 μM. Cytotoxicity of all tested compounds was evaluated using neutral red uptake assay. Briefly, cells were seeded onto 96-well plates, incubated with tested compounds for 24 h and then with neutral red (final concentration 40 μg/ml) for 3 h. Following the medium removal, cells were fixed in 0.5% formaldehyde (containing 1 mM $CaCl_2$), washed twice with PBS and lysed in 1% acetic acid/50% ethanol for 15 min. Absorbance was then measured at 540 nm; 10 μM doxorubicin (Sigma-Aldrich; #D1515) was used as a positive control, and the respective solvents as negative controls. The concentrations of BaP and PUFAs used in the present study (with exception of a minor cytotoxicity observed for combination of 100 μM DHA and 10 μM BaP) were not toxic to the cells, as established by the neutral red assay in the present study (Supplementary Fig. 1), or in our previous work (Hofmanová et al., 2017). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; CAS No. 1746-01-6, Cambridge Isotope Laboratories, Andover, MA, USA) was dissolved in DMSO and used at 1 nM final concentration. The control cells were treated with respective solvents (their concentration did not exceed 0.05% v/v). None of the parameters tested in this study was significantly influenced by vehicle at this concentration as compared with untreated cells.

2.3. Analysis of BaP metabolites

BaP metabolite standards: (BaP-r-7,t-8,t-9,c-10-tetrahydrotetrol (±) (BaP-tetrol I-1), BaP-r-7,t-8,t-9,t-10-tetrahydrotetrol(±) (BaP-tetrol I-2), BaP-r-7,t-8,c-9,t-10-tetrahydrotetrol(±) (BaP-tetrol II-1), BaP-trans-7,8-dihydrodiol(±) (BaP-7,8-DHD), BaP-trans-9,10-dihydrodiol (BaP-9,10-DHD), BaP-trans-4,5-dihydrodiol(±) (BaP-4,5-DHD), BaP-3,6-dione, BaP-1,6-dione, BaP-6,12-dione, 1-OH-BaP, 3-OH-BaP, 7-OH-BaP, 8-OH-BaP and 9-OH-BaP) were all obtained from the National Cancer Institute (NCI), Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO, USA). Ethyl acetate p.a. ACS, methanol p.a. ACS and methanol HPLC gradient grade were purchased from Merck (Merck, Ricany-Jazlovice, Czech Republic). Ultrapure water was obtained from a Milli-Q UF Plus water system (Millipore, Molsheim, France).

Cells were washed with phosphate-buffered saline (PBS), scraped into 1.5 ml Eppendorf tubes and cell pellets were stored at −80 °C. Determination of BaP metabolites was performed as described previously (Kabátková et al., 2015). Briefly, cell pellets were extracted twice with 700 μl of ethyl acetate, combined extracts were dried under a stream of nitrogen, re-dissolved in 50 μl of methanol, and an aliquot of 10 μl was injected into the HPLC column. Solid-phase-extraction was used for extraction of BaP metabolites from cell culture medium after hydrolysis of glucuronidated and sulfated analytes. Conjugated metabolites were hydrolyzed with a mixture of β-glucuronidase and arylsulphatase (Sigma-Aldrich). The pH of cell culture medium (1 ml) was adjusted by diluted acetic acid to pH 5.5 and mixed with 0.1 ml of 1 M sodium acetate buffer, pH 5.5. Mixture was incubated overnight (for 16 h) at 37 °C with 20 μl of enzyme mixture. After cooling and acidifying with 20 μl of acetic acid, the samples were applied to primed Supel-Select HLB SPE tubes 30 mg/1 ml (Sigma–Aldrich). The SPE columns were then washed with 2 ml of 1% acetic acid and analytes were eluted with 1.5 ml of methanol. Finally, all media extracts were dried under a stream of nitrogen, re-dissolved in 60 μl of methanol, and an aliquot of 5 μl was injected into the HPLC column. The Agilent 1200 chromatographic system (Agilent Technologies, Santa Clara, CA), consisting of a binary pump, vacuum degasser, autosampler and thermostated column compartment was used for the LC–MS/MS analyses. The

conditions for analysis of BaP metabolites have been described previously (Kabátková et al., 2015). The levels of BaP metabolites were normalized to total protein concentrations; protein concentrations were estimated using DC assay protein kit (Bio-Rad, Prague, Czech Republic).

2.4. Detection of DNA adducts

Cells were washed with PBS, scraped into Eppendorf tubes and the pellets were stored at −80 °C. DNA was isolated using RNAse A, RNAse T1 and proteinase K treatment followed by phenol/chloroform/isoamyl alcohol extraction. DNA samples were stored at −80 °C until analysis. DNA samples (6 μg) were digested with a mixture of micrococcal endonuclease (Sigma-Aldrich, USA) and spleen phosphodiesterase (MP Biomedicals, France) for 4 h at 37 °C. Nuclease P1 (Yamasa Corporation, Japan) was used for adduct enrichment. Labelled DNA adducts were resolved via multidirectional TLC on 10 cm × 10 cm PEI-cellulose plates. Autoradiography was carried out at −80 °C for 24 h. Radioactivity of distinct adduct spots was measured using liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (1 μg of DNA hydrolysate) were analyzed for nucleotide content using reverse-phase High Performance Liquid Chromatography with UV detection, which simultaneously controlled for DNA purity. DNA adduct level was expressed as relative DNA adduct levels per 10^8 nucleotides. A BPDE-DNA adduct standard was run in a parallel sample, in order to determine variability between experiments.

2.5. Western blotting analysis

The extracts of total proteins obtained from treated cells were washed with PBS and prepared in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10% glycerol, 100 mM Tris pH 7.4, protease inhibitor mix, 1 mM Na_3VO_4, 1 mM NaF), heated for 10 min at 90 °C and sonicated (Branson Sonifier B-12A, Emerson Automation Solutions, Marshalltown, IA, USA). Protein concentrations were estimated using DC assay protein kit (Bio-Rad, Hercules, CA, USA), proteins were diluted to an equal concentration and separated by SDS-PAGE and transferred into polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany) by semi-dry blotting and the membranes were blocked in 5% non-fatty milk. Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): phospho-p53 (#2984; 1:500), phospho-Chk1 (#2349; 1:500), phospho-Chk2 (#2661; 1:500), Chk2 (#3440; 1:1000) or Santa Cruz Biotechnology (Santa Cruz, CA, USA) – Chk1 (#8408; 1:1000), p53 (#126; 1:1000). Primary antibodies were then detected using horseradish peroxidase-labelled anti-rabbit (Amersham, #NA934V), or anti-mouse (Amersham, #NA931V), secondary antibodies at dilution 1:3000 and chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore, #WBKLS0500) using ChemiDoc™ MP system (Bio-Rad). Anti-β-actin (antibody #A5441, Sigma–Aldrich) was used as a loading control. Densitometry was performed using Image J software.

2.6. Flow cytometric detection of DNA damage

Cells (5×10^5 per sample) were fixed in 4% formaldehyde for 15 min, washed with PBS, permeabilized in 0.25% Triton X for 15 min and washed in PBS. Cells were stained with anti-γH2AX phycoerythrin (PE)-conjugated antibody (Cell Signaling Technology; #5763) or isotype control for PE (Cell Signaling Technology; #5742) at 1:50 dilution in PBS with sodium azide for 1 h, washed with PBS and analyzed on BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

2.7. Flow cytometric analysis of S-phase

5-Ethynyl-2-deoxyuridine (EdU; 10 μM) was added to the medium 1 h prior to processing cells. Cells (5×10^5 per sample) were fixed in 4% formaldehyde for 15 min, washed with PBS, permeabilized in 0.25% Triton X for 15 min and again washed with PBS. Cells were stained with Click-iT EdU flow cytometry assay kit (Molecular Probes, Thermo Fisher Scientific; #C10420) according to the manufacturer's protocol for 30 min, washed with PBS and stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; AppliChem, Darmstadt, Germany; #A4099-0025) at dilution 1:1000 for 30 min. Stained cells were analyzed on BD FACSVerse flow cytometer.

2.8. Measurement of 7-ethoxyresorufin-O-deethylase (EROD) activity

EROD activity was determined as described previously (Kabátková et al., 2015). At the end of treatment, cells were washed with PBS, incubated 15 min in 150 μl ultra-pure water and then frozen at −80 °C for 30 min to lyse the cells. Resorufin production was measured in 96-well plate using 20 μl of cell lysate, 50 μl of 2 mM 3,3′-methylene-bis (4-hydroxycoumarin) (Sigma-Aldrich) in Tris-sucrose buffer pH 8.0 and 25 μl of 20 μM 7-ethoxyresorufin (Sigma-Aldrich) per each sample. Plates were pre-incubated for 20 min at 37 °C and the reaction was initiated by adding 25 μl of 1 mM NADPH per sample. Plates were incubated for 1 h at 37 °C. The resorufin production was measured in Fluostar Galaxy (BMG Labtech, Ortenberg, Germany) with an excitation filter set at 530 nm and an emission filter at 590 nm. Relative EROD activities were established as ratio between EROD activity and protein concentration, and expressed relative to control. Protein concentrations were estimated using DC assay protein kit (Bio-Rad).

2.9. Quantitative real-time RT-PCR

Total RNA was isolated using High Pure RNA Isolation Kit (#11 828 665 001, Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The primers and probes were designed with ProbeFinder (Roche Diagnostics) or provided by Generi Biotech (Hradec Kralove, Czech Republic). Where indicated, the primers were used with probes from the Universal Probe Library (UPL, Roche Diagnostics): CYP1A1 (NM_000499.3) F: 5′-CACCATCCCCCACAGCAC-3′, R: 5′-TTAC AAAGACACAACGCCCC-3′, probe: 5′-CAAGTTTGAAAGGCTTTTACATC CCC-3′; CYP1A2 (NM_000761.3) F: 5′-AGACCTTCCGACACTCCTC-3′, R: 5′-GTTTACGAAGACACAGCATTTC-3′, probe: 5′-CACCATCCCCCACAGC ACAA-3′; CYP1B1 (NM_000104.3) F: 5′-GCTTTTTCTCTTCATCTCC ATC-3′, R 5′-TTCATTTTCGCAGGCTCATTTG-3′, probe: 5′-CTCACCAGT GCGATTTCAGGGCCAAC-3′; TBP (NM_003194.4) F: 5′-GAACATCATG GATCAGAACAACA-3′, R: 5′-ATAGGGATTCCGGGAGTCAT-3′, UPL probe #87. TATA-Box Binding Protein (TBP) was used as a reference gene. The amplifications of samples were carried out using Superscript III Platinum One-Step Quantitative RT-PCR System (#11732-088, Thermo Fisher Scientific) on the RotorGene 6000 (Corbett Life Science, Qiagen, Germantown, MD, USA) real-time cycler, using the following program: reverse transcription at 50 °C for 15 min and initial activation step at 95 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s.

2.10. Statistical analysis

Results were expressed as means ± S.D. of three independent experiments. One-way ANOVA followed by a Tukey test or a nonparametric Mann–Whitney test were used for statistical analyses. Differences in % of max induction were assessed using one-sample *t*-test in case of comparison with a variable with no variation.

3. Results

3.1. n-3 PUFAs modulate BaP metabolism in human colon cell models

In order to evaluate the effects of two major dietary n-3 PUFAs - EPA and DHA on BaP metabolism in colon cells, we used two colon adenocarcinoma cell lines HT-29 and HCT-116 as colon epithelial cell

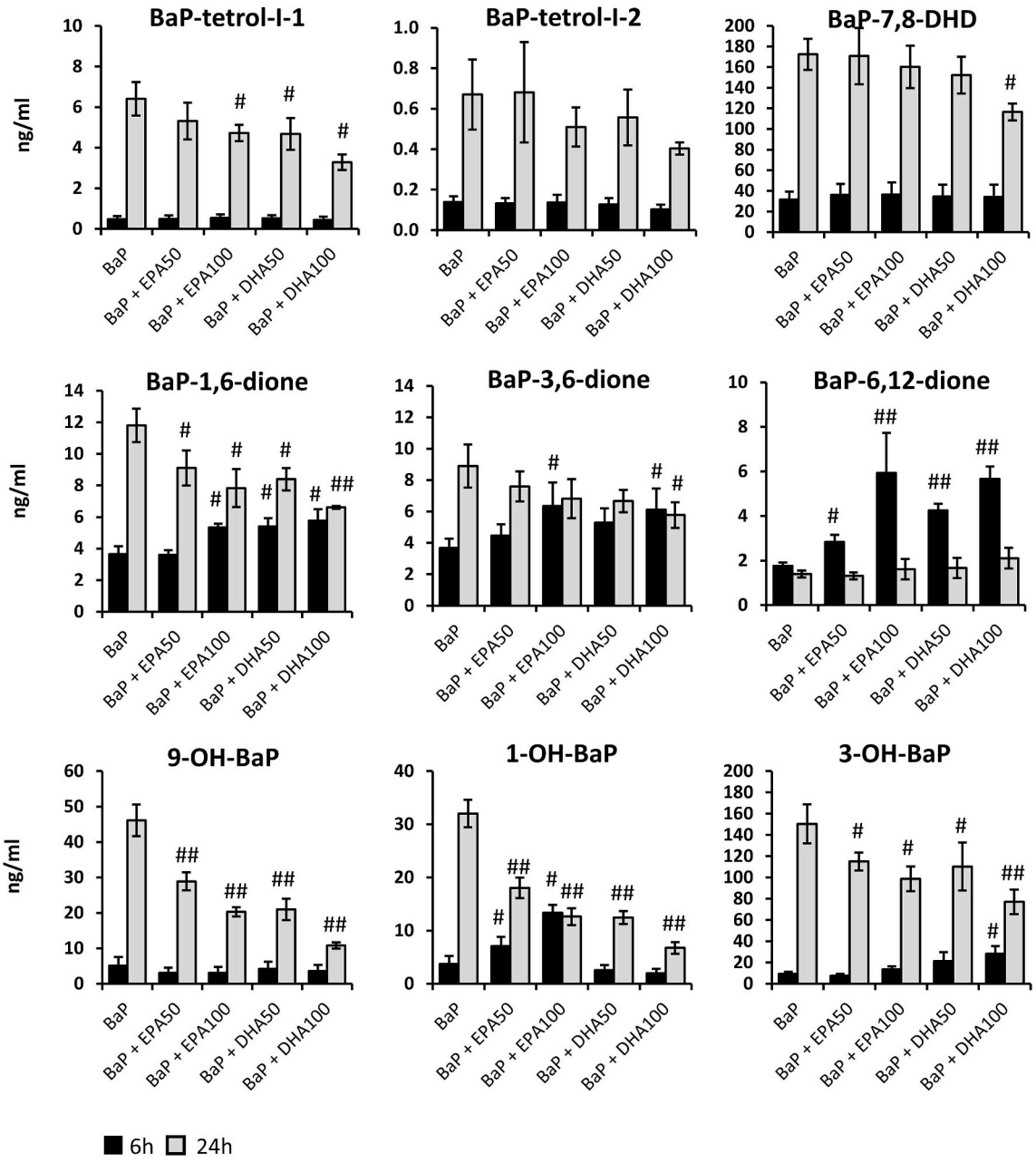

Fig. 1. Effects of EPA and DHA on levels of BaP metabolites in cell culture medium. HT-29 cells were treated with BaP (10 μM) alone or in combination with EPA (50 or 100 μM) or DHA (50 or 100 μM) for 6 and 24 h, respectively. Cell culture media were collected and frozen until further analyses. Following the hydrolysis of conjugated BaP metabolites with a mixture of β-glucuronidase and arylsulphatase, BaP metabolites were analyzed by LC-MS/MS, and the results were expressed as means ± S.D. of three independent experiments. Symbols '#' and '##' denote significant difference between BaP group and the respective treatment ($P < 0.05$ and $P < 0.01$, respectively).

models. Our previous study has indicated that 10 μM concentration of BaP is sufficient to induce a significant amount of DNA damage (formation of DNA adducts), as well as to induce expression of major BaP-bioactivating enzymes (CYP1) in both cell lines (Zapletal et al., 2017). For EPA and DHA, we used final concentration 50 μM (physiological, non-toxic dose), and also a higher 100 μM concentration – this was based on studies performed previously in our laboratory.

We investigated the impact of PUFAs on production of major BaP metabolites in cell culture medium following hydrolysis of conjugated metabolites with a mixture of β-glucuronidase and arylsulphatase. We focused on detection of signature BaP metabolites: BaP-tetrols (BaP-tetrol I-1 and BaP-tetrol I-2), BaP-7,8-dihydrodiol (BaP-7,8-DHD), BaP-diones (BaP-1,6-dione, BaP-3,6-dione and BaP-6,12-dione) and BaP-OHs (1-OH-BaP, 3-OH-BaP and 9-OH-BaP), which were all measured by LC-MS/MS method. The results are summarized in Fig. 1 (for HT-29 cells) and in Supplementary Fig. 2 (for HCT-116 cells). The overall impact of PUFAs on BaP metabolism was similar in both cell lines, with exception of production of BaP-diones (promoted by PUFAs in particular in HCT-116 cells) and 3-OH-BaP, as this particular metabolite was not detected in cell culture medium collected from HCT-116 cells. In general, HT-29 cell line appeared to be more sensitive to BaP exposure than HCT-116 cells, and significantly higher levels of all major BaP metabolites were formed in HT-29 cells. At 6 h, PUFAs mostly did not affect formation of BaP-tetrols, BaP-7,8-DHD or OH-BaPs. By contrast, both PUFAs promoted formation of BaP-diones at the same time point (which was even more evident in case of HCT-116 cells; Suppl. Figure 2). By 24 h, however, both PUFAs significantly reduced levels of major BaP metabolites being released into cell culture medium, again with

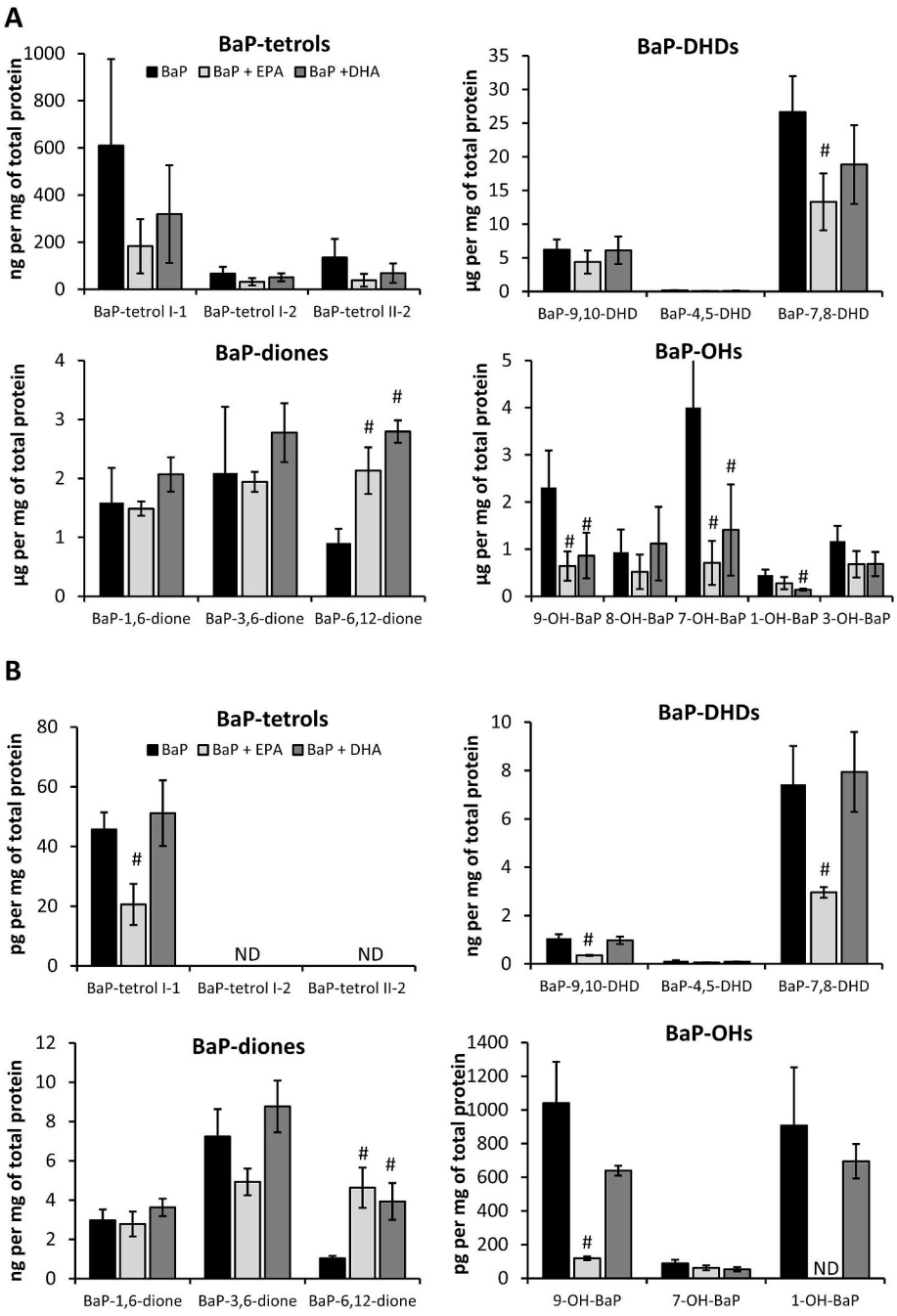

Fig. 2. Effects of EPA and DHA on formation of intracellular BaP metabolites. HT-29 cells **(A)** and HCT-116 **(B)** cells were treated with BaP (10 µM) alone or in combination with EPA (50 µM) or DHA (50 µM) for 24 h. Cell pellets were collected and BaP metabolites were analyzed by LC-MS/MS. The results were normalized to total protein levels and expressed as means ± S.D. of three independent experiments. Symbol '#' denotes significant difference between BaP alone, and the samples treated with combination of BaP and the respective PUFA ($P < 0.05$); ND, levels of the respective metabolite were below detection limit.

exception of BaP-diones (in particular BaP-6,12-dione). Importantly, both PUFAs reduced levels of BaP-tetrols in cell culture medium, the signature metabolites of CYP1-dependent metabolism (Lu et al., 2011), in a similar manner in both cell lines.

We also evaluated levels of BaP metabolites directly in cells after 24-h treatment. Here we found that, in contrast with a similar impact of both PUFAs on levels of BaP metabolites in cell culture medium, EPA more potently reduced levels of a majority of metabolites of BaP in both HT-29 (Fig. 2A) and HCT-116 (Fig. 2B) cells, including BaP-tetrols and BaP-7,8-diol. Here, DHA primarily decreased levels of only some BaP-OHs in both colon cell lines. By contrast, and in agreement with the above data on extracellular BaP metabolites, PUFAs did not reduce formation of final metabolites of the radical cation pathway (BaP-diones), and again even promoted formation of BaP-6,12-dione (Fig. 2).

3.2. PUFAs reduce formation of DNA adducts in human colon cell models

Since the above data indicated that PUFAs may significantly reduce BaP metabolism in colon cells after 24 h, we next focused on determination of BaP-induced genotoxicity in cells treated with both BaP and PUFAs at this time point. BaP induced formation of DNA adducts, as measured by ^{32}P-postlabeling method – on average 200 DNA adducts per 10^8 nucleotides in HT-29 cells vs. 20 DNA adducts per 10^8 nucleotides in HCT-116 cells. Here, both EPA and DHA were able to

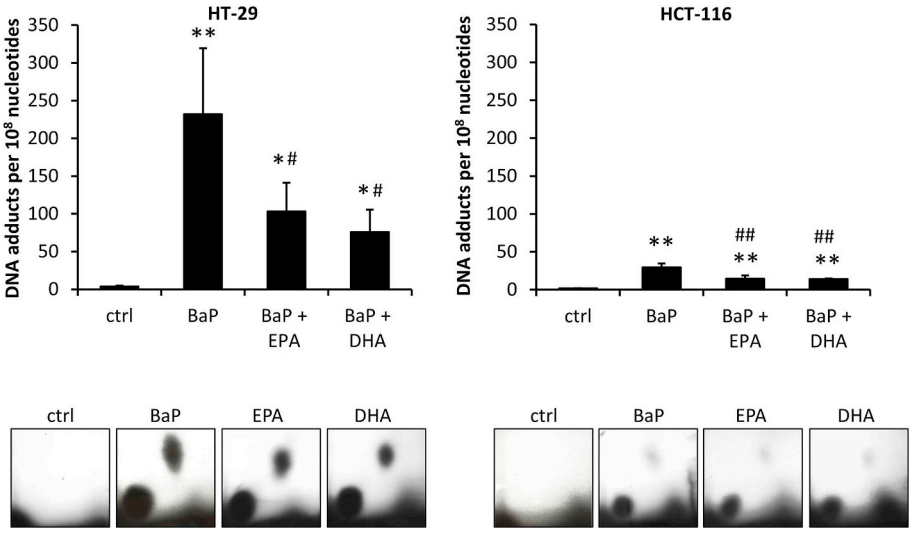

Fig. 3. EPA and DHA reduce formation of covalent DNA adducts. DNA adducts detected by P^{32} post-labeling in HT-29 (left) and HCT-116 (right) cells treated with BaP (10 μM) alone or in combination with EPA (50 μM) or DHA (50 μM) for 24 h. Results are expressed as means ± S.D. of three independent experiments. Symbols '*' and '**' denote significant difference between control and the respective treatment ($P < 0.05$ and $P < 0.01$, respectively). Symbols '#' and '##' denote significant difference between BaP alone, and the samples treated with combination of BaP and the respective PUFA ($P < 0.05$ and $P < 0.01$, respectively). Representative chromatograms of DNA adducts are shown at the bottom of each graph.

significantly decrease amount of DNA adducts in both cell lines, as compared with BaP induction (Fig. 3).

3.3. EPA may inhibit further forms of DNA damage in colon cells

Since the above results (production of BaP metabolites and formation of DNA adducts) suggested that HT-29 cells could be more sensitive to genotoxic effects of BaP, we next compared DNA damage response in both colon cell models. Here, we used two different BaP concentration 10 μM and 30 μM, as previous studies have indicated that 30 μM concentration of BaP induces maximum response of endpoints linked with genotoxicity in some human cell models (Staal et al., 2006). We first analyzed both total levels and the levels of active phosphorylated forms of proteins involved in DNA damage response – checkpoint kinases (Chk) 1 and 2, and p53. In HT-29 cells, BaP induced major phosphorylation of Chk1 (Ser296), Chk2 (Thr68) and p53 (Ser15), while in HCT-116 cells the response was much weaker (Fig. 4A). This partly corresponded with lower levels of DNA adducts being formed in HCT-116 cells. A similar trend was observed also when we evaluated effects of BaP on levels of histone H2AX phosphorylated at Ser139 (γH2AX). Again, a higher percentage of HT-29 cells was positive for γH2AX, as compared with HCT-116 cells (Fig. 4B). The higher (30 μM) concentration of BaP did not further increase activation of Chk1, Chk2 and p53, or H2AX phosphorylation, as compared with 10 μM concentration, thus suggesting that the lower concentration was sufficient to induce a significant DNA damage in our experiments.

Therefore, we next focused on HT-29 cells, which had more active BaP metabolism, and which appeared to be more sensitive to the genotoxic effects of BaP. We investigated whether EPA and DHA are able to further decrease DNA damage (detected as phosphorylation of DNA damage response proteins) induced by BaP. We found that both PUFAs only slightly reduced phosphorylation of Chk2 (Fig. 5A). Other BaP-induced DNA damage response parameters (Chk1 and p53 phosphorylation) were not affected by either PUFA. Finally, we observed that EPA significantly reduced γH2AX levels, a marker of formation of DSBs, as detected by flow cytometry (Fig. 5B).

3.4. BaP-induced S phase cell cycle arrest is not prevented by PUFAs

The accumulation of cells in S-phase is another consequence of substantial DNA damage induced by genotoxic PAHs (Topinka et al., 2008). Therefore, we next investigated the impact of PUFAs on the BaP-induced accumulation of HT-29 cells in S-phase. We analyzed cell cycle distribution by flow cytometry using double staining with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) and 5-ethynyl-2-deoxyuridine (EdU), nucleoside analog of thymidine, which is incorporated into DNA during active DNA synthesis. This enabled us to determine the percentage of S-phase cells in the respective cell population. We found that exposure to BaP induced accumulation of cells in S-phase (an increase from 40 to 60% of cells in S-phase, as compared with control cells); however, neither EPA nor DHA prevented this increase (Fig. 6).

3.5. EPA and DHA inhibit, to a different extent, expression and/or activity of CYP1A enzymes in HT-29 cells

CYP1 enzymes play a major role in the detoxification, as well as in bioactivation of BaP to the highly carcinogenic BPDE. In particular, CYP1A1 has been found to contribute to DNA adducts formation in HCT-116 cells (Kabátková et al., 2015; Wohak et al., 2016). Therefore, we next evaluated effects of EPA and DHA on induction of CYP1 activity in HT-29 and HCT-116 cells. We determined 7-ethoxyresorufin-*O*-deethylase (EROD) activity, as a marker of CYP1 activity, in colon cancer cell lines. We used TCDD as a model aryl hydrocarbon receptor (AhR) ligand and CYP1 inducer for this purpose, because BaP has been found to inhibit catalytic activities of human CYP1 enzymes towards 7-ethoxyresorufin (Shimada and Guengerich, 2006), and, because our previous work has also indicated that TCDD is a more reliable EROD inducer in colon cells (Kabátková et al., 2015; Zapletal et al., 2017). As shown in Fig. 7A, both EPA and DHA significantly decreased EROD activity induced by TCDD in HT-29, as well as in HCT-116 cells (Fig. 7A).

Finally, we measured induction of CYP1A1, 1A2 and 1B1 mRNA levels, using qRT-PCR, in both HT-29 and HCT-116 cells. All CYP1 enzymes were inducible by BaP in HT-29 and HCT-116 cells; however, PUFAs differentially modulated their induction in a cell-specific manner. In HT-29 cells, EPA significantly decreased mRNA levels of CYP1A1, 1A2 and 1B1 mRNAs, while DHA only partly reduced CYP1A2 in this cell line (Fig. 7B). In contrast, both PUFAs prevented CYP1A1 and 1A2 induction in HCT-116, whereas only DHA reduced CYP1B1 induction in this cell line (Fig. 7B).

4. Discussion

Cancer chemoprevention is one of promising strategies aiming to reduce both cancer incidence and mortality (Umar et al., 2012). Nutrition optimization and dietary supplementation with natural chemoprotective agents may offer significant health benefits. In recent *in vivo* and *in vitro* experimental studies, diets rich in n-3 PUFAs have been shown to provide significant anti-tumor protection (Fuentes et al.,

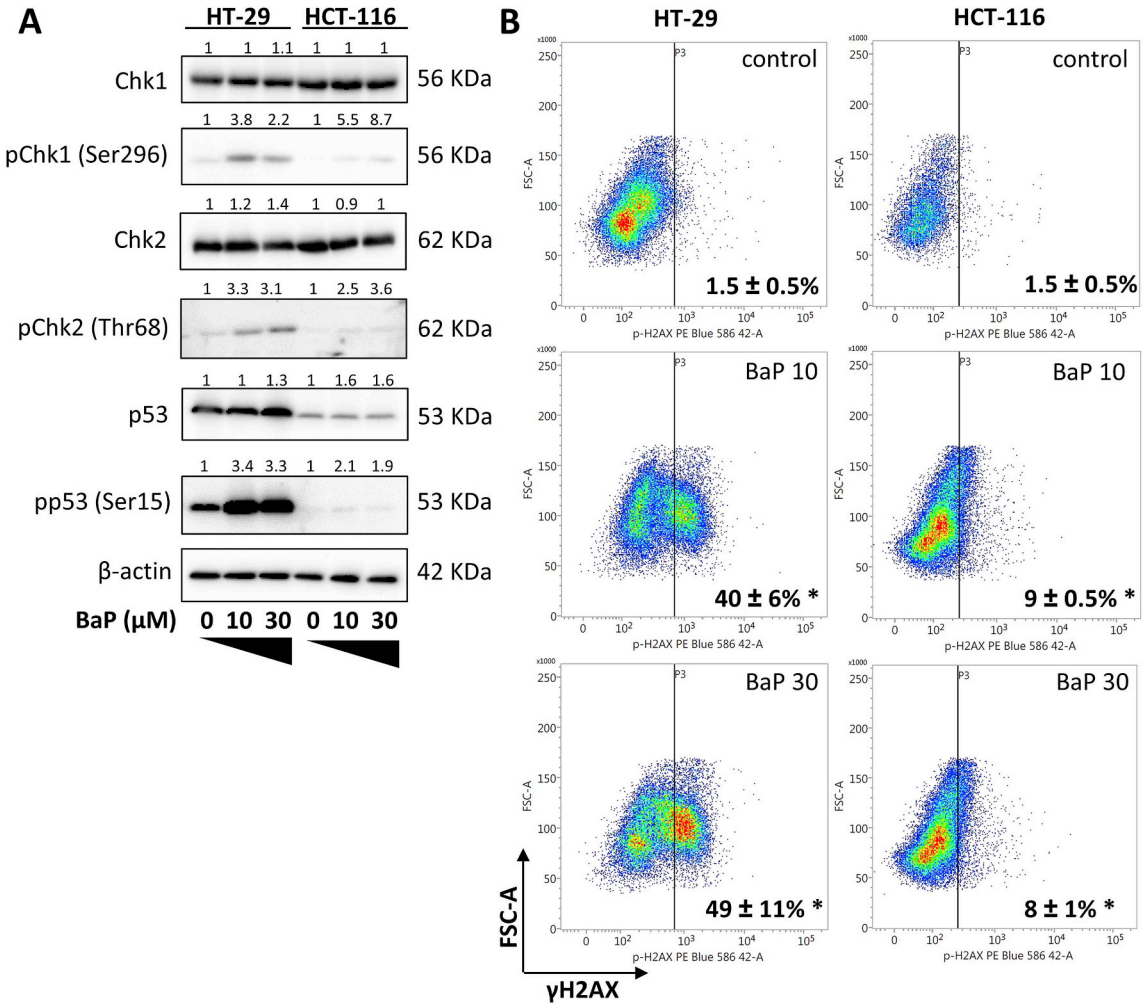

Fig. 4. Induction of DNA damage response in HT-29 and HCT-116 cells treated with BaP. HT-29 cells and HCT-116 cells were treated with BaP (10 μM; 30 μM) for 24 h. **(A)** Levels of total and phosphorylated proteins involved in DNA damage response (Chk1, Chk2 and p53) were determined by Western blotting analysis. Representative results from three independent experiments are shown; β-actin was used as a loading control. Densitometry was performed using Image J software and protein levels were expressed relative to control; the results are expressed as means of three independent experiments and shown above each Western blot. **(B)** Percentage of γH2AX-positive cells as measured by flow cytometry. The numbers in dot plots represent means ± S.D. of three independent experiments. Symbol '*' denotes significant difference between control and the respective treatment ($P < 0.05$).

2018). Modulation of expression of enzymes involved in bioactivation and/or detoxification of BaP, such as CYP1A1, is an approach with a potential to reduce BaP-induced carcinogenesis (Mescher and Haarmann-Stemmann, 2018). In the present study, we evaluated the hypothesis that interaction between dietary n-3 PUFAs, EPA and DHA, and BaP, a well-known dietary carcinogen, may alter its metabolism and genotoxicity in human colon cell models.

We used HT-29 and HCT-116 colon adenocarcinoma cell lines, which are well characterized in terms of expression of enzymes involved in metabolism of chemical carcinogens and other xenobiotics. In particular, HT-29 cells have been proposed to have enzymatic profile corresponding to normal human colonic tissue (Bourgine et al., 2012). Their response to n-3 PUFAs has been also extensively characterized in our laboratory (Hofmanová et al., 2017; Tylichová et al., 2017). The principal finding of our study is that n-3 PUFAs were able to alter metabolism and reduce genotoxic effects of BaP in colon cell models. Interestingly, just recently, DHA has been reported to inhibit genotoxicity and production of several BaP metabolites in human lung adenocarcinoma A549 cells (Barhoumi et al., 2014). DHA-treated cells exhibited significantly lower amount of 3-OH-BaP, BaP-7,8-diol or pyrene-like metabolites, as compared with oleic acid or linoleic acid (n-9 monounsaturated fatty acid or n-6 PUFA) treated cells, which suggests that this inhibitory activity could be specific for n-3 PUFAs (Barhoumi et al., 2014). Nevertheless, DHA has been reported to increase BaP metabolism in a model of rat liver epithelial cells (Dendelé et al., 2014), which suggests that effects of PUFAs on BaP metabolism could be also species- or tissue-specific.

Our experiments have indicated that PUFAs acted in both time and concentration-dependent manner in colon cell models. At the earlier 6-h time point, we observed potentiation of formation of some signature BaP metabolites, such as BaP-diones, in particular BaP-6,12-dione, while others, including BaP-tetrols, were not affected. By contrast, levels of most of the evaluated BaP metabolites in cell culture medium decreased after 24-h exposure. Importantly, both PUFAs decreased levels of extracellular BaP-tetrols in colon cell lines after 24 h, which seemed to correspond well with their effects on CYP1 expression/activity and with formation of DNA adducts at this time point. We also observed that after 24 h, intracellular levels of metabolites were more reduced by EPA. Our results thus suggest that EPA could be even more potent inhibitor of BaP metabolism than previously reported DHA (Barhoumi et al., 2014). The effects of PUFAs on formation of BaP-diones (especially in case of BaP-6,12-dione) also indicated that metabolism of BaP via the radical cation pathway (which is mediated by peroxidases) could be potentiated when production of BaP metabolites by CYP1 enzymes is compromised.

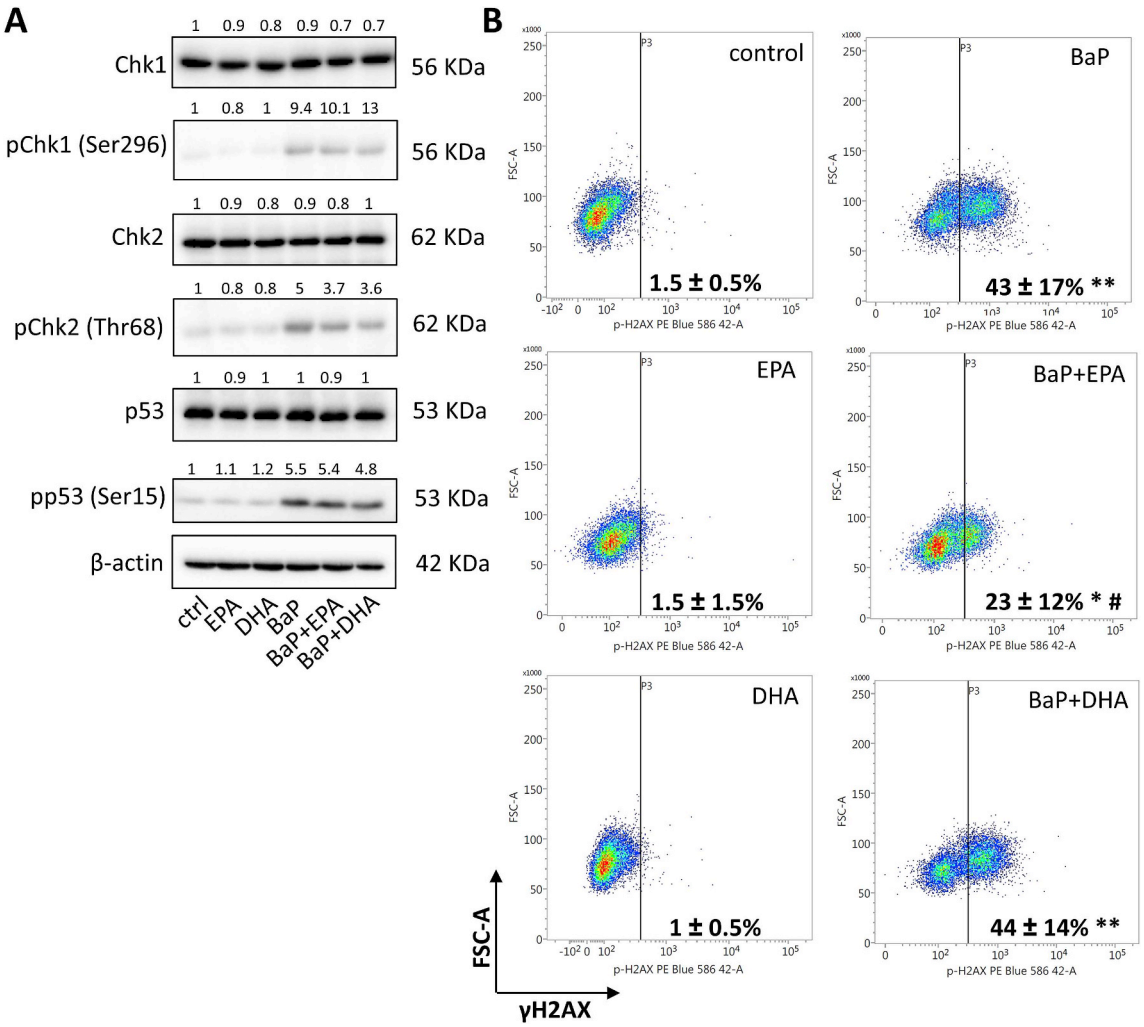

Fig. 5. EPA reduces γH2AX levels following BaP treatment. HT-29 cells were treated with BaP (10 μM) alone or in combination with EPA (50 μM) or DHA (50 μM) for 24 h. **(A)** Levels of total and phosphorylated proteins involved in DNA damage response (Chk1, Chk2 and p53) were determined by Western blotting analysis. Representative results from three independent experiments are shown; β-actin was used as a loading control. Densitometry was performed using Image J software and protein levels were expressed relative to control; the results are expressed as means of three independent experiments and shown above each Western blot. **(B)** Percentage of γH2AX-positive cells was determined by flow cytometry, using anti-γH2AX PE-conjugated antibody. The numbers in dot plots represent means ± S.D. of three independent experiments. Symbols '*' and '**' denote significant difference between control and the respective treatment ($P < 0.05$ and $P < 0.01$, respectively). Symbol '#' denotes significant difference between BaP alone, and the samples treated with combination of BaP and the respective PUFA ($P < 0.05$).

The n-3 PUFAs have been previously suggested to inhibit *in vivo* the genotoxic effects of BaP in mice, or 7,12-dimethylbenz[*a*]anthracene (a highly genotoxic and carcinogenic PAH) in rat (Manna et al., 2008). Interestingly, dietary fish oil, which contains high levels of n-3 PUFAs has been shown to play a protective role in PAH-induced hepatocarcinogenesis and to significantly reduce levels of DNA adducts in mice (Zhou et al., 2011). The major DNA adduct has been previously identified as 10-(deoxyguanosin-N2-yl)-BPDE (Arlt et al., 2008). Here, we observed that EPA and DHA were able to decrease amount of BPDE-DNA adducts, as well as that of one additional adduct, which identity is pending. Just recently, two additional BaP-derived DNA adduct spots, similar to the one observed in our experiment, have been detected in mouse embryonic fibroblasts that have not been structurally identified so far (Krais et al., 2015). DHA and EPA were found to reduce levels of DNA adducts in a similar manner in both HT-29 and HCT-116 cell lines. Again, as mentioned above, in the study of Dendelé et al., DHA has actually increased levels of DNA adducts in rat liver epithelial F258 cells, which had also corresponded with increased BaP metabolism and CYP1 expression (Dendelé et al., 2014). This suggests that effects of PUFAs could be specific for different cell types, and that more attention should be paid to cell types derived from other cell populations representing human target tissues for BaP and related dietary carcinogens, such as colon epithelium. As mentioned above, the chemopreventive potential of lipid mixtures containing high levels of PUFAs, linked to inhibition of metabolism of PAHs, is supported by further *in vivo* studies. Fish oil significantly reduces levels of DNA adducts in the liver of mice treated with various PAHs (Zhou et al., 2011). Another *in vivo* study showed that treatment of mice with BaP and olive oil significantly altered the expression of xenobiotic-metabolizing enzymes CYP1A1 and CYP1B1 in both colon and liver tissue. Olive oil has been reported to promote detoxification of BaP and decrease the extent of DNA damage to colon and liver tissues, and it has been shown to suppress intestinal polyp growth through additional mechanisms (Banks et al., 2016; Barone et al., 2014). Suppression of bioactivation of dietary carcinogens, such as BaP, may thus contribute to protective effects of n-3 fatty acids.

DSBs are the most deleterious DNA lesions, which, if left unrepaired, may have severe consequences for cell survival and carcinogenesis, as they lead to chromosome aberrations, genomic instability, or cell death. Cells respond to DNA damage by activating the DNA damage response, a complex molecular mechanism developed to detect and repair DNA damage. It is well established that, upon formation of DSBs, histone

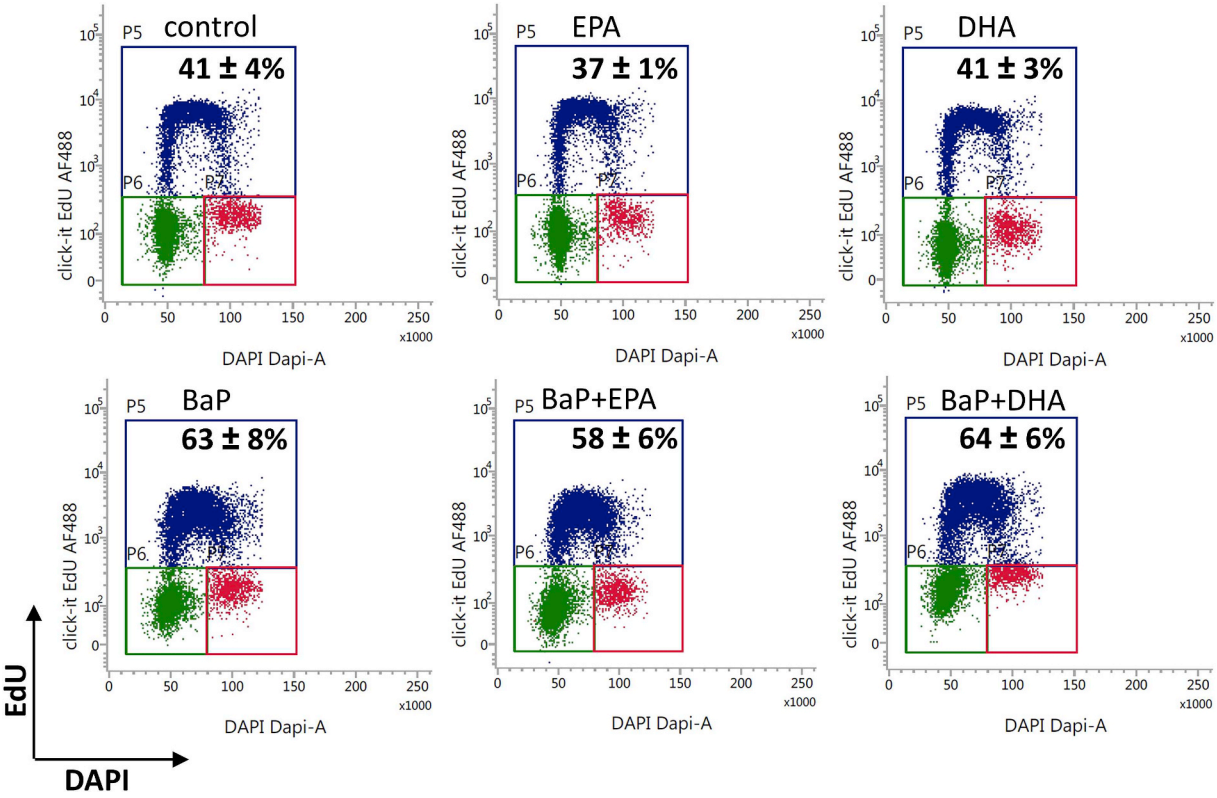

Fig. 6. Effects of BaP and PUFAs on cell cycle progression. HT-29 cells were treated with BaP (10 μM) alone or in combination with EPA (50 μM) or DHA (50 μM) for 24 h. EdU was added 1 h before processing the cells. Percentage of cells in S-phase was determined by flow cytometry. Figure combines DNA content (DAPI staining) with EdU (Alexa Fluor 488 staining). Cells positive for both labels are in S-phase of the cell cycle (marked blue area). Dot plots are representative of three independent experiments; numbers represent means ± S.D. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

H2A variant H2AX becomes phosphorylated at Ser139 (here denoted as γH2AX). This phosphorylation increases during cell cycle progression, and, in particular, in response to major DNA damage. It is mediated by ataxia-telangiectasia mutated (ATM) protein kinase, or other kinases (Rogakou et al., 1998). Apart from H2AX, the target substrates of ATM include also checkpoint kinases, Chk1 and, in particular Chk2, and ATM contributes also to p53 stabilization and phosphorylation. Activation of Chk1, Chk2 and p53 inhibits cell cycle progression and activates further proteins responsible for DNA repair (Shanmugam et al., 2018). In our experiments, BaP significantly induced phosphorylation of Chk1, Chk2 and p53 tumor suppressor, and it increased γH2AX levels in HT-29 cells. Previous studies have correlated BaP exposure to the formation of DSBs, as e.g. human lung A549 cells exposed to BPDE exhibit increased levels of γH2AX (Mattsson et al., 2009). Together, this suggests that BaP induced DSBs in HT-29 cells (and to a lesser extent also in HCT-116 cell line) and that formation of DSBs in cells exposed to BaP was partly prevented by EPA. EPA was also a more potent inhibitor of formation of some intracellular BaP metabolites, including BaP-7,8-DHD. This dihydrodiol metabolite is not only a precursor for BPDE formation, but its metabolization via cytosolic aldo-keto reductases may yield further toxic metabolites leading to oxidative DNA damage and formation of DNA double strand breaks (Park et al., 2008, 2009). This may in part explain the differential effects of EPA on endpoints linked with formation of DNA strand breaks, such as γ-H2AX, which reflects DSB formation, as opposed to similar effects of both PUFAs on formation of DNA adducts.

The activity of CYP1 enzymes plays a key role in BaP metabolism and its bioactivation (Mescher and Haarmann-Stemmann, 2018; Nebert and Dalton, 2006). BaP was a potent inducer of CYP1A1, 1A2 and 1B1 mRNAs in both colon cell lines used in the present study. DHA has been previously shown to downregulate CYP1A1, which contributes to alleviation of toxic effects caused by PAHs in endothelial cells (Gdula-Argasinska et al., 2015). Resolvin D1, a product of sequential oxygenation of DHA by 15- and 5- lipoxygenases, has been proposed to mediate this effect of DHA on toxicity of BaP in endothelium (Gdula-Argasinska et al., 2016). Just recently, Cyp1a1 has been also found to protect against non-alcoholic fatty liver disease caused by Western diet containing BaP in mice, or BaP-induced hepatic inflammation in Cyp1a1(−/−) mice, but not in wild-type mice (Uno et al., 2018). The protective effects of PUFAs in the present study could thus be partly explained by inhibition of CYP1 induction. Here, the effects of PUFAs appeared to be both PUFA- and cell-specific, which may seem to be in contrast with their effects on DNA adduct formation. However, both PUFAs may also directly modify activities of CYP1 enzymes. It has been shown that some n-3 PUFAs may inhibit activities of CYP enzymes (Yao et al., 2006), and importantly, that high levels of PUFAs may act as substrates for CYP1 enzymes (Divanovic et al., 2013; Mescher and Haarmann-Stemmann, 2018), which could in turn limit their activity towards BaP as a substrate. Additionally, it has been proposed that DHA may reduce the abundance of BaP-7,8-dihydrodiol and the 3-OH-BaP metabolites through its effects on the physicochemical properties of cell membranes, thus leading to altered activity of membrane-associated enzymes, including CYP1 enzymes (Barhoumi et al., 2014). Here, we used TCDD to stimulate EROD activity in colon cell lines, in order to evaluate the impact of PUFAs on CYP1 activity. While these results should be interpreted with caution, as PUFAs may differentially alter the BaP-induced CYP1 activity, we have observed that both EPA and DHA inhibited the TCDD-induced EROD activity in HT-29 and HCT-116 cells. This corresponded well with our observations that EPA and DHA similarly inhibit both production of extracellular BaP-tetrols and formation DNA adducts in colon cell models. Together, this seems to indicate that multiple mechanisms may contribute to inhibition of CYP1 expression/activity by n-3 PUFAs in colon epithelial cells.

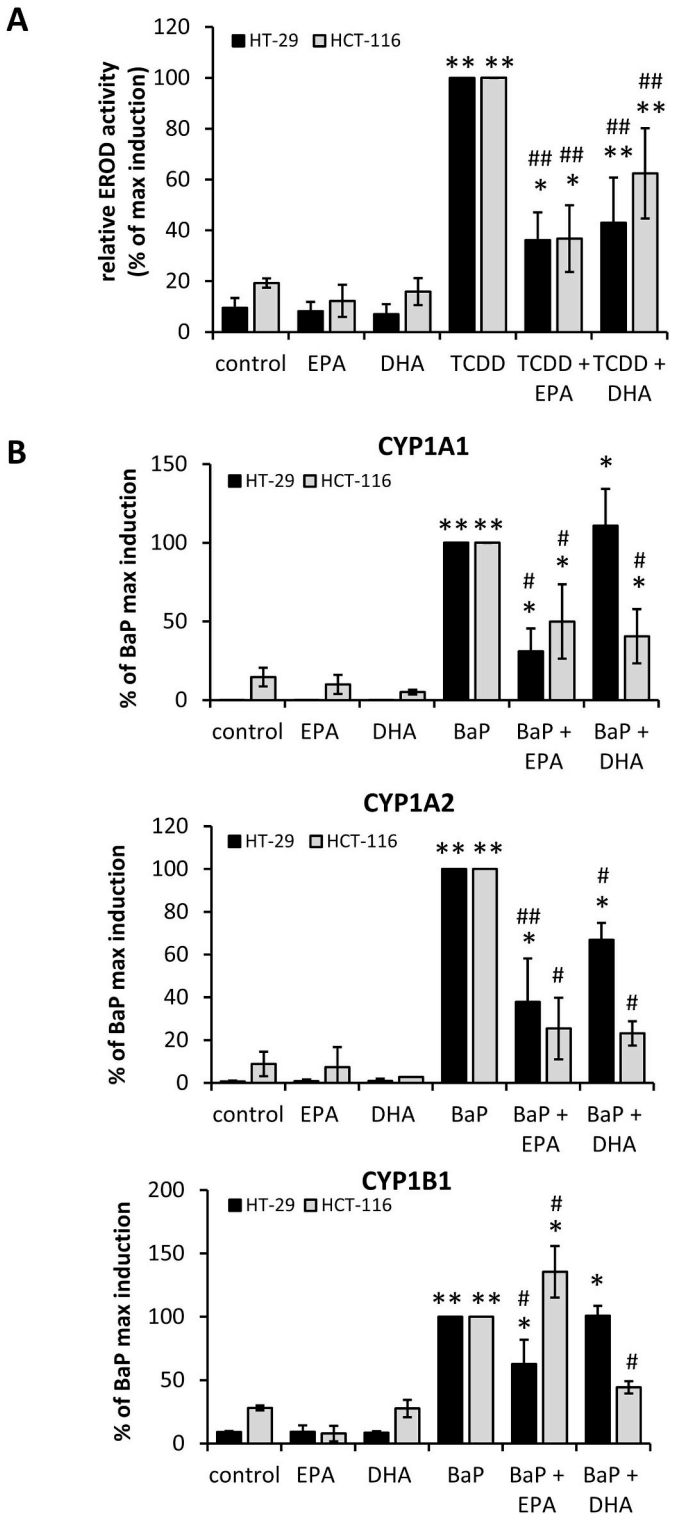

Fig. 7. Effects of EPA and DHA on expression/activity of CYP1 enzymes. **(A)** CYP1 activity was measured by 7-ethoxyresorufin-O-deethylase (EROD) activity in HT-29 cells treated with EPA (50 μM), DHA (50 μM) and TCDD (1 nM) alone, or in combination, for 24 h. **(B)** CYP1A1, CYP1A2 and CYP1B1 mRNA expression was detected by qRT-PCR in HT-29 cells treated with BaP (10 μM), EPA (50 μM) and DHA (50 μM) alone, or in combination, for 24 h. Results are expressed as means ± S.D. of three independent experiments. Differences in % of max induction were assessed using one-sample *t*-test in case of comparison with a variable with no variation. Symbols '*' and '**' denote significant difference between control and the respective treatment ($P < 0.05$ and $P < 0.01$, respectively). Symbols '#' and '##' denote significant difference between BaP alone, and the samples treated with combination of BaP and the respective PUFA ($P < 0.05$ and $P < 0.01$, respectively).

In summary, the data presented here suggest that genotoxic effects of BaP can be ameliorated by n-3 PUFAs, in particular by EPA, in colon epithelial cell models *in vitro*. This seems to be at least partly linked to reduced BaP metabolism via CYP1 enzymes due to inhibition of their expression and/or activity. Our data suggest that inhibition of metabolism of dietary carcinogens could be yet another mode of action contributing to chemopreventive effects of dietary n-3 PUFAs against colorectal carcinogenesis. Future studies should establish the relevance of the observed phenomenon *in vivo*, as well as to determine the precise role of PUFAs in control of metabolism of BaP (or other dietary carcinogens) within human colon epithelium.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.021.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.021.

Conflicts of interest

The authors declare that they have no conflict of interest.

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