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Journal of Nutritional Biochemistry 23 (2012) 539–548

Journal of Nutritional Biochemistry

Lipid alterations in human colon epithelial cells induced to differentiation and/or apoptosis by butyrate and polyunsaturated fatty acids☆

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Received 25 August 2010; received in revised form 24 November 2010; accepted 21 February 2011

Abstract

The present study highlights the important association between lipid alterations and differentiation/apoptotic responses in human colon differentiating (FHC) and nondifferentiating (HCT-116) cell lines after their treatment with short-chain fatty acid sodium butyrate (NaBt), polyunsaturated fatty acids (PUFAs), and/or their combination. Our data from GC/MS and LC/MS/MS showed an effective incorporation and metabolization of the supplemented arachidonic acid (AA) or docosahexaenoic acid (DHA), resulting in an enhanced content of the respective PUFA in individual phospholipid (PL) classes and an altered composition of the whole cellular fatty acid spectrum in both FHC and HCT-116 cells. We provide novel evidence that NaBt combined with PUFAs additionally modulated AA and DHA cellular levels and caused their shift from triacylglycerol to PL fractions. NaBt increased, while AA, DHA and their combination with NaBt decreased endogenous fatty acid synthesis in FHC but not in HCT-116 cells. Fatty acid treatment also altered membrane lipid structure, augmented cytoplasmic lipid droplet accumulation, reactive oxygen species (ROS) production and dissipation of the mitochondrial membrane potential. All these parameters were significantly enhanced by combined NaBt/PUFA treatment, but only in FHC cells was this accompanied by highly increased apoptosis and suppressed differentiation. Moreover, the most significant changes of ROS production, differentiation and apoptosis among the parameters studied, the highest effects of combined NaBt/PUFA treatment and a lower sensitivity of HCT-116 cells were confirmed using two-way ANOVA. Our results demonstrate an important role of fatty acid-induced lipid alterations in the different apoptotic/differentiation response of colon cells with various carcinogenic potential.
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Keywords: Colon cancer; Polyunsaturated fatty acids; Butyrate; Lipid analyses; Apoptosis; Differentiation

1. Introduction

Experimental and epidemiological studies support the idea that dietary factors, particularly fat and fiber, play an important role in the development and progression of colon cancer [1]. Omega-6 (*n*-6) and omega-3 (*n*-3) essential polyunsaturated fatty acids (PUFAs), whose availability depends on external supply in the diet, differentially influence induction of inflammation and the balance between cell growth and death in the colon [2,3]. The most physiologically important PUFAs are represented by arachidonic (AA, 20:4, *n*-6), eicosapentaenoic (EPA, 20:5, *n*-3) and docosahexaenoic (DHA, 22:6, *n*-3) fatty acids. They are obtained directly from the diet or produced by desaturation and chain elongation of precursor linoleic acid (LA, 18:2, *n*-6) or α-linolenic acid (ALA, 18:2, *n*-3).

Ingestion of PUFAs influences the lipid profile and fatty acid composition of cell membranes [4]. This consequently alters specific lipid membrane microdomains (rafts), lipid structure and fluidity, thus influencing ligand–receptor interactions and other membrane-mediated processes [5]. Other effects include changes of the expression and activities of lipid metabolism enzymes, production of various biologically active PUFA metabolites (eicosanoids) and other lipid signaling molecules, changes of redox balance, and modulation of the intracellular signaling pathways and transcription factors leading to altered gene expression [6,7]. These effects on various levels of cell organization and interaction of PUFAs with other endogenous or exogenous factors can finally significantly affect cell proliferation, differentiation and apoptosis [8,9] and are supposed to play a role in malignant transformation of cells.

Short-chain fatty acid butyrate, produced in the gastrointestinal tract by anaerobic microbial fermentation of dietary fiber, exerts beneficial effects on the colon, mainly inducing differentiation and apoptosis of neoplastic cells [10]. It was reported by ourselves and others using experimental systems *in vitro* as well as *in vivo* that fat and fiber (PUFAs and butyrate) mutually interact, enhance colon cell apoptosis and can protect against colon cancer development [11–13].

☆ Grants: This work was supported by grants 524/07/1178, P301/11/1730 (The Czech Science Foundation), AVOZ50040507 (Acad Sci Czech Republic) and MZe0002716202.
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doi:10.1016/j.jnutbio.2011.02.010

Nutrition containing these compounds was reported to be a useful adjuvant therapy improving clinical response in patients with inflammatory bowel disease [14].

Despite the progress in the knowledge of cellular and molecular mechanisms of PUFA effects, not much is known about (i) the association of specific changes of cellular lipid content and composition with subsequent cellular events influencing cell growth, differentiation and death, (ii) interactive effects with other dietary components such as butyrate and (iii) response of cells on various levels of neoplastic transformation.

Based on our previous results and on the literature data [15], we suggested that cells with different carcinogenic potential differ in lipid composition and metabolism providing a distinct environment for the effects of the fatty acids studied. Thus, the objective of our study was to verify the response of two human colon cell lines with different differentiation and apoptotic ability to supplementation with butyrate, AA, DHA alone and especially their combination and to investigate lipid alterations accompanying these effects.

2. Materials and methods

2.1. Chemicals and cell cultures

Both human colon cell lines were obtained from ATCC (Rockville, MD). The fetal colon FHC cells (CRL-1831) were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (Sigma-Aldrich, Prague, Czech Republic) containing HEPES (25 mM), cholera toxin (10 ng/ml; Calbiochem-Novabiochem, La Jolla, CA), insulin (5 μg/ml), transferrin (5 μg/ml) and hydrocortisone (100 ng/ml; all three from Sigma-Aldrich) and supplemented with 10% fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany). Colon cancer HCT-116 cells (derived from poorly differentiated, invasive adenocarcinoma) were cultured in McCoy's 5A medium (Sigma-Aldrich) supplemented with gentamycin (50 mg/l; Serva Electrophoresis, Heidelberg, Germany) and 10% FCS. The cultures were passaged twice a week and maintained at 37 °C in 5% CO_2 and 95% humidity. All lipid standards, chemicals and solvents were obtained from Sigma-Aldrich.

2.2. Cell treatment

Seventy-two hours after seeding (6×10^3 cells/cm^2), the medium was exchanged and the subconfluent cells were treated with 50 μM AA or DHA, sodium butyrate (NaBt, 3 mM) or their combination for 24, 48 or 72 h. The concentration of FCS during the treatments was 5%. AA and DHA (Sigma-Aldrich) were dissolved in 96% ethanol and stored as stock solutions (100 mM) under nitrogen at −80°C. In all types of experiments, fatty acids were diluted in the growth medium. The control cells were treated with ethanol, which did not exceed 0.05%. This concentration was proven not to significantly influence any of the parameters tested. NaBt (Sigma-Aldrich) was dissolved in PBS and then diluted to the growth medium.

2.3. Lipid analyses—sample preparation and purification

Sample preparation was based on a modified Bligh–Dyer extraction procedure designed for the analysis of cell pellets containing 1×10^6–1×10^7 cells [16,17]. The total cell extract was divided into three same aliquots for separation of polar and nonpolar lipids by solid-phase extraction, GC/MS analysis of fatty acid methyl esters (FAMEs) and LC/MS/MS analysis of phospholipids (PLs) [18].

2.4. GC/MS analysis of FAMEs

Aliquots of Bligh–Dyer cell extracts were evaporated to dryness under a stream of nitrogen, and transesterification of fatty acids was performed according to a slightly modified method of Kang and Wang [17], and FAMEs were analyzed by GC/MS. The average degree of unsaturation of all unsaturated fatty acids (unsaturation index) determined in the whole-cell lipid extract was calculated by multiplying the number of double bonds in fatty acids by the percentage of those compounds and dividing by 100.

2.5. LC/MS/MS analysis of PL classes

The analysis of PLs was performed on a triple-quadrupole mass spectrometer TripleQuad 6410 (Agilent, Santa Clara, CA) equipped with an electrospray ion source (ESI) using an HPLC column Supelcosil LC-SI (25 cm×2.1 mm, 5 μm) [19].

2.6. Detection of plasma membrane lipid structure

Plasma membrane lipid structure (lipid packing/unpacking) was detected using the lipophilic negatively charged heterocyclic chromophore merocyanine 540 (MC540) [20]. Briefly, 0.5×10^6 cells/sample, suspended in PBS, were treated with 5 μg/ml MC540 (stock solution 1 mg/ml in ethanol at −30°C). After 10-min incubation with gentle shaking at room temperature, the cells were pelleted in a centrifuge (200*g*), washed once and resuspended in PBS. Fluorescence (2×10^4 cells in each sample) was measured with 585 nm emission wavelength (FL-2) using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA) equipped with an argon ion laser at 488-nm wavelength for excitation. The CellQuest software (Becton Dickinson) was used to generate histograms distinguishing MC540-dim and MC540-bright cells.

2.7. Detection of lipid droplets

Accumulation of lipid droplets was detected by flow cytometry after staining of the cells with Nile red. The cells treated with the appropriate agent were harvested, washed twice and resuspended in PBS. Nile red (stock solution 1 mg/ml in acetone) was added in a final concentration of 0.1 μg/ml. The samples (5×10^5 cells per sample) were then incubated for 5 min at RT in the dark. Fluorescence was measured in 2×10^4 cells per sample with a 530/30 (FL-1) optical filter.

2.8. Detection of mitochondrial membrane potential

The changes of mitochondrial membrane potential were analyzed by flow cytometry using tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Eugene, OR) as described previously [12]. The data were evaluated (CellQuest software, Becton Dickinson) as a percentage of the cells with decreased mitochondrial membrane potential.

2.9. Determination of reactive oxygen species production

The intracellular production of reactive oxygen species (ROS) after treatments with the appropriate agents was detected by flow cytometry using dihydrorhodamine-123 (DHR-123; Fluka, Buchs, Switzerland), which reacts with intracellular hydrogen peroxide and/or (partly) with other ROS, as described previously [12].

2.10. Cell proliferation, differentiation and death evaluation

The cells were counted using a Coulter Counter (model ZM; Beckman Coulter, Fullerton, CA) and cell viability was determined microscopically by eosin (0.15%) dye exclusion assay. Cell differentiation was detected by the activity of the marker enzyme alkaline phosphatase in a lysate of sonicated cells (5×10^5 per sample) after incubation with its substrate (4-*p*-nitrophenylphosphate; Fluka) in a 96-well plate at 37°C for 30 min, as described previously [21]. The optical densities were measured at 405 nm (DigiScan Reader). The reading values (units $\times10^{-6}$/50,000 cells) were converted to the percentage of control.

Apoptosis was detected by fluorescence microscopy (Olympus IX-70; Olympus) after staining of the cells with a 4,6-diamidino-2-phenyl-indole (DAPI; Fluka) solution (1 μg DAPI/ml ethanol) at room temperature in the dark for 30 min. After mounting in Mowiol, the percentage of cells with chromatin condensation and fragmentation was determined from a total number of 200 cells.

2.11. Statistical analysis

The results of at least three times repeated experiments are expressed as arithmetic mean supported by S.E. of its estimate. The data of experiments testing the influence of DHA, AA, NaBt and their combinations were entered into a one-way ANOVA model (comparing the main effect of a preselected concentration of the compounds). Furthermore, the analyses adopted a two-way ANOVA mixed model that also involved two tested cell lines as a random-block factor. The ANOVA approach was used on the basis of verified normality of sample distributions (Shapiro–Wilk's test) and homogeneity of variance (Levene's test). To reach it, the primary data were log-transformed before the analyses: $X_{tr}=\ln [X+1]$. The total variability of the parameters measured in the experiments was described by a standard coefficient of variance on the data transformed. Each ANOVA model was evaluated using standard set global *F* tests; Tukey HSD tests were used for the post hoc comparisons of the experimental variants. The relative contribution of the factors tested to the overall experimental variability was described by a variance ratio calculated as a proportion of the sum of squares belonging to the effect of a given factor in the model to the total sum of squares.

3. Results

Human colon epithelial FHC and HCT-116 cells were treated with previously chosen concentrations of NaBt (3 mM), AA and DHA (50 μM) used individually or in combination (AA/NaBt or DHA/NaBt) [12,13]. After selected intervals, the cells were harvested for lipid content and composition analyses and evaluated for other individual parameters.

3.1. Polyunsaturated fatty acids, NaBt and their combination significantly modulate fatty acid content in FHC and HCT-116 cells

Forty-one fatty acids were quantitatively determined in the FHC and HCT-116 cells untreated or treated with AA, DHA, NaBt or their combinations after 24 and 48 h. Twenty-one fatty acids were detected with a relative content of over 1% of the total fatty acid content in cellular lipids (supplementary data in Tables S1 and S2). As expected, oleic acid (OA, 18:1, *n*-9) and palmitic acid (PA, 16:0) were the most abundant ones in both untreated cell lines, followed by palmitoleic acid (PO, 16:1, *n*-7), stearic (SA, 18:0) and LA (18:2, *n*-6) (Fig. 1).

Importantly, NaBt treatment increased the concentrations of saturated (SFAs) and monounsaturated (MUFAs) fatty acids in both cell lines. On the other hand, both PUFAs and their combination with NaBt reduced the relative content of MUFAs in both cell lines. While in the PUFA-treated FHC cells we also detected an apparent decrease in the SFA content (approximately up to one half of the control values or more), their content in HCT-116 cells was increased (Fig. 1).

The treatment of FHC as well as HCT-116 cells with AA or DHA considerably increased the amount of the respective PUFAs (about 10- to 14-fold for AA, about 23-fold for DHA). A combined treatment with AA/NaBt additionally increased incorporation of AA to both FHC and HCT-116 cells up to about 40% after 24 h (Fig. 1A,B) and more (to 95%) after 48 h (Tables S1 and S2). However, a combination of DHA/NaBt increased DHA incorporation in HCT-116 cells (45% in 24 h) (Fig. 1D) but decreased it strongly in FHC cells (to about 60%–70%) (Fig. 1C). The ratio of *n*-3/*n*-6 fatty acids was lowered after AA (or AA/NaBt) or increased after DHA (or DHA/NaBt) exposure. Moreover, the treatment with both PUFAs and their combination with NaBt increased the unsaturation index (more so after DHA treatment) and increased the SA/OA ratio (about two-fold or more), which reflects changes of membrane properties (especially its fluidity). The changes detected seem to be stable and some of them were more pronounced in a longer time interval of 48 h (Tables S1 and S2).

3.2. De novo fatty acid synthesis is differently modulated by PUFAs and/or NaBt in FHC and HCT-116 cells

Palmitic acid (16:0) is a key metabolite of *de novo* biosynthesis of fatty acids in mammalian cells. This compound can be further elongated, desaturated or β-oxidized producing other SFAs or MUFAs as shown in Fig. 2A. We used the sum of the relative concentrations of these fatty acids as a general marker for evaluation of the overall activity of *de novo* fatty acid biosynthesis. The treatment of the cells with NaBt, PUFAs or their combination had no significant effects on this parameter in HCT-116 cells. On the other hand, in FHC cells NaBt alone increased, while AA, DHA or their combination with NaBt significantly suppressed *de novo* fatty acid synthesis compared with the untreated control (Fig. 2B).

3.3. Exogenous AA and DHA are further metabolized by colon cell lines

Both series of *n*-3 and *n*-6 PUFAs are metabolized by the same set of enzymes. The pathways for their biosynthesis are shown in Fig. 3. Solid arrows denote anabolic reactions localized in the endoplasmatic reticulum, dashed arrows show partial degradative reactions (β-oxidation) taking place in peroxisomes [22]. From Fig. 1 and Suppl. Table S1, we can acquire additional data showing modulations of these pathways. A certain part of incorporated AA and/or DHA was subsequently metabolized to products with higher or lower molecular weight by elongation and β-oxidation reactions, respectively. Elongation of AA (20:4, *n*-6) to 22:4, *n*-6 and 24:4, *n*-6 products was more intensive in HCT-116 cells than in FHC cells (Fig. 1A,B). Docosahexaenoic acid (22:6, *n*-3) was further elongated to a 24:6, *n*-3 product and β-oxidized particularly to EPA (20:5, *n*-3) and further to an 18:4, *n*-3 product (Fig. 1C,D). EPA was the second more abundant fatty acid in the *n*-3 cascade and the 18:4, *n*-3 and 24:6, *n*-3 fatty acids were found as newly produced substances in the treated samples but not detected in controls.

3.4. Phospholipids are highly enriched with AA- or DHA-containing species after treatment with the respective PUFA and their combination with NaBt

Using LC/MS/MS, we analyzed the composition of major PL classes (*i.e.*, diacylglycero-phosphocholines (PCs), diacylglycero-ethanolamines (PEs), diacylglycero-serines (PSs), diacylglycero-inositols (PI) as well as sphingomyelins and ceramides in FHC and HCT-116 cells untreated or treated for 24 or 48 h with NaBt, AA, DHA and their combinations. We evaluated the changes after individual treatments, particularly with respect to AA- or DHA-containing species, comparing the relative individual species peak areas in control samples (100%) and the adequate peak areas in the treated samples. Fig. 4 generally depicts the sums of the total peak areas of all main PL species containing AA or DHA evaluated after 24 h treatment of FHC or HCT-116 cells. While NaBt alone induced only minor changes compared with control, both PUFAs alone and in combination with NaBt markedly enhanced the detected values. The modulations observed were most pronounced in PC and PE, lower but with a similar tendency in PI and PS and minor in ceramide or sphingomyelins (not shown). Representative mass spectra of FHC cells untreated or treated with AA or DHA showing highly increased amount of species containing the respective fatty acid are shown in Suppl. Fig. S1. As an example, a detailed pattern of fatty acid structures of PC after all types of treatments (24 h) in both FHC and HCT-116 cells is documented in Suppl. Tables S3 and S4. Generally, treatments with AA, DHA and their combination with NaBt decreased the content of species containing fatty acids dominant in the control and simultaneously increased AA- or DHA-containing species, respectively. A brief overview of the modulation of the most representative species content is shown in Table 1.

3.5. NaBt combined with AA or DHA affects distribution of the respective PUFAs in polar and neutral lipids

In FHC cells, where more apparent effects were detected after treatment with fatty acids, we made additional detailed analyses verifying the distribution of AA and DHA in PLs and triacylglycerols (TAGs) lipid fractions. Compared with control, no significant changes of fatty acid distribution in both classes were found after NaBt treatment (not shown). On the contrary, after treatment with AA or DHA, a considerable percentage of the respective fatty acid was detected in both polar lipids and TAGs (Fig. 6). Only about 2%–4% of these compounds were found in diacylglycerols (not shown). After treatment with PUFA alone, relatively more AA was identified in PLs, whereas DHA was incorporated predominantly to TAGs. Importantly, AA or DHA combination with NaBt decreased the content of both PUFAs in the PL fraction and increased their accumulation in TAGs (Fig. 5) which are supposed to be mostly located in cytoplasmic lipid droplets (see below).

3.6. Combination of AA or DHA with NaBt caused membrane lipid structure changes and accumulation of cytoplasmic lipid droplets

The lipophilic fluorescent dye MC540 can be used for detection of subtle changes in the membrane PL arrangement, namely, enhanced interlipid spacing (lipid unpacking) [23]. Particularly, in FHC cells, we detected a significant increase of the percentage of MC540 bright cells (detecting increased lipid unpacking) after treatment with AA, DHA

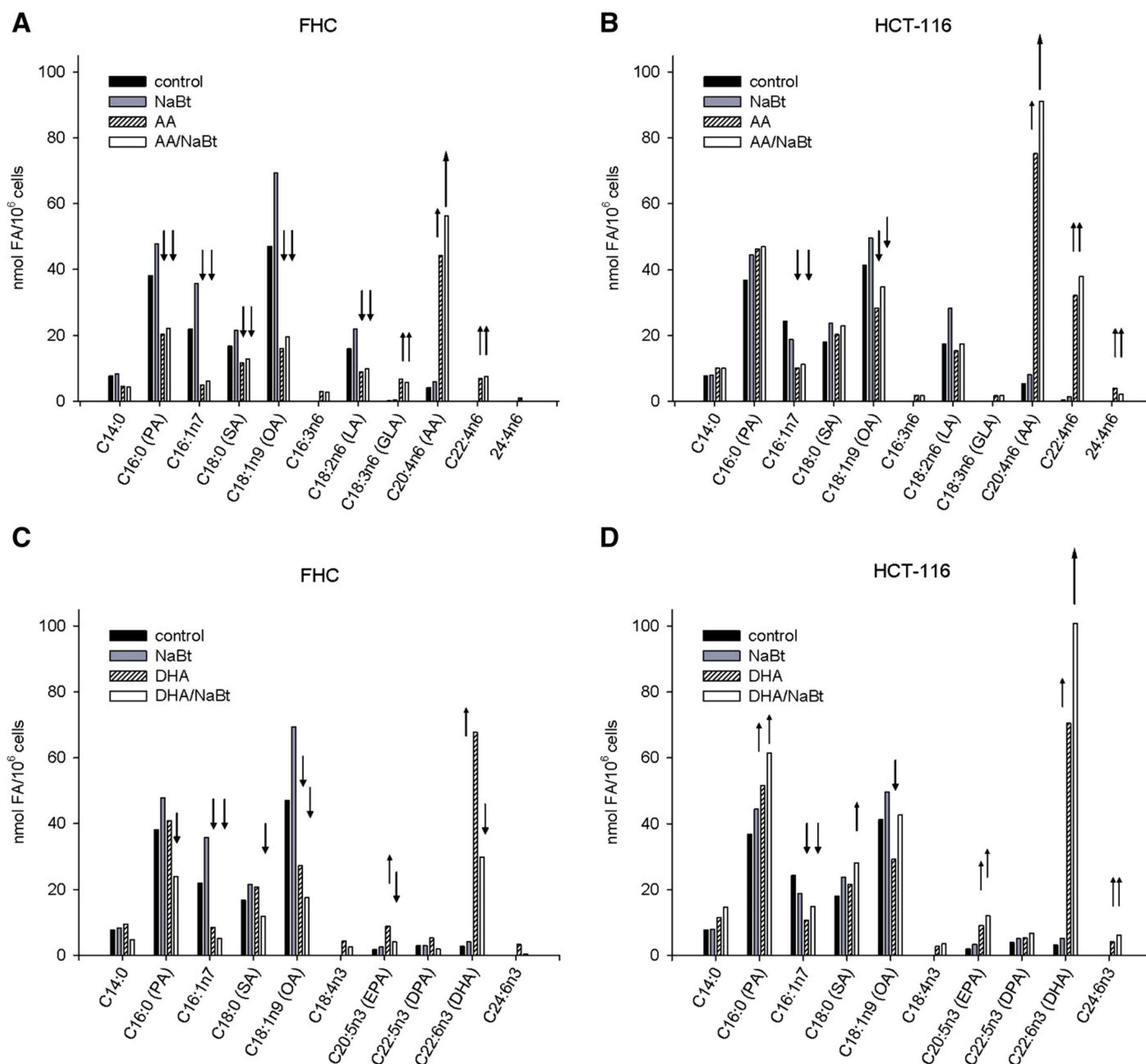

Fig. 1. Content of prevalent fatty acids (mmol FA/10^6 cells) in FHC (A, C) and HCT-116 (B, D) cells treated with vehicle (control), NaBt (3 mM), AA (50 μM), DHA (50 μM) or their combination AA/NaBt or DHA/NaBt for 24 h. GLA, gamma-linoleic acid; DPA, docosapentaenoic acid. Important ↑ increase or ↓ decrease of specific fatty acid after AA, DHA or combined AA/NaBt or DHA/NaBt treatment.

or NaBt, which was additionally enhanced by their combination after 24 (data not shown) and 48 h (Fig. 6A).

All types of treatments also led to an increased amount of cytoplasmic lipid droplets (containing mostly TAGs) detected by Nile red fluorescence after 24 h (data not shown) and more efficiently after 48 h (Fig. 6B) in both FHC and HCT-116 cell lines. Moreover, in FHC cells, this effect was most pronounced by a combination of NaBt with DHA and was also detected in early time intervals of 6 and 12 h (data not shown).

3.7. Cell growth, differentiation and apoptosis are differentially affected in FHC and HCT-116 cells by NaBt and its combination with AA or DHA

HCT-116 cells underwent apoptosis after NaBt treatment (about 30% after 72 h), as demonstrated by evaluating apoptotic nuclear morphology after DAPI staining (Fig. 6C). On the other hand, we observed only a low level of apoptosis in FHC cells (about 10%), which are induced to differentiation as detected by the increase of alkaline phosphatase activity, an enterocyte differentiation marker. No changes of alkaline phosphatase activity were found in HCT-116 cells after any type of treatment (Fig. 6D). Both differentiation and apoptosis were accompanied by a significantly enhanced proportion of cells with decreased mitochondrial membrane potential (Fig. 6E) and ROS production (Fig. 6F).

The apoptotic response of FHC but not HCT-116 cells to NaBt was strongly potentiated by combination with AA (up to about 30%) and especially with DHA (up to 60%, Fig. 6C), which alone caused about 20% of the apoptosis. On the contrary, about a ninefold increase of ALP activity induced by NaBt (48 h) in FHC cells was significantly suppressed by both PUFAs (Fig. 6D). These effects were accompanied

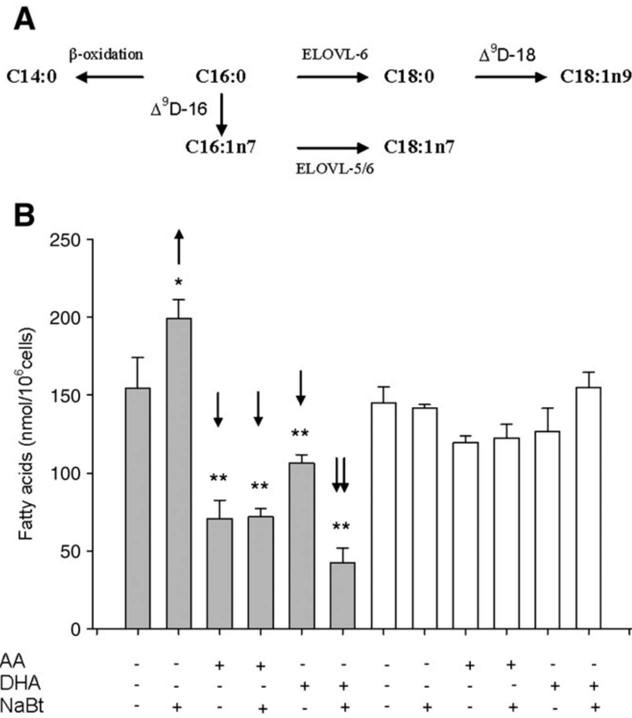

Fig. 2. (A) Pathways of *de novo* fatty acid synthesis started from key metabolite (16:0 PA) to its β-oxidation (14:0 myristic acid), elongation (18:0 SA) and desaturation (18:1 *n*-9 OA, 16:1 *n*-7 PO, 18:1 *n*-7 vaccenic acid) products. ELOVL-6, ELOVL-5/6, elongases; Δ9D-16 and Δ9D-18, desaturases. (B) The sum of *de novo* synthesized fatty acids (nmol FA/10^6 cells) in FHC and HCT-116 cells treated with vehicle (control), NaBt (3 mM), AA (50 μM), DHA (50 μM) or their combination AA/NaBt or DHA/NaBt for 24 h. Statistical significance: *P<.05; **P<.01 in comparison with control. ↑ increase; ↓ and ↓↓ decrease.

by an additional enhancement of the proportion of cells with decreased mitochondrial membrane potential (about twofold) and high ROS production (Fig. 6E,F). In FHC cells, production of ROS increased during cultivation and was detected also in early time intervals (after 6 and 12 h, data not shown).

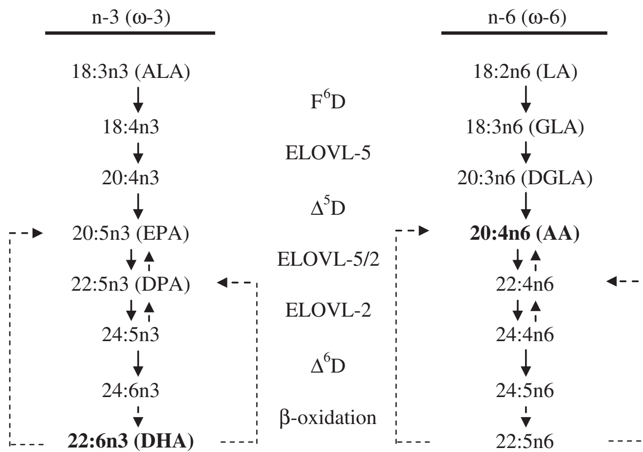

Fig. 3. Pathways for biosynthesis of n(ω)-3 or n(ω)-6 PUFAs. Solid arrows denote reactions localized in the endoplasmatic reticulum, dashed arrows show partial degradative reactions taking place in peroxisomes (adapted according to Sprecher [22]). ELOVL-5, ELOVL-5/2, ELOVL-2, elongases; Δ6D and Δ5D, desaturases; DPA, docosapentaenoic acid; GLA, gamma-linoleic acid; DGLA, dihomo-gamma-linoleic acid.

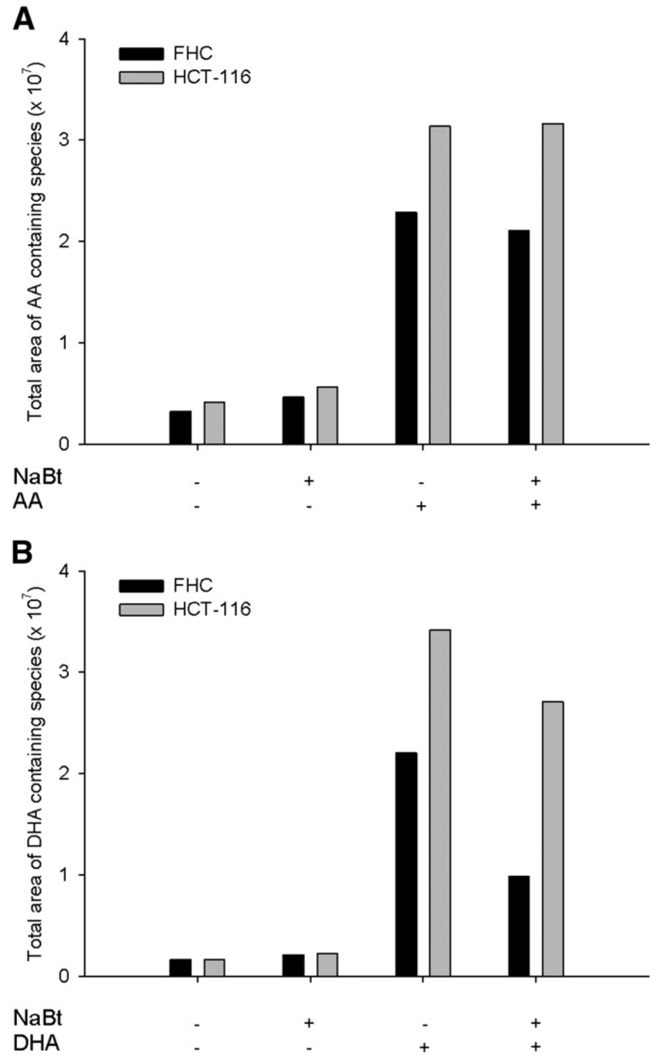

Fig. 4. The sum of PL classes containing AA (A) or DHA (B) species detected in total cell lipid extracts from FHC or HCT-116 cells after treatment with vehicle (control), NaBt (3 mM), AA (50 μM), DHA (50 μM) or their combination AA/NaBt or DHA/NaBt for 24 h. Data represent sums of total areas of chromatographic peaks analyzed in a particular sample of cell PLs detected by HPLC–tandem MS/MS method (normalized to 10^6 cells).

3.8. The ANOVA model confirmed lower sensitivity to fatty acids in HCT-116 cells compared with FHC cells

Table 2 summarizes the outcomes of two-way ANOVA models as well as the results of post hoc tests comparing some experimental variants. This evaluation confirmed a different reaction of the cell lines tested, HCT-116 being the less significant responder in a quantitative sense. A global difference was observed in ROS production and lipid unpacking (MC540). The main effect of the experimental factors (AA, DHA, NaBt, AA/NaBt and DHA/NaBt) was statistically significant in all end-points measured, except for the alkaline phosphatase in nondifferentiating HCT-116 cells. The interaction between the main effects and the cell line (incorporated as a random block effect in the ANOVA model) was statistically significant for ROS production, alkaline phosphatase activity and apoptotic cells (DAPI). This fact indicates a different response of FHC and HCT-116 cells to the experimental factors, namely on the combined treatment with AA/NaBt and DHA/NaBt. The most significant effect of the cell line x factors interaction was observed in the induction of apoptosis (P<.01), which was significantly

Table 1
Example of fatty acid pattern and percentage of decrease (−) or increase of selected phosphatidylcholine species relative to control samples (proportion 1% or more) after treatment of FHC or HCT-116 cells with 50 μM of AA or DHA or their combination with 3 mM NaBt (AA/NaBt, DHA/NaBt)

FHC	AA	AA/NaBt
P-O, Po-S	−13.6	−13.2
G-Po, S-L, di-O	−13.2	−12.8
P-Po, M-O	−11.2	−10.3
P-L, Po-O	−9.0	−8.4
AA-P	15.0	18.3
AA-O	9.3	10.6
AA-S	6.3	7.0
AA-24:4	4.2	2.3
di-P, M-S	3.6	2.2
La-S, M-P	1.7	0.2
FHC	**DHA**	**DHA/NaBt**
G-Po, S-L, di-O	−13.5	−12.3
P-Po, M-O	−10.6	−10.3
P-O, Po-S	−10.1	−8.2
P-L, Po-O	−10.0	−8.5
DHA-P	12.1	11.2
di-P, M-S	7.6	6.2
La-S, M-P	5.4	3.5
DHA-S	4.7	3.6
DHA-O	3.8	3.6
DHA-A	1.9	1.1
DHA-E	1.6	1.1
DHA-G	1.3	1.0
di-DHA	1.3	0.8
HCT116	**AA**	**AA/NaBt**
P-Po, M-O	−17.7	−17.1
P-O, Po-S	−17.2	−14.1
P-L, Po-O	−9.4	−8.5
G-Po, S-L, di-O	−8.1	−7.2
AA-P	13.9	16.9
di-P, M-S	9.5	6.7
AA-O	6.2	7.1
AA-S	8.2	9.1
AA-22:4	3.9	2.0
AA-24:4	3.3	1.8
di-AA	2.2	1.2
HCT116	**DHA**	**DHA/NaBt**
P-Po, M-O	−17.4	−15.6
P-O, Po-S	−15.1	−10.7
P-L, Po-O	−9.2	−7.5
G-Po, S-L, di-O	−7.8	−6.7
DHA-P	13.8	12.8
di-P, M-S	12.6	8.1
DHA-O	5.8	3.9
di-DHA	4.1	1.6
DHA-S	3.8	4.0
DHA-L	1.3	0.7

Data were evaluated as the chromatographic peak area of particular lipid species (normalized to 10^6 cells) obtained by HPLC–tandem MS/MS method

increased after DHA/NaBt treatment in the FHC but not in the HCT-116 cell line.

4. Discussion

Our previous results using human colon cells *in vitro* [12,13] as well as the results of other authors using a mouse or rat model *in vitro* and *in vivo* [11] support the concept of a coordinated action of *n*-3 PUFAs and butyrate protecting against colon carcinogenesis. Our results comparing two distinct human colon cell lines newly document their different response to butyrate, PUFAs and their combination considering cellular lipid alterations, oxidative metabolism and cell proliferation, differentiation and apoptosis.

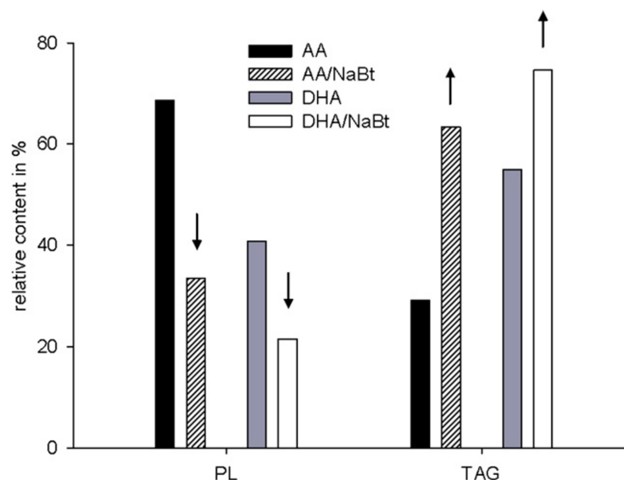

Fig. 5. Distribution of AA or DHA (percentage from all detected in total cellular lipids) in cellular polar lipid (PL) and neutral lipid fractions (triacylglycerol, TAG) after treatment of FHC cells with 50 μM AA or DHA and their combination with NaBt (3 mM) for 24 h.

4.1. HCT-116 cells are less responsive to NaBt/PUFA treatment than FHC cells

As we reported previously [13] and as the evidence provided in the present study shows, NaBt alone induced G_0/G_1 arrest, differentiation and a low level of apoptosis in FHC cells, while G2/M arrest, no differentiation and a high degree of apoptosis were detected in HCT-116 cells. Moreover, its combination with relatively low physiologically relevant doses of PUFAs, which alone presented only minor effects, turned the differentiation response of FHC cells to strong apoptosis. No such effects were detected in HCT-116 cells.

Here, we further demonstrated a significantly increased level of the respective PUFA after supplementation with AA or DHA and the ability of both cell lines to metabolize them. Our findings are in agreement with other reports using cultured cells treated with PUFAs [24,25]. Moreover, experimental animal and human studies clearly document a good bioavailability of DHA and EPA in pure form or from fish and algal oils and their dose-dependent increase in plasma lipids (up to 400 μM), erythrocyte membranes as well as in various tissues including the colon and tumors [26–28]. We newly demonstrated that NaBt further markedly increased the cellular *n*-3 or *n*-6 PUFA content when combined with DHA or AA, in both FHC and HCT-116 cells, respectively, except for the decrease of the *n*-3 PUFA content in FHC cells. Based on our data detecting a higher oxidative response of FHC cells to the treatment, we suggest that there might be a more effective and rapid metabolization (oxidation) of DHA supported by NaBt in these cells, resulting in its decreased detectable level.

It was reported that butyrate served as a substrate for *de novo* lipogenesis in normal rat colonic cells [29] and could influence lipid composition and metabolism of colon cancer cell lines [30,31]. This corresponds with the enhanced *de novo* fatty acid synthetic activity after NaBt treatment in FHC cells induced to differentiation. On the contrary, suppression of *de novo* fatty acid synthesis accompanied suppressed differentiation and induction of apoptosis in FHC cells after treatment with PUFAs and especially after combination of NaBt/PUFA. These differences in endogenous fatty acid synthesis between FHC and HCT-116 cells correlated with their distinct sensitivity to PUFAs. Overexpression of fatty acid synthase and elevated fatty acid synthetic activity was reported during colon cancer development [32]. Malignant cells develop the so-called lipogenic character associated with unresponsiveness of lipogenesis to nutritional regulation [33].

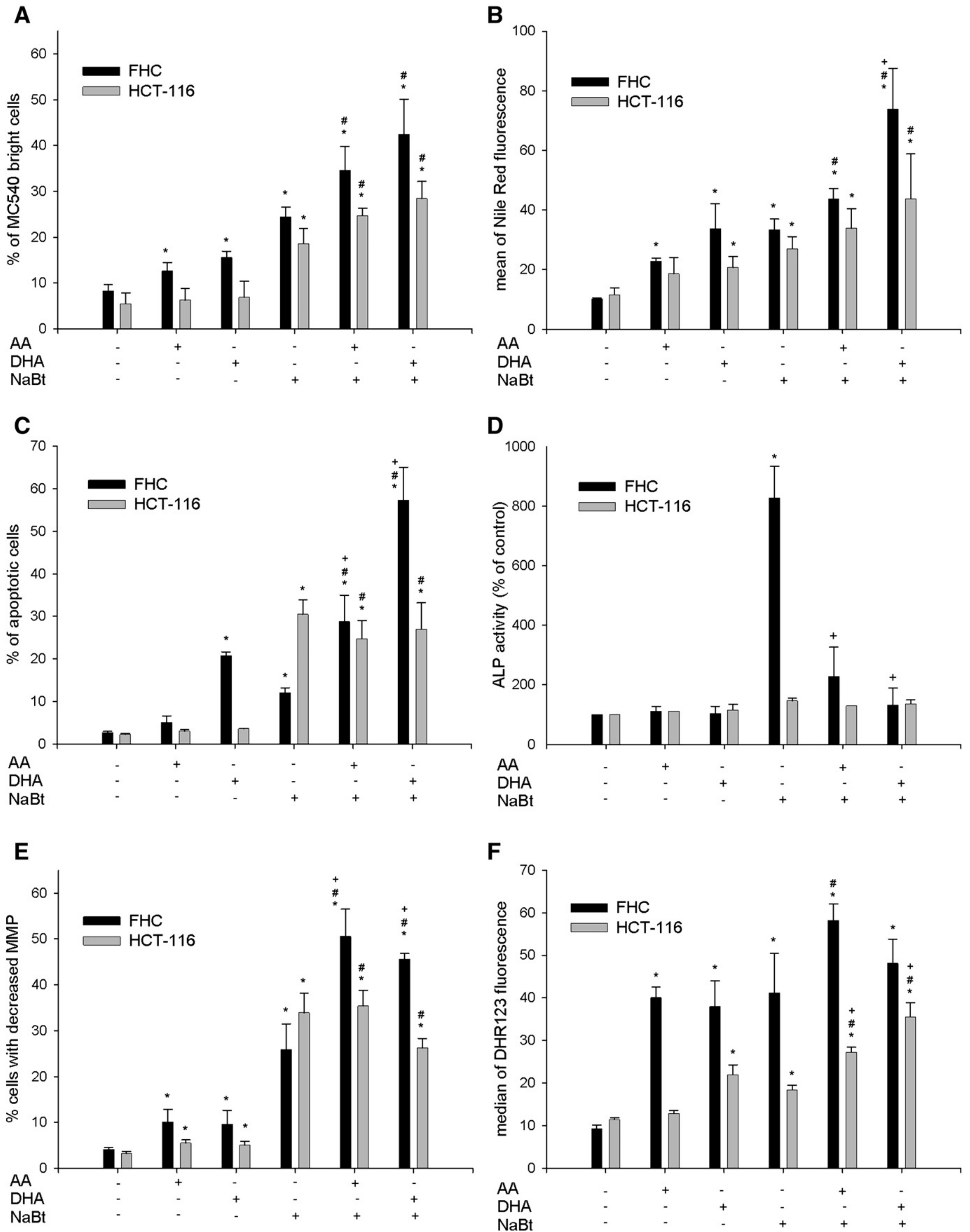

Fig. 6. Plasma membrane lipid unpacking measured as MC-540 bright cells (A), cytoplasmic lipid droplets accumulation detected as median of Nile red fluorescence (B), percentage of cells with apoptotic nuclear morphology (DAPI staining) (C), differentiation (ALP activity, % of control) (D), ROS production detected as median of DHR-123 fluorescence (E) and mitochondrial membrane potential (MMP) detected by TMRE fluorescence (F) of FHC and HCT-116 cells treated with NaBt (3 mM), AA or DHA (50 μM) and their combination for 48 h or 72 h (for apoptosis). Statistical significance: $P<.05$ *vs*. control (*), *vs*. the respective PUFA (#), *vs*. NaBt (+).

Table 2
Results of experiments and corresponding ANOVA models

Experimental variant	Cell counts[a]		Viability[a]		ROS[a]		MC540[a]	
	FHC	HCT-116	FHC	HCT-116	FHC	HCT-116	FHC	HCT-116
Control	89.4 (85.6–93.4)	94.5 (92.9–96.0)+	Baseline (100)	Baseline (100)	9.2 (7.7–10.9)+	11.2 (10.3–12.2)+	8.0 (5.9–10.9)+	5.0 (2.7–9.3)+
AA	76.6 (66.8–87.9)	94.1 (91.7–96.5)+	93.5 (78.3–111.5)+	98.4 (90.2–107.4)+	39.9 (35.7–44.6)*	13.7 (13.2–14.3)	12.2 (9.4–15.9)+	5.6 (2.9–10.8)
DHA	60.7 (49.2–74.8)*	91.3 (85.2–97.7)+	83.7 (66.1–105.9)+	87.3 (80.4–94.6)+	36.8 (28.0–48.5)*	21.4 (16.2–28.2)*	15.4 (12.7–18.6)+	5.8 (2.6–13.0)
NaBt	74.1 (60.2–91.2)	78.3 (73.2–83.7)*	32.1 (26.2–39.3)*	30.3 (25.7–35.8)*	38.3 (23.8–61.7)*	19.8 (17.8–22.0)*	29.5 (21.9–39.7)*	18.2 (13.5–24.5)*
NaBt+AA	63.6 (52.7–76.7)	73.8 (68.6–79.3)*	50.5 (39.1–65.2)*+	27.7 (21.1–36.2)*	57.9 (54.7–61.9)*+	27.6 (23.4–32.4)*	33.4 (25.8–43.3)*	24.6 (22.1–27.5)*
NaBt+DHA	48.8 (40.7–58.5)*+	65.9 (61.3–70.9)*+	54.7 (39.7–75.3)*+	28.1 (23.2–33.9)*	47.4 (39.3–57.2)*	38.2 (33.9–43.2)*+	42.3 (34.2–53.6)*+	28.1 (24.6–32.9)*+
ANOVA model[b]								
Effect of experimental factors[c] (%)	51.2*		75.8**		61.5**		68.7**	
Effect of cell lines[c] (%)	6.5		3.3		16.7*		12.6*	
Interaction[b] (%)	7.0		7.1		14.7*		1.8	
P	<.001		<.001		<.001		<.001	

Experimental variant	MMP[a]		Nile red[a]		ALP[a]		DAPI[a]	
	FHC	HCT-116	FHC	HCT-116	FHC	HCT-116	FHC	HCT-116
Control	4.1 (3.6–4.7)+	3.1 (2.5–3.9)+	10.3 (9.8–10.8)+	11.1 (8.2–15.0)+	Baseline (100)	Baseline (100)	2.9 (2.6–3.1)+	2.3 (2.0–2.6)+
AA	9.6 (6.3–14.7)+	5.4 (4.2–7.0)+	22.8 (21.0–24.7)*	16.8 (10.1–28.0)	110.6 (93.1–131.4)+	104.1 (94.8–114.4)	5.6 (4.0–7.9)*+	3.0 (2.4–3.7)+
DHA	8.9 (5.1–15.6)+	4.9 (3.7–6.4)+	31.4 (20.3–48.6)*	19.9 (15.4–26.6)*	102.4 (79.6–131.6)+	106.1 (94.8–118.8)	17.1 (13.0–22.6)*+	3.6 (3.5–3.7)+
NaBt	21.9 (13.3–36.2)*	33.0 (26.2–41.5)*	33.0 (27.5–39.7)*	26.4 (20.8–33.4)*	856.2 (677.2–1 082.5)*	107.4 (90.2–127.9)	10.7 (9.0–12.7)*	30.2 (25.1–36.4)*
NaBt+AA	50.1 (41.1–61.0)*+	35.2 (30.1–41.0)*	43.5 (39.3–48.4)*+	33.1 (24.8–44.3)*	216.8 (139.0–338.1)*+	124.7 (105.0–148.1)	27.2 (17.5–42.2)*+	24.2 (18.5–31.8)*
NaBt+DHA	45.6 (43.5–47.7)*+	26.1 (23.1–29.6)*	70.6 (49.4–100.8)*+	40.8 (24.6–67.7)*	125.0 (79.5–196.8)+	119.0 (95.7–147.8)	57.4 (47.3–69.7)*+	25.9 (17.1–39.3)*
ANOVA model[b]								
Effect of experimental factors[c] (%)	88.3**		72.0**		43.6*		70.0**	
Effect of cell lines[c] (%)	2.6		5.4		9.2		3.0	
Interaction[b] (%)	3.3		2.1		27.0*		23.0**	
P	<.001		<.001		<.001		<.001	

All models were constructed using data after 48 h of follow-up except for % of cells with apoptic nuclear morphology (72 h).

[a] Geometric mean (95% CI): statistically significant difference ($P<.05$) *vs.* control (*) or *vs.* NaBt (+) based on Tukey HSD post hoc test.

[b] Parameters of the two-way ANOVA model based on log transformed data: *P* value of the global ANOVA F test.

[c] Variance ratio expressed in percentage of total sum of squares; percentage of experimental variance explained by the effect of examined factors (AA, DHA, NaBt, NaBt +AA, NaBt +DHA), cell lines (FHC, HCT-116) or by interaction term of these two factors. Statistical significance of the components: *$P<.05$; **$P<.01$.

4.2. NaBt/PUFA supplementation increased PUFA content in TAGs, lipid droplet accumulation and ROS production

Our detailed analysis of the fatty acid profile of individual PL classes provided evidence of incorporation of AA or DHA substituting SFA or MUFAs in various positions, which was most pronounced in PC and PE. However, no significant differences after combination with NaBt and only slight variations between FHC and HCT-116 cells were observed (Table 1). Using mouse colon adenocarcinoma and rat neuronal cell lines, other authors reported preferential incorporation of AA, EPA or DHA into the particular PL species [34]. Interestingly, DHA at low concentrations was shown to prefer PE over neutral lipids, but in DHA excess accumulation in neutral lipids outstripped that of PLs. However, these effects depend on cell types and our data (evaluation of only relative changes after fatty acid treatments) do not enable us to make similar quantitative conclusions.

Our results using more responsive FHC cells newly showed the important ability of NaBt to shift both AA and DHA from the PL fraction in the TAG fraction when combined with these PUFAs. This correlates well with the significant increase of lipid droplets in the cytoplasm reflecting the accumulation of TAGs. The formation and metabolism of lipid droplets appear to be directly involved in membrane trafficking and PL recycling and are closely connected with mitochondria and altered ROS production supposing association with apoptosis [35]. Thus, increased intracellular PUFA content in the form of TAGs in lipid droplets, enhanced ROS production and dissipation of mitochondrial membrane potential after combined treatment of AA or DHA with NaBt are in agreement with this hypothesis and suggest participation of the intrinsic mitochondrial pathway in the FHC cell apoptotic response. This is also in accord with the reported DHA ability to specifically enhance unsaturation and the oxidative status of the mitochondrial membranes by incorporation into cardiolipin—a specific mitochondrial PL [36] that makes the cells more susceptible to apoptosis [37].

Thus, we suggest that NaBt together with PUFAs, which are incorporated into cellular neutral lipids and specific PLs, primed the cells toward oxidative stress induced by butyrate, and this played a role in the transition from differentiation to apoptotic response in sensitive FHC cells. These conclusions are supported by ANOVA results showing a significant interaction of the effects of experimental factors and of the cell line for ROS production, differentiation (activity of ALP) and apoptosis (DAPI). The role of mitochondria and ROS was also supported by our previous results showing caspase-3 and caspase-9 activation, dynamic modulation of the expression of antiapoptotic Mcl-1 protein and cleavage of proapoptotic Bid by NaBt, PUFA and their combination. Moreover, the antioxidant Trolox decreased apoptosis and restored differentiation in FHC cells ([13] and our unpublished results).

4.3. NaBt and PUFAs enhanced lipid unpacking more effectively in FHC than in HCT-116 cells

In addition to biochemical changes, our results also indicate altered physical membrane properties. We newly demonstrated the increased ratio of SA/OA and an increased unsaturation index (Table S1 and S2) together with changes of membrane lipid structure (lipid unpacking) after treatment with PUFAs, NaBt and particularly with their combination. This implies changes of membrane fluidity and other properties more effective in FHC cells, which could affect the signaling associated with regulation of proliferation, differentiation and apoptosis [38]. The increase of MC540 binding was shown to precede phosphatidylserine exposure and correlate with the elevated membrane fluidity during apoptosis initiated by certain agents [39].

In conclusion, our results brought further evidence of PUFA and butyrate interaction influencing the behavior of human colon cells and highlight the important role of lipid composition and structure changes, oxidative metabolism and apoptosis in their effects. These *in vitro* data are in agreement with those obtained by other authors using normal mouse or rat colonocytes *in vitro* as well as *in vivo* [11] and with the presumption that dietary modulation of PUFA levels may be more efficient in the initiation and promotion stage of colon carcinogenesis [40,41]. Thus, our data suggest that supplementation with PUFAs (especially DHA) together with fiber (or butyrate) may represent a nontoxic way of colon cancer prevention and supportive therapy and may call attention to the possible different response of colon cells with various carcinogenic potential.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.02.010.

Acknowledgments

The authors thank J. Netíková and I. Lišková for technical assistance and L. Červený for the English revision.

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