Research Article

Human fetal colon cells and colon cancer cells respond differently to butyrate and PUFAs

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We verified the hypothesis suggesting modulation of the effects of sodium butyrate (NaBt) by ω−3 or ω−6 PUFAs. Comparing the response of human colon epithelial cell lines of fetal (FHC) and adeno-carcinoma (HT-29, HCT-116) origin, we detected significant differences in proliferation, differentiation and apoptotic response to the treatment of NaBt, arachidonic or docosahexaenoic acids and their combination. While in FHC and HT-29 cells NaBt induced G0/G1 arrest, differentation and low level of apoptosis, in HCT-116 cells G2/M arrest, no differentiation and high degree of apoptosis were detected. Moreover, in FHC cells significant potentiation of apoptosis accompanied by increased arrest in the cell cycle, cell detachment and decrease in differentiation were detected after combined treatment with NaBt and both PUFAs. Changes in cytokinetics induced by fatty acids were accompanied by membrane lipid unpacking, reactive oxygen species (ROS) production, and decrease in mitochondrial membrane potential (MMP). Detection of caspase-3 activation and dynamic modulation of Mcl-1 protein expression imply their possible role in both cell differentiation and apoptotic response. Our results support the concept of modulation of NaBt effects by PUFAs, especially of ω−3 type, in colonic cells *in vitro* with diverse impact in cell lines derived from normal or neoplastic epithelium.

1 Introduction

In the continuously renewing colon epithelium, a proper balance between cell growth and death is a prerequisite for maintenance of homeostasis of healthy tissue [1]. Dysregulation of proliferation, differentiation and apoptosis substantially contributes to colorectal carcinogenesis (CRC). An intriguing hypothesis suggests that diet plays a key role in the development and progression of this disease [2]. Particularly, the type of dietary fat and fibre are thought to have the most significant impact on CRC [3]. These compounds are sources of important fatty acids – PUFAs and

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Abbreviations: AA, arachidonic acid; **ALP,** alkaline phosphatase; **CRC,** colorectal carcinogenesis; **CEA,** carcinoembryonic antigen; **DAPI,** 4,6-diamidino-2-phenyl-indole; **DHA,** docosahexaenoic acid; **FCM,** flow cytometry; **FCS,** fetal calf serum; **MMP,** mitochondrial membrane potential; **MC540,** merocyanine 540; **NaBt,** sodium butyrate; **PARP,** poly(ADP-ribose) polymerase; **ROS,** reactive oxygen species

SCFAs like butyrate. In addition to their nutritional value, they are required for a wide range of physiological functions.

Essential PUFAs of ω−6 and ω−3 types and their metabolites function as inter- and intracellular mediators of cell signalling networks, together with cytokines and hormones [4]. In general, studies using *in vitro* and *in vivo* systems showed that ω−3 PUFAs appear to affect some of the cellular functions more effectively, and sometimes also in opposite direction, compared to the ω−6 PUFAs [5]. Many experimental studies support the idea of protective effects of ω−3 eicosapentaenoic and docosahexaenoic (DHA) acids (rich in fish oil) against colon inflammation and cancer [6, 7]. However, data from clinical trials and epidemiological studies of the effects of ω−3 or fish oil consumption on inflammatory bowel disease or cancer risk are inconsistent [8–10]. In therapeutic strategies using PUFAs it is important to consider seriously their multitarget effects, ω−3:ω−6 ratio, influence of genetic background, and an interaction with other exogenous and endogenous factors [11].

The protective role of dietary fibre is partially associated with butyrate, produced in the gastrointestinal tract by anaerobic fermentation of fibre and resistant starch [12]. Butyrate can promote or inhibit proliferation as well as apoptosis

Mol. Nutr. Food Res. 2009, *53*, S102−S113

S 103

in dependence upon the cell type, the stage of differentiation, concentration, physiological conditions during the study, and the presence of other factors [13, 14]. It is generally accepted that butyrate serves as the main oxidative fuel for normal colonic epithelium, has immunomodulatory and anti-inflammatory effects, and plays an important role in the maintenance of colonic health [15]. On the other hand, sodium butyrate (NaBt) can decrease the proliferation of neoplastic colonocytes, and induces their differentiation as well as apoptosis *in vitro* and *in vivo* [16]. This response characterizes epithelial cells migrating from the end to the surface of colonic crypts. NaBt-induced enterocyte-like differentiation is accompanied by an increase in specific markers such as brush border enzyme activities (especially alkaline phosphatase (ALP)) or carcinoembryonic antigen (CEA) expression. CEA is an intercellular adhesion glycoprotein which is expressed in embryonic as well as adult colon mucosa, and its deregulated overexpression can have tumorigenic effects [17]. On the other hand, it has been shown to be upregulated with differentiation induction of colon cancer cells [18]. The most commonly reported mechanism by which butyrate modulates gene expression involves alteration of chromatin structure through increased histone acetylation [19]. A recent gene array and proteome analysis of human colon cancer cell lines treated with NaBt revealed that the genes (mainly transcription factors) and proteins linked to the cell growth, apoptosis, and oxidative metabolism are most significantly affected [20].

It has been proposed by ourselves and others that the effects of butyrate and PUFAs on the metabolism and kinetics of colon epithelial cell population may be mutually influenced [21, 22]. Our previous results also showed that these fatty acids can modulate the effects of endogenous regulators such as cytokines and apoptotic inductors [23–25]. The dietary combination of fish oil, but not corn oil, and the fermentable fibre pectin (a significant butyrate source) has been found to be more efficient (compared to the agents used alone) in the upregulation of apoptosis in a rat model of experimentally induced colon cancer [26]. There are also clinical trials showing that oral supplementation with fish oil together with soluble fibre and antioxidants enhanced the therapeutic efficacy in patients with ulcerative colitis [27].

There is still not enough information about the effects of dietary fatty acids on colonic cell differentiation and especially about the mechanisms of how they can modify the pathways governing both cell maturation and apoptosis [26]. A particularly important issue related to CRC is represented by the deregulation of anoikis (cell detachment-induced apoptosis) associated with colonocyte exfoliation on the top of the crypts [28]. Members of the Bcl-2 family are essential regulators for homeostasis determining cell survival and differentiation. The antiapoptotic short-half-life protein Mcl-1 has been identified as a key prosurvival molecule during hematopoietic cell development and dif-

ferentiation [29]. Rapid induction of the immediate early Mcl-1 gene prevents cells from undergoing apoptosis, thereby supporting viability during the early stage of differentiation [30].

It has been reported that cancer cells differ from normal ones in fatty acid composition, enzyme activities, and deregulation of many signalling pathways important for balanced cell growth and death [31]. Thus, distinct effects of dietary fat and fibre on normal and transformed cells should be considered. To contribute to this issue, we compared the proliferative, differentiation, and apoptotic response of the cell lines derived from normal human fetal colon (FHC), well-differentiated (HT-29), and poorly differentiated (HCT-116) colon adenocarcinoma cells to treatment with NaBt and $\omega-3$ (DHA, 22:6) or $\omega-6$ (arachidonic acid (AA), 20:4) PUFAs alone or in combination. We focused our attention on the role of membrane lipid structure changes, mitochondrial pathway, reactive oxygen species (ROS) production, caspase activation, and Mcl-1 protein in the regulation/modulation of apoptotic *versus* differentiation cell response.

2 Materials and methods

2.1 Cell cultures

All human colon cell lines were obtained from ATCC (Rockville, MD, USA). The human fetal colon FHC cells (CRL-1831) were cultured in a 1:1 mixture of Ham's F12 and DMEM (Sigma−Aldrich; Prague, Czech Republic) containing HEPES (25 mM), cholera toxin (10 ng/mL; Calbiochem-Novabiochem Corporation; La Jolla, CA, USA), insulin (5 µg/mL), transferrin (5 µg/mL) and hydrocortisone (100 ng/mL; all Sigma−Aldrich; Prague), and supplemented with 10% fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany). Human colon adenocarcinoma HT-29 and HCT-116 cells 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.

2.2 Application of the agents

Seventy-two hours after seeding (6×10^3 cells/cm²), the medium was exchanged and the subconfluent cells were treated with AA or DHA (50 µM), NaBt (3 mM) or their combination for 24, 48, or 72 h. The resulting concentration of FCS during the treatments was 5%. AA and DHA (Sigma−Aldrich) were dissolved in 96% ethanol and stored as stock solution (100 mM) under nitrogen at −80°C. For the experiments, fatty acids were diluted in the growth medium. In all types of experiments, the control cells were treated with ethanol (0.05%; the concentration used in samples treated with PUFAs). This ethanol dose was also used when

the cells were treated with NaBt as single agent. Any of the parameters tested in this study was not significantly influenced by ethanol concentration used. NaBt (Sigma−Aldrich) was dissolved in PBS and then diluted to the growth medium. Trolox (a water-soluble analogue of vitamin E, 100 µg/mL) used as an antioxidant was added to the cells 1 h before the application of NaBt and/or PUFAs. A general caspase inhibitor Z-VAD-FMK (BD PharMingen, 20 µM in DMSO) was added 30 min before the application of NaBt and/or PUFAs. The concentrations of Trolox and Z-VAD-FMK were chosen on the basis of previous experiments testing their ability to efficiently inhibit ROS production and caspase-3 activity in the cell lines studied, respectively.

2.3 Cell counts, floating cell quantification and viability assays

Floating and adherent cells were counted separately using a Coulter counter (model ZM; Beckman Coulter, Fullerton, CA, USA), and the amount of floating cells was expressed as a percentage of the total cell number. Cell viability was determined microscopically by eosin (0.15%) dye exclusion assay.

2.4 Cell cycle analysis

Fixed cells (70% ethanol) were washed with PBS, low molecular weight fragments of DNA were extracted in citrate buffer, RNA was removed by ribonuclease A, and DNA was stained with propidium iodide as described previously [22]. Fluorescence was measured using a flow cytometer (FCM; FACSCalibur; Becton Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength for excitation. A total of 2×10^4 cells was analysed in each sample. The ModFit 2.0 software (Verity Software House; Topsham, CA, USA) was used to generate DNA content frequency histograms.

2.5 Fluorescence microscopy

The cells were stained with a 4,6-diamidino-2-phenylindole (DAPI; Fluka; Buchs, Switzerland) solution (1 µg DAPI/mL ethanol) at room temperature in the dark for 30 min. They were then mounted in Mowiol, and the percentage of apoptotic cells (with chromatin condensation and fragmentation) was determined using a fluorescence microscope (Olympus IX-70; Olympus, Prague, Czech Republic) from a total number of 200 cells.

2.6 Detection of mitochondrial membrane potential (MMP)

The changes of MMP were analysed by FCM using tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Eugene, OR, USA) as described previously [22].

The data were evaluated (Cell Quest software, BD) as a percentage of the cells with decreased MMP.

2.7 Production of reactive oxygen species (ROS)

The intracellular production of ROS after treatments with the appropriate agents was detected by FCM analysis using dihydrorhodamine-123 (DHR-123, Fluka, Switzerland), which reacts with intracellular hydrogen peroxide as described previously [22].

2.8 Lipid packing of plasma membranes in live cells

Plasma membrane lipid packing was detected using the lipophilic negatively charged heterocyclic chromophore merocyanine 540 (MC540) [32]. 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 R.T., the cells were pelleted in a centrifuge (200 g), washed once, and resuspended in PBS. Fluorescence was measured by FCM (at 488 nm excitation and 585 nm emission wavelength). A total of 2×10^4 cells was analysed in each sample. The Cell-Quest software (BD) was used to generate histograms distinguishing MC540-dim and MC540-bright cells. The results are expressed as means of percentage of MC540-bright cells of three independent experiments.

2.9 Alkaline phosphatase (ALP) activity

ALP activity was determined in a lysate of sonicated cells (5×10^5 *per* sample) after incubation with ALP substrate (4-p-nitrophenylphosphate; Fluka) in a 96-well plate at $37°C$ for 30 min as described previously [33]. The optical densities were measured at 405 nm (DigiScan Reader). The reading values (units $\times 10^{-6}/5 \times 10^4$ cells) were converted to the percentage of control.

2.10 Expression of carcinoembryonic antigen (CEA)

The cells treated with the appropriate agents were harvested, washed twice in azide buffer (PBS with 0.1% sodium azide), resuspended in 100 µL of this buffer, and 5 µL of anti-CD66e-FITC mAb (SEROTEC) was added. As an isotype control, mouse IgG1-FITC (Pharmingen) was used. The samples were incubated 30 min on ice in the dark, washed twice, and resuspended in 0.5 mL of azide buffer. Fluorescence was detected using FCM in 2×10^4 cells *per* sample with a 530/30 (FL-1) optical filter.

2.11 Caspase activities

The cells were lysed in lysis buffer (250 mM HEPES, 25 mM CHAPS, 25 mM DTT, 40 µM protease inhibitor

Mol. Nutr. Food Res. 2009, *53*, S102–S113

S 105

cocktail; Sigma–Aldrich) on ice for 20 min and then centrifuged at $15\,000 \times g$ for 15 min in 4°C. The proteins acquired (equal concentrations) were incubated with caspase-3 (Ac-DMQD-AMC; 50 µM; Alexis; Carlsbad, CA, USA) and caspase-9 (Ac-LEHD-AMC; 50 µM; Alexis) substrates overnight in assay buffer (40 mM HEPES, 20% glycerol, 4 mM DTT). Fluorescence was measured (355/460 nm) using a Fluostar Galaxy fluorometer (BMG Labtechnologies; Offenburg, Germany).

2.12 Immunoblotting

The cells lysed in a Laemmli sample buffer and diluted to an equal concentration were processed and subjected to SDS-PAGE as described previously [34]. The membranes were probed with anti-poly(ADP-ribose) polymerase (anti-PARP) (SC-7150, Santa Cruz Biotechnology; Santa Cruz, CA, USA) or anti-Mcl-1 (M8434, Sigma-Aldrich) antibodies overnight. The proteins recognized were detected using horseradish peroxidase-labelled rabbit (1:6000, #NA934, Amersham Biosciences; Buckinghamshire, UK) or mouse (1:3000, #NA931, Amersham Biosciences) anti-IgG secondary antibody and an enhanced chemiluminescence kit (ECL; Amersham Biosciences). An equal loading was verified using β-actin (A5441, Sigma–Aldrich) quantification.

2.13 Statistical analysis

The results of at least three independent experiments were expressed as the means ± SEM. Statistical significance ($p < 0.05$) was determined by one-way ANOVA followed by a Tukey test or a nonparametric Mann–Whitney test.

3 Results

3.1 Cell proliferation and apoptosis

The doses of compounds used for combined treatment in this study were chosen on the basis of previous experiments testing the range of concentrations for NaBt (1–5 mM) and PUFAs (10–200 µM) on various types of colon cell lines. The selected doses of single agents are relevant to physiological concentrations of fatty acids and have only moderate short-time effects on cell growth and death. The data in Table 1 summarize the effects of these relatively low concentrations of AA, DHA (50 µM) and NaBt (3 mM), used individually or in combination, on cell growth (total cell number) and death (per cent of floating cells, cell viability) of FHC, HT-29 and HCT-116 cells after 24, 48 and 72 h of treatment. Furthermore, we detected changes of cell cycle parameters (Table 2) and the percentage of apoptotic cells (Fig. 1).

NaBt time-dependently decreased the total cell number (to 30–40% of control) as well as viability, and increased the percentage of floating cells and apoptosis in all cell lines
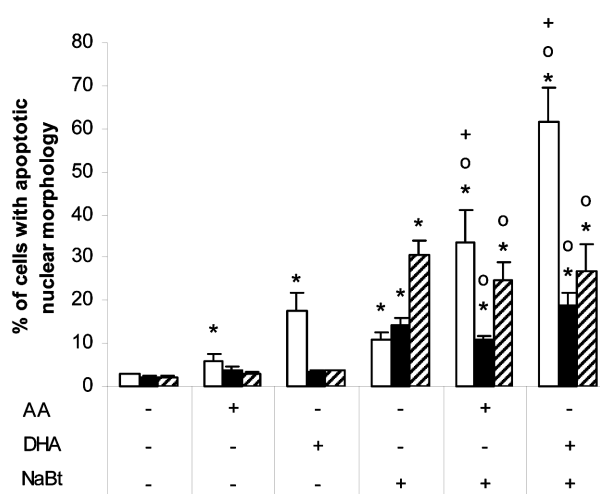

Figure 1. FHC, HT-29 and HCT-116 cells (%) with apoptotic nuclear morphology (DAPI staining). The cells were treated (72 h) with 50 µM AA or DHA, NaBt (3 mM) and their combination. Statistical significance: $p < 0.05$ *versus* control (*), *versus* respective PUFA (o), *versus* NaBt (+).

(Table 1). Compared to FHC and HT-29 cells, these effects were markedly stronger in HCT-116 cells (54% of floating cells and 63% viability after 72 h). Cell cycle analysis performed after 24 h (Table 2) and 48 h (data not shown) showed that NaBt caused G0/G1 arrest in FHC and HT-29 cells and G2/M arrest in HCT-116 cells.

AA or DHA in the concentration used did not significantly affect cell cycle parameters or the cell number (except DHA after 72 h in FHC cells). However, in FHC cells we detected a significant time-dependent increase in floating cells (30–45% after 72 h), decreased cell viability (70% for AA and about 50% for DHA after 72 h), and apoptosis. DHA had more considerable long-term apoptotic effects (about 18%) compared to AA (about 6%) (Fig. 1).

After a combined treatment of FHC cells with NaBt and AA or especially DHA, significantly higher effects on cell death were achieved compared to the agents used alone. In addition to a significant time-dependent increase in the proportion of floating cells and a decrease in viability (Table 1), we detected a strong increase in apoptotic cells especially after 72 h (up to 34% with AA and 62% with DHA) (Fig. 1). In parallel, a significant increase in the number of cells in G0/G1 and a decrease in S and G2/M phases was observed compared to NaBt alone. In HT-29 and HCT-116 cells, the changes of cell growth and death parameters detected after a combined treatment with NaBt and PUFA were generally nonsignificant compared to single NaBt treatment.

Furthermore, we verified cell apoptotic response by detection of PARP cleavage and caspase-3 activity. The PARP cleavage pattern (Fig. 2A) corresponded with other cell death parameters and was the most pronounced after 72 h. NaBt alone caused the highest time-dependent effect

Table 1. The effects of 50 µM AA or DHA, NaBt (3 mM) and their combination on FHC, HT-29 and HCT-116 cell growth (total cell number) and death (proportion of floating cells and viability) after 24, 48 and 72 h of treatment

Treatment		Total cell number (× 10³)			Floating cells (%)			Viable cells (%)		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Control	FHC	2315.0 ± 274.1	2843.6 ± 253.2	4684.0 ± 86.1	4.6 ± 0.6	4.6 ± 0.8	4.3 ± 0.8	90.4 ± 2.0	89.5 ± 2.2	89.5 ± 3.3
	HT-29	1941.2 ± 293.8	3711.2 ± 224.6	5825.9 ± 754.3	6.2 ± 1.5	3.4 ± 0.7	3.8 ± 1.5	89.7 ± 0.4	90.4 ± 2.0	88.0 ± 4.1
	HCT-116	3043.6 ± 255.7	6003.8 ± 299.2	6753.5 ± 532.8	3.4 ± 0.6	4.2 ± 0.7	4.3 ± 0.4	94.5 ± 1.1	94.5 ± 0.8	95.5 ± 1.1
AA 50	FHC	2363.7 ± 326.8	2581.3 ± 196.7	4360.7 ± 279.8	4.6 ± 0.4	30.2 ± 7.3[a]	28.9 ± 2.7[a]	87.6 ± 2.6	77.5 ± 5.3[a]	70.3 ± 10.2[a]
	HT-29	2079.4 ± 288.1	3470.8 ± 156.1	5779.9 ± 488.4	5.9 ± 0.8	4.5 ± 1.0	8.5 ± 3.1	82.3 ± 6.2	86.6 ± 3.2	82.5 ± 4.7
	HCT-116	3055.0 ± 353.2	5622.1 ± 404.6	6466.0 ± 867.7	3.2 ± 0.7	3.6 ± 0.5	4.2 ± 0.7	91.8 ± 2.3	94.1 ± 1.3	93.5 ± 1.5
DHA 50	FHC	2363.9 ± 315.4	2453.1 ± 225.4	3402.9 ± 138.1[a]	10.1 ± 2.7[a]	35.4 ± 6.4[a]	44.2 ± 6.7[a]	89.2 ± 3.8	62.3 ± 6.7[a]	47.5 ± 8.6[a]
	HT-29	2161.9 ± 136.9	3364.7 ± 331.2	5723.3 ± 544.7	9.0 ± 4.4	4.7 ± 1.0	5.4 ± 1.4	83.7 ± 7.6	86.2 ± 1.5	86.0 ± 3.2
	HCT-116	3006.6 ± 236.0	5122.7 ± 405.3	5544.7 ± 998.4	3.9 ± 0.5	3.4 ± 0.4	5.2 ± 0.8	94.8 ± 0.6	91.6 ± 3.1	86.3 ± 7.8
NaBt 3	FHC	1284.5 ± 119.2[a]	1085.1 ± 112.5[a]	1682.6 ± 88.9[a]	12.4 ± 1.2[a]	20.7 ± 1.9[a]	27.4 ± 6.0[a]	82.4 ± 3.3	76.0 ± 7.8[a]	69.0 ± 10.6
	HT-29	1489.9 ± 218.3[a]	1545.8 ± 158.3	2005.4 ± 178.3[a]	12.0 ± 1.6[a]	18.8 ± 3.6	23.3 ± 7.2[a]	84.3 ± 9.0[a]	84.0 ± 1.9	76.0 ± 8.4
	HCT-116	1541.3 ± 128.1[a]	1802.4 ± 146.3[a]	2169.7 ± 150.7[a]	13.9 ± 1.5[a]	34.6 ± 1.8[a]	53.9 ± 2.3[a]	88.7 ± 3.5	78.6 ± 2.9[a]	64.5 ± 3.9[a]
AA50 + NaBt3	FHC	1444.0 ± 145.6[a,b]	1467.2 ± 139.5[a,b,c]	2233.8 ± 130.6[a,b,c]	12.6 ± 1.3[a,b,c]	34.9 ± 2.7[a,c]	67.2 ± 9.7[a,b,c]	84.0 ± 4.0	65.0 ± 6.6[a,c]	44.3 ± 15.1[a,b,c]
	HT-29	1361.1 ± 205.4[a,b]	1584.5 ± 121.8	2042.7 ± 243.2[a,b]	10.7 ± 1.6	20.9 ± 3.6	27.7 ± 4.9[a,b]	79.3 ± 3.6[a]	76.6 ± 5.1	69.0 ± 4.8[a,b]
	HCT-116	1357.2 ± 82.3[a,b]	1643.9 ± 135.2[a,b]	2045.2 ± 157.2[a,b]	16.2 ± 1.3[a,b]	36.3 ± 1.6[a,b]	59.7 ± 0.8[a,b]	85.3 ± 4.7	74.1 ± 3.0[a,b]	50.3 ± 0.3[a,b,c]
DHA50 + NaBt3	FHC	1441.7 ± 132.1[a,b]	1620.1 ± 157.8[a,b,c]	2027.1 ± 79.3[a,b]	22.8 ± 3.0[a,b,c]	47.6 ± 3.6[a,b]	75.5 ± 4.0[a,b,c]	84.8 ± 1.3	49.8 ± 4.9[a,b,c]	22.0 ± 3.1[a,b,c]
	HT-29	1422.2 ± 191.0[a,b]	1488.1 ± 107.2	2045.0 ± 242.1[a,b]	15.3 ± 1.4[a,b]	19.3 ± 1.2	32.2 ± 4.0[a,b]	76.0 ± 6.5[a]	76.0 ± 1.1	65.7 ± 9.2[a]
	HCT-116	1482.1 ± 126.3[a,b]	1647.0 ± 151.5[a,b]	2089.1 ± 159.6[a,b]	14.2 ± 1.4[a,b]	37.2 ± 1.9[a,b]	63.3 ± 0.9[a,b]	78.0 ± 7.0[a,b]	66.2 ± 2.7[a,b]	51.5 ± 8.3[a,b]

a) Statistical significance $p < 0.05$ *versus* control.
b) Statistical significance $p < 0.05$ *versus* respective PUFA.
c) Statistical significance $p < 0.05$ *versus* NaBt.

Mol. Nutr. Food Res. 2009, *53*, S102–S113

S107

Table 2. The effects of 50 µM AA or DHA, NaBt (3 mM) and their combination on FHC, HT-29 and HCT-116 cell cycle parameters after 24 h of treatment

Treatment		% of cells		
		G_0/G_1	S	G_2/M
Control	FHC	55.8 ± 1.5	30.3 ± 1.9	13.8 ± 0.8
	HT-29	56.0 ± 6.0	29.7 ± 3.6	14.2 ± 4.0
	HCT-116	48.7 ± 2.7	32.5 ± 3.4	15.3 ± 1.9
AA 50	FHC	53.7 ± 2.5	32.5 ± 2.5	13.7 ± 1.0
	HT-29	53.0 ± 6.1	32.0 ± 4.4	14.9 ± 4.7
	HCT-116	47.2 ± 2.4	31.9 ± 5.2	14.3 ± 2.3
DHA 50	FHC	59.6 ± 1.8	26.8 ± 1.5	13.6 ± 0.8
	HT-29	52.8 ± 4.7	32.2 ± 4.2	14.9 ± 3.4
	HCT-116	46.3 ± 1.9	34.1 ± 2.6	16.6 ± 1.4
NaBt 3	FHC	73.1 ± 0.9[a]	4.1 ± 1.0[a]	22.8 ± 1.0[a]
	HT-29	80.6 ± 7.6[a]	6.3 ± 1.2[a]	13.0 ± 7.9
	HCT-116	46.1 ± 1.1	21.7 ± 4.4[a]	29.4 ± 4.9[a]
AA50 + NaBt3	FHC	81.2 ± 1.3[a,b,c]	2.4 ± 0.7[a,b]	16.7 ± 0.9[a,b,c]
	HT-29	79.5 ± 7.8[a,b]	6.5 ± 2.0[a,b]	13.9 ± 9.5
	HCT-116	47.3 ± 5.1	16.3 ± 1.5[a,b]	35.3 ± 3.2[a,b]
DHA50 + NaBt3	FHC	79.2 ± 0.9[a,b,c]	2.3 ± 0.8[a,b]	18.4 ± 1.2[a,b,c]
	HT-29	86.4 ± 3.0[a,b]	5.2 ± 1.2[a,b]	8.3 ± 1.0
	HCT-116	47.6 ± 2.1	18.8 ± 1.3[a,b]	32.4 ± 3.0[a,b]

a) Statistical significance $p < 0.05$ *versus* control.
b) Statistical significance $p < 0.05$ *versus* respective PUFA.
c) Statistical significance $p < 0.05$ *versus* NaBt.
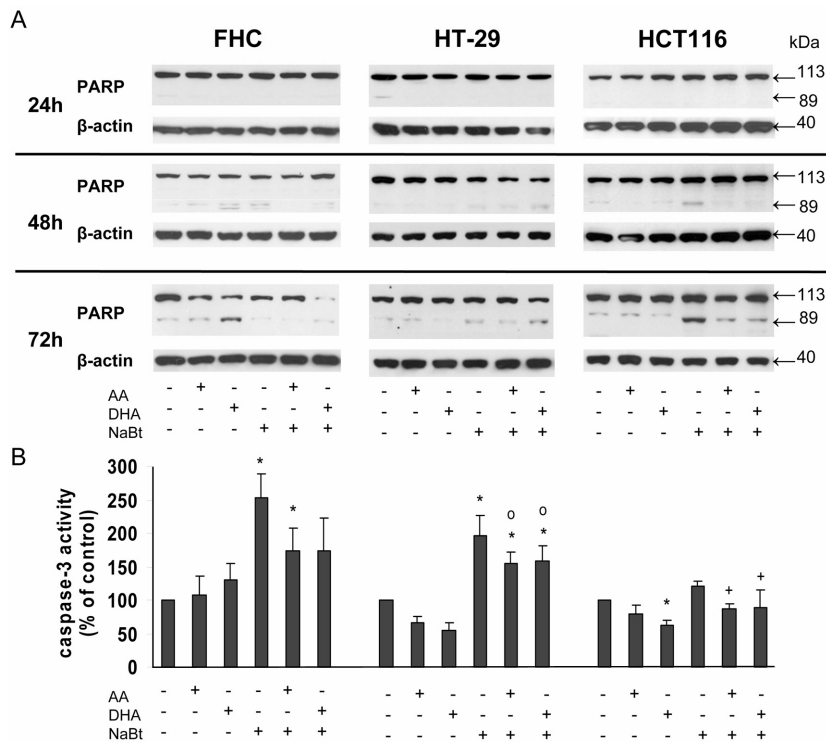
Figure 2. Cleavage of PARP and caspase-3 activation in FHC, HT-29, and HCT-116 cells treated with 50 µM AA or DHA, NaBt (3 mM) and their combination. (A) Detection of full-length PARP and its 89 kD fragment after 24, 48 and 72 h. Protein levels were determined by Western immunoblotting and β-actin served as control of equal protein loading. Representative results from three independent experiments. (B) Caspase-3 activity expressed as percentage of the control value after 48 h. Statistical significance: $p < 0.05$ *versus* control (*), *versus* respective PUFA (o), *versus* NaBt (+).

on PARP cleavage in HCT-116 cells, and this effect was slightly attenuated by the combination of NaBt with either AA or DHA. On the other hand, AA and especially DHA alone caused a strong decrease in full-length PARP only in FHC cells. Moreover, this effect was potentiated after the combined treatment of FHC cells with NaBt and DHA. A less yet considerable effect was also observed in HT-29 cells.

Compared to the control, we ascertained an increase in caspase-3 activity after 48 h of NaBt treatment in all the
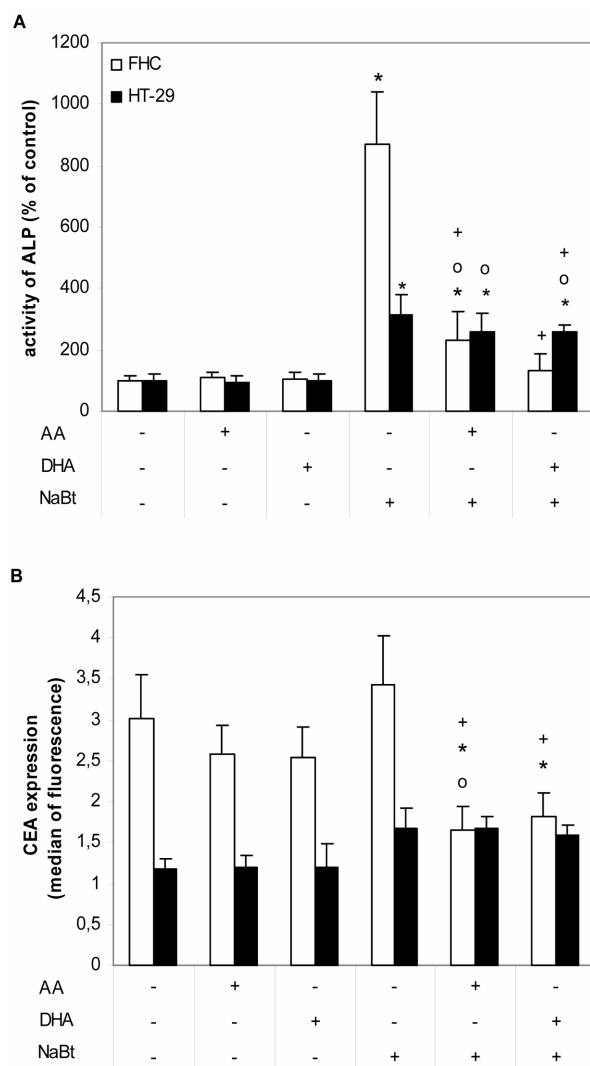
A

B

Figure 3. The activity of ALP and expression of CEA of FHC and HT-29 cells treated with 50 μM AA or DHA, NaBt (3 mM) and their combination. (A) Activity of ALP expressed as per cent of control (=100%) after 48 h. Adherent and floating cells were evaluated together. (B) Fluorescence after staining with anti-CEA mAb (FITC) was analysed after 72 h by FCM (FL1-H) in viable cell population (PI-negative) and expressed as MFI (median fluorescence intensity index). Statistical significance: $p < 0.05$ *versus* control (*), *versus* respective PUFA (o), *versus* NaBt (+). p 0.01 (#) compared FHC *versus* HT-29 cells.

three cell lines, with the most prominent effect in FHC cells (Fig 2B). There was a tendency of attenuation of caspase-3 activity after combination of NaBt with AA or DHA compared to NaBt alone (significant only in HCT-116 cells).

3.2 Differentiation

FHC and HT-29 cells, but not HCT-116 cells, were able to differentiate after treatment with NaBt as detected by enter-

ocyte differentiation markers – ALP activity and CEA expression. Compared to the control, the activity of ALP increased nine-fold in FHC cells and three-fold in HT-29 cells after 48 h (Fig. 3A). This effect was significantly suppressed by PUFAs, especially DHA. On the other hand, we did not observe any changes either in FHC or HT-29 cells after treatment with PUFAs used alone. No changes of ALP activity were demonstrated in HCT-116 cells after any type of treatment (not shown).

Both untreated HT-29 and FHC cells expressed CEA, but this basal expression was markedly higher in FHC cells (Fig. 3B). NaBt increased CEA expression in FHC cells not before 72 h, and this expression was suppressed after combined treatment with AA or DHA. We did not find any CEA expression in HCT-116 cells.

3.3 Lipid packing in plasma membrane

An analysis of structural changes in the phospholipid bilayer (lipid packing) reflected by MC540 binding in the plasma membrane (Fig. 4A) showed that NaBt significantly increased the proportion of MC540 bright cells in all cell lines. However, only in FHC cells this effect was apparent after AA and DHA treatment and was moderately enhanced after combined treatment with NaBt and PUFAs.

3.4 Mitochondrial membrane potential and ROS production

NaBt and, to a lesser extent also both AA and DHA, increased the proportion of HCT-116, HT-29, and especially FHC cells with decreased MMP after 24 h (not shown) and especially 48 h (Fig. 4B) treatment. However, only in FHC cells we detected an about two-fold significant enhancement of this proportion after combined treatment with NaBt and AA or DHA (about 50 and 45%, respectively).

NaBt, AA, DHA and their combinations induced production of ROS measured after both 24 h (not shown) and 48 h of treatment (Fig. 4C). The intensity of this effect was time-dependent and differed between the individual colon cell lines used. Generally, we detected the highest ROS induction in FHC cells and the tendency of additional enhancement of this effect after combined treatment with NaBt and both PUFAs in FHC and HCT-116 cells.

3.5 The effects of antioxidant treatment

The antioxidant Trolox functions as a ROS scavenger and stops lipid peroxidation by reaction with peroxyl radicals. To clarify the role of ROS in apoptotic *versus* differentiation response we added Trolox to the most responding FHC cells 1 h before treatment with NaBt, DHA or their combination. Trolox significantly suppressed the amount of apoptotic cells after all types of treatment (Fig. 5A), simultaneously preventing the decrease in ALP activity after com-
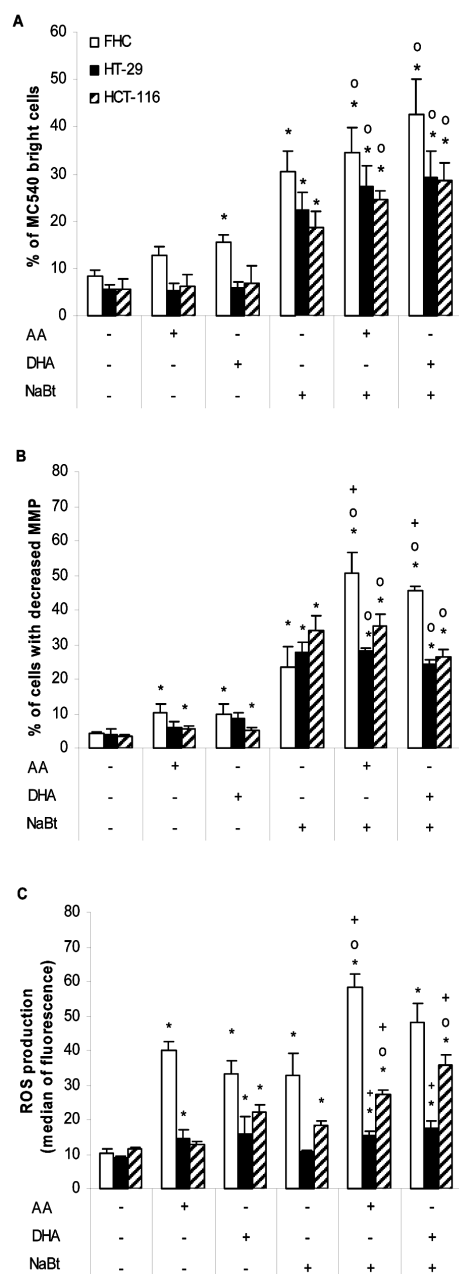
Mol. Nutr. Food Res. 2009, 53, S102–S113

S109

Figure 4. Changes of MMP, production of ROS and proportion of MC540-bright cells in FHC, HT-29 and HCT-116 cell lines after 48 h of treatment with 50 μM AA or DHA, NaBt (3 mM) and their combination. (A) Percentage of MC540-bright cells after staining with MC540 detected by FCM (FL2-H). (B) Percentage of cells with decreased MMP detected by FCM after staining with TMRE (FL2-H). (C) Production of ROS (median of fluorescence) detected by FCM after staining with DHR-123 (FL-1H). Statistical significance: $p < 0.05$ *versus* control (*), *versus* respective PUFA (o), *versus* NaBt (+).

bined treatment of cells with NaBt and DHA (Fig. 5B). However, Trolox had negligible effects on the percentage of cells with decreased MMP (not shown).
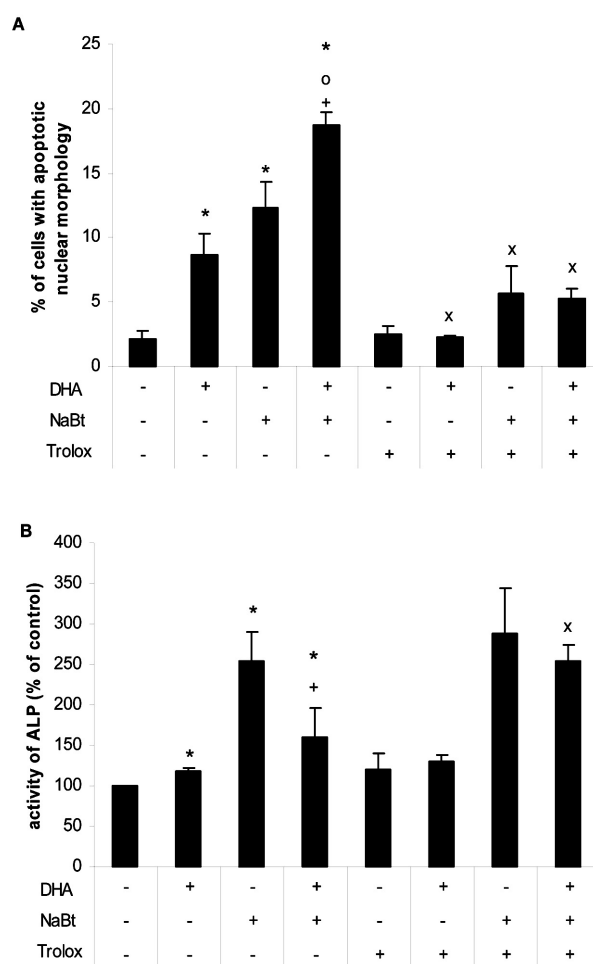

Figure 5. The effects of Trolox (100 ug/mL added 1 h before other compounds) on (A) apoptosis (% of cells with apoptotic nuclear morphology, DAPI staining) and (B) differentiation (ALP activity,% of control) of FHC cells treated 48 h with 50 μM DHA, NaBt (3 mM) and their combination. Statistical significance: $p < 0.05$ *versus* control (*), *versus* DHA (o), *versus* NaBt (+), and *versus* the respective sample without Trolox (×).

3.6 Expression of Mcl-1 protein

The role of antiapoptotic Mcl-1 protein in both apoptosis and differentiation is supposed. Thus, we compared its time-course changes in FHC (differentiated) and HCT-116 (nondifferentiated) cells after treatment with NaBt, PUFAs or their combination (Fig. 6). In FHC cells, we detected a strong increase in the Mcl-1 protein level after NaBt treatment already after 24 h, which remained high up to 72 h. On the other hand, in HCT-116 cells, the NaBt-induced increase in the Mcl-1 level was apparent after longer treatments, especially 72 h. AA and DHA increased Mcl-1 expression in both cell lines after 24 h and this effect was attenuated during cultivation. Considerable differences in the Mcl-1 protein level after combined NaBt and PUFA treatment were apparent between FHC and HCT-116 cells
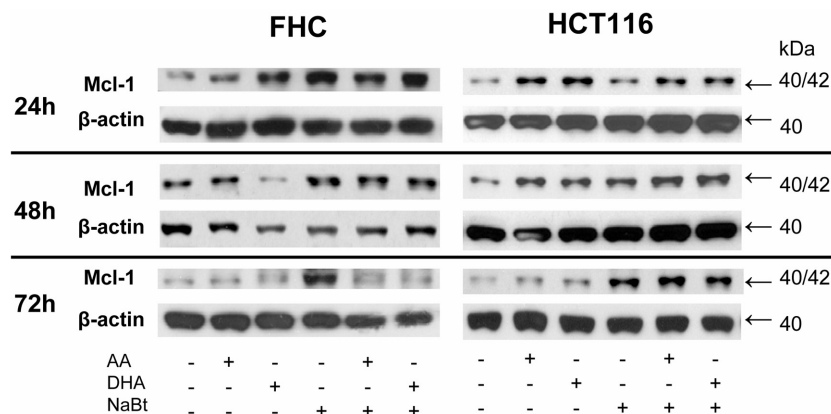
Figure 6. The levels of Mcl-1 protein during 24–72 h of cultivation of FHC and HCT-116 cells treated with 50 µM AA or DHA, NaBt (3 mM) and their combination. Protein levels were determined by Western immunoblotting and β-actin served as control of equal protein loading. Representative results from three independent experiments.

after 72 h. While we detected a strong downregulation of Mcl-1 expression compared to NaBt alone in FHC cells, a relatively high level of Mcl-1 protein was still present in HCT-116 cells.

4 Discussion

Dietary ω−3 PUFAs and NaBt from fibre have been shown to induce both differentiation and apoptosis of colonic cells and are considered as chemoprotective nutrients in colon cancer. We report that NaBt effects are modulated by combination with PUFAs of both ω−3 and ω−6 type and these effects are different in the cell lines derived from human normal fetal tissue (FHC), well-differentiated (HT-29) and poorly differentiated (HCT-116) adenocarcinomas.

In our experiments, these cell lines differed in the type of their response to NaBt (3 mM). While FHC as well as HT-29 cells accumulated in the G0/G1 phase were induced to differentiation with a relatively low level of apoptosis, HCT-116 cells accumulated in the G2/M phase showed high apoptotic response and no differentiation. We confirmed that NaBt-induced apoptosis closely follows anoikis [35] influencing cell adhesion and thus increasing the proportion of floating cells, especially in HCT-116 cells. Floating cells are supposed to be primed for apoptosis because their amount correlated with PARP cleavage and with the increased amount of apoptotic cells.

Although FHC and HT-29 cells present similar growth characteristics and are able to differentiate to enterocyte-like cells after NaBt treatment, induction of ALP activity induced in subconfluent HT-29 cells is only about one half of the values observed in FHC cells. Moreover, compared to HT-29 cells, untreated FHC cells present a higher basal expression of CEA than do HT-29 cells, probably due to their embryonal origin. However, CEA expression was only slightly increased after NaBt treatment, probably due to a short time of cultivation [36].

Previously, we reported that the differences between FHC and HT-29 cell lines could be associated with a faster

downregulation of telomerase activity in FHC cells [33]. However, our investigation of the changes in histone acetylation and methylation pattern showed a similar dynamic chromatin reorganization in both FHC and HT-29 cells in parallel with changes in its epigenetic modifications [37].

Contrary to our results, Comalada *et al.* reported that FHC cells are not affected by NaBt (2–8 mM). However, they evaluated only ³H-thymidine incorporation and viability in subconfluent cells after 24 h [38]. Recent evidence indicates that the response of proliferating cells to butyrate depends upon its concentration, time, the physiological conditions during the study, the cell phenotype and confluence, differentiation status and other factors present [14, 39]. Differences in butyrate metabolism and transport mechanisms could also be suggested in normal and adenocarcinoma colon cells [40] and may contribute to the differences in apoptotic *versus* differentiation response between the colon cell lines used in our experiments.

FHC cells are the only cell line responding to the relatively low PUFA concentration used (50 µM). In spite of the fact that tumour cell-specific effects and a lower sensitivity of normal cells to PUFAs were reported [41], there are several studies showing a higher PUFA sensitivity of normal cells than in cancer or transformed cells of the same type, which is in concert with our findings [42, 43]. In our study, DHA suppressed FHC cell growth, decreased adhesion and induced apoptosis more effectively than AA. Epidemiological and *in vivo* studies reported a promoting role of ω−6 PUFAs and their metabolites in colon cancer development [6]. However, AA exogenously added to cultured cells inhibits proliferation and induces cell death (apoptosis and often necrosis) in various cell types, but mostly at concentrations higher than those used by ourselves [44]. Moreover, literature data have documented differences between AA and DHA with regard to incorporation into various types of cell lipids, influence on oxidative metabolism, and other cell functions [45]. After DHA treatment more complex mechanisms involving modification of membrane properties [46], activation of specific intracellular receptors, altered expression of transcription factors, cell cycle and

Mol. Nutr. Food Res. 2009, *53*, S102−S113

S 111

apoptosis regulating proteins as well as inactivation of prostaglandin family genes and lipoxygenases [47] should be considered.

The most important finding of the present study is the modulation of NaBt effects on colonic cells by PUFA cotreatment. We newly demonstrated a significant potentiation of antiproliferative and apoptotic response accompanied by suppression of differentiation in noncancer human colon FHC cells after cotreatment with NaBt and AA, and especially DHA. In HT-29 cells, moderate effects were detected only in combination of NaBt with DHA, and no such effects were observed in HCT-116 cells. Our results are in accordance with those obtained by other authors using normal mouse colonocytes *in vitro* as well as *in vivo*. It has been shown that bioactive components of fermentable fibre (butyrate) and fish oil (DHA) work co-ordinately to protect against colon tumorigenesis. These effects were primarily caused by enhancing apoptosis rather than decreasing proliferation in a rat model of experimentally induced colon cancer [26, 48]. Using young adult mouse colonocytes and *ex vivo* intact rat colon crypts it was demonstrated that these cells treated with DHA, but not linoleic acid, were primed for butyrate-induced apoptosis. The mechanisms of these effects include mitochondrial lipid oxidation, resulting in an increase in ROS, dissipation of MMP, and initiating apoptotic cascade [21, 49]. Moreover, expression of the antiapoptotic Bcl-2 protein, which possesses antioxidant properties suppressing membrane lipid oxidation, was decreased by DHA and butyrate [50]. Hossain *et al.* [51] reported enhancing effects of phospholipids containing DHA on butyrate-induced growth inhibition, differentiation and apoptosis of colon cancer cells.

Our results suggest that changes in plasma membrane lipid packing, ROS production and dissipation of MMP play an important role during the apoptotic *versus* differentiation effects of NaBt and PUFA in the colon cell lines used. However, the precise relationship still needs to be fully investigated. Previously, MC540 has been reported as a useful probe for monitoring structural changes in the phospholipid bilayer during apoptosis, differentiation or activation of various types of hemopoietic cells [52, 53]. Our results newly indicate a significant increase in MC540 fluorescence (lipid unpacking) after NaBt treatment in all the three colon cell lines despite various differentiation *versus* apoptotic response. Additionally, increased lipid unpacking in FHC cells after combined NaBt and PUFA treatment implies the association of this effect particularly with apoptotic response.

Our presented data do not quite confirm the suggested correlation of cell ROS production and the proportion of cells with dissipated MMP with apoptotic response. In spite of significantly increased ROS production and dissipation of MMP, FHC and HT-29 cells differentiated after single NaBt treatment. We suppose that the quantitative aspect of these changes shifting redox balance to the pro-oxidative

environment may play a role in the transition from differentiation to apoptotic response after combined treatment with NaBt and PUFAs in sensitive FHC cells. It was confirmed by the reversing effect of the antioxidant Trolox, which suppressed apoptosis and enhanced differentiation.

Increased production of ROS by NaBt was reported to contribute to sensitization of HT-29 cells to apoptosis induced by TNF or Fas [25, 54]. In our previous papers, we showed that processes induced by pretreatment with PUFAs including ROS production, lipid peroxidation and MMP changes could promote the apoptotic sensitivity of HT-29 cells to other regulatory factors operating in the colon such as NaBt or TNF family apoptotic inducers [23, 24]. The different oxidative response of normal and cancer cell lines may be related to the reported altered membrane lipid composition, different activity of antioxidant defence as well as dysregulation of certain signalling pathways in cancer cells [31].

In addition to their role in the regulation of apoptosis, a possible role of caspases and Mcl-1 protein in differentiation has been suggested [55, 56]. Our results support this idea showing (i) a higher increase in caspase-3 activity during NaBt-induced differentiation of FHC and HT-29 cell lines compared to HCT-116 cells and (ii) declined trends of caspase activity together with decreased FHC and HT-29 cell differentiation after combined NaBt and PUFA treatment observed in our experiments. On the other hand, in spite of induction of apoptosis, PUFAs in 50 µM concentration do not induce caspase-3 activity in FHC cells. The ability of AA or DHA to activate caspases is probably concentration-dependent, because in colon cancer cells we detected caspase-3 and -9 activation after higher DHA or AA concentrations [24]. The addition of a pan-caspase inhibitor together with NaBt caused a significant decrease in floating cells and apoptosis in all cell lines, but no significant changes of differentiation. The same effects were detected after combination of NaBt and DHA in HT-29 and HCT-116 cells (data not shown). In FHC cells the combination of Z-VAD-FMK with PUFAs was cytotoxic.

Increased Mcl-1 protein expression after NaBt treatment of differentiating FHC colon cells is in accordance with the reported effects of differentiation agents in other cell types [57]. Furthermore, after a combined treatment with PUFAs, where a high apoptotic response of FHC cells was detected, Mcl-1 expression was significantly downregulated. These changes were not observed in nondifferentiating HCT-116 cells which were induced only to apoptosis.

In summary, our results suggest the interaction of the effects of short- and long-chain fatty acids, which may influence differentiation and/or apoptotic response of colonic cells. We showed significant differences between colon cell lines derived from normal fetal and cancer tissues. The most pronounced effects of NaBt and PUFA combination significantly potentiating cell cycle arrest, cell detachment and apoptosis and decreasing differentiation

were detected in the fetal FHC cell line. Changes of cytokinetic parameters were accompanied by membrane lipid unpacking, decrease in MMP and increased ROS production. Detection of caspase-3 activation and dynamic modulation of Mcl-1 protein expression imply their possible role in both differentiation and apoptotic response. Thus, our results agree with the concept that the effects of fermentable fibre or butyrate in the colon are modulated by dietary unsaturated fatty acids. Moreover, different effects of these compounds on cell lines of noncancer and cancer origin may imply a possible diverse impact in normal and neoplastic epithelia.

The authors have declared no conflict of interest.

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S113

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