Eur J Nutr (2005) 44 : 40–51
DOI 10.1007/s00394-004-0490-2

ORIGINAL CONTRIBUTION

Jiřina Hofmanová
Alena Vaculová
Antonín Lojek
Alois Kozubík

Interaction of polyunsaturated fatty acids and sodium butyrate during apoptosis in HT-29 human colon adenocarcinoma cells

Summary *Background* Dysregulation of the balance between cell growth and death in the colonic epithelium is associated with cancer promotion. Understanding how cell death in this self-renewing tissue is regulated and how it is influenced by interaction of specific dietary components, especially fat and fibre, could lead to improved treatment and prevention strategies for cancer. *Aim of the study* The effects of two types of polyunsaturated fatty acids (PUFAs) – arachidonic (AA, 20:4, n-6) or docosahexaenoic (DHA, 22:6, n-3) – on the response of human colon adenocarcinoma HT-29 cells to sodium butyrate (NaBt) were investigated. *Methods* The parameters reflecting cell proliferation and cell death were studied together with oxidative response, mitochondrial membrane potential (MMP) and changes of selected regulatory molecules associated with cell cycle ($p27^{Kip1}$ and $p21^{Cip1/WAF1}$) and apoptosis (caspase-3, caspase-9, poly (ADP-ribose) polymerase – PARP, Bcl-2, Bax, Bak, Mcl-1). *Results* We demonstrated that pre-treatment with either AA or DHA attenuated cell cycle arrest caused by NaBt which is associated with modulation of $p27^{Kip1}$, but not $p21^{Cip1/WAF1}$ protein expression. On the other hand, PUFAs sensitised HT-29 cells to NaBt-induced apoptosis. An increased amount of floating cells and cells in the $subG_0/G_1$ population was associated with increased reactive oxygen species production, lipid peroxidation, decrease of MMP, activation of caspase-3 and -9, PARP cleavage, and decrease in the expression of anti-apoptotic Mcl-1 protein. The observed effects were modulated by the addition of a protein synthesis inhibitor, cycloheximide, and partially reversed by the antioxidant Trolox. *Conclusions* PUFAs may have beneficial effects in the colon enhancing apoptosis induced by NaBt. Alteration of cell membrane lipid composition and potentiation of oxidative processes accompanied by changes in mitochondria followed by stimulation of apoptotic cascade components play a role in these effects.

Key words colon cancer – diet – polyunsaturated fatty acids – butyrate – apoptosis

Received: 17 February 2003
Accepted: 12 January 2004
Published online: 10 March 2004

RNDr. J. Hofmanová, PhD (✉) · A. Vaculová · A. Kozubík
Laboratory of Cytokinetics
Institute of Biophysics,
Academy of Sciences of the Czech Republic
Královopolská 135
61265 Brno, Czech Republic
E-Mail: hofmanova@ibp.cz

A. Lojek
Laboratory of Free Radical Pathophysiology
Institute of Biophysics, Academy of Sciences of the Czech Republic
Brno, Czech Republic

Abbreviations

AA	arachidonic acid
CHX	cycloheximide
CL	cardiolipin
DAPI	4.6-diamidino-2-phenyl-indole
DHA	docosahexaenoic acid
DHR-123	dihydrorhodamine
FCM	flow cytometry
FCS	foetal calf serum
MMP	mitochondrial membrane potential
NaBt	sodium butyrate
PARP	poly(ADP-ribose) polymerase
PL	phospholipids
PUFAs	polyunsaturated fatty acids
ROS	reactive oxygen species
TBARs	thiobarbituric acid reactive substances

EJN 490

TMRE tetramethylrhodamine ethyl ester perchlorate

Introduction

The colonic epithelium is in direct contact with dietary factors present in the lumen, and these factors may affect the pattern of growth, differentiation and cell death within the tissue [1]. Apoptotic cell death is one of the principal mechanisms by which cell numbers are controlled within the crypt of a healthy colon. However, the regulation of this event is often altered in transformed cells [2].

The important modulators of cytokinetics in the colonic epithelium are short-chain fatty acids, particularly butyrate, produced during microbial fermentation of dietary fibre. Butyrate provides the preferred oxidative fuel for the normal colonocytes [3] but it has also been shown to affect gene expression, cell growth regulation, and differentiation in colon cancer cells. Although the molecular mechanisms by which butyrate mediates its effects are not well understood, it is known to induce a variety of changes within the nucleus, including histone hyperacetylation and DNA methylation [4]. A recent gene array and proteome analysis of human colon adenocarcinoma HT-29 cells treated with sodium butyrate (NaBt) revealed that the genes (mostly transcription factors) and proteins linked to the cell growth, apoptosis and oxidative metabolism appeared as the most significantly affected [5, 6]. Butyrate or its analogues are used as anticancer drugs and have been shown to potentiate TNF-α or Fas-induced apoptosis [7, 8]. However, cancer cells develop mechanisms by which they can escape the beneficial effects of butyrate, particularly the induction of apoptosis [5, 9].

Quantitative and qualitative supply of essential polyunsaturated fatty acids (PUFAs) of n-6 and n-3 families from dietary fat is proposed to be linked with the risk of colorectal cancer [10]. Some types of PUFAs (particularly n-3 series) have antiproliferative effects, enhance apoptosis, and are protective against colon cancer in experimental systems both *in vitro* [11] and *in vivo* [12]. PUFAs, and especially the large family of their oxygenated metabolites, eicosanoids, act as modulators or messengers of the intra- and intercellular information network and regulate various aspects of inflammatory and immune responses [13, 14]. They may influence gene expression and cellular processes such as proliferation, differentiation, and apoptosis [15]. However, the precise cellular and molecular mechanisms by which PUFAs modulate tumour growth remain to be detected.

Tumour cells, due to their selective uptake of fatty acids from the circulation, may be specifically targeted by dietary supplementation with PUFAs [16]. The accumulation of PUFAs and increased lipid peroxidation may cause cell death or modulate the sensitivity of tumour cells to the action of other factors operating in the colon [17, 18]. We hypothesised that interaction of butyrate with PUFAs might influence the cytokinetic response, particularly apoptosis, of the colonic epithelium. Therefore, in our study, the response of the human colon adenocarcinoma cell line HT-29 to NaBt after pretreatment with arachidonic (AA, 20:4, n-6) or docosahexaenoic (DHA, 22:6, n-3) acids was investigated.

Material and methods

Cell culture

The HT-29 human colon adenocarcinoma cells obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) were cultured in McCoy's 5A medium supplemented with 50 μg/ml gentamicin (both from Sigma-Aldrich Corp., St. Louis, MO, USA) and 10 % foetal calf serum (FCS, PAN Biotech GmbH; Aidenbach, Germany) in a humidified incubator at 37 °C in 5 % CO_2. For the experiments, the cells were seeded in 40x10 mm ($1x10^5$ cells per dish) or 60x15 mm ($5x10^5$ cells per dish) dishes (TPP; Trasadigen, Switzerland) and allowed to attach for 24 h.

Experimental design

AA and DHA (Sigma-Aldrich Corp., St. Louis, MO, USA) were dissolved in 96 % ethanol and stored as stock solution (100 mM) under nitrogen at –80 °C. For the experiments, fatty acids were freshly prepared from stock solutions. To simulate physiological conditions, PUFAs were bound to fatty acid-free bovine serum albumin (BSA, SERVA Electrophoresis GmbH; Heidelberg, Germany) at a molar ratio of 1:2.5, and then diluted with the growth medium. The resulting concentration of FCS during PUFA treatment was 5 %. The control cells were treated with BSA and an appropriate concentration of ethanol. This type of treatment did not influence any of the parameters tested. After 48 h cultivation of cells with AA or DHA the medium was exchanged for a fresh serum-free McCoy's 5A medium supplemented with 1 % ITS (insulin, transferrin, sodium selenite) without PUFAs and with or without 5 mM of NaBt. Then the cells were cultivated for 24 h. In the experiments using cycloheximide (CHX), the cells were treated with 5 μg/ml CHX added 3 h before the application of NaBt. Trolox (a water-soluble analogue of vitamin E, 50 μg/ml) used as an antioxidant was added to the cells simultaneously with PUFAs for 48h or with NaBt for 24h. All the chemicals described in this paragraph were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA.

European Journal of Nutrition (2005) Vol. 44, Number 1

Analysis of fatty acid composition of cellular lipids

The content of selected fatty acids in cell lipids after 48 h treatment with AA or DHA was determined. Cells were washed twice with phosphate-buffered saline (PBS), resuspended in freezing medium (cultivation medium containing 10 % FCS and 5 % DMSO) and aliquots were frozen in –80 °C. After thawing total lipids were extracted from the samples and fatty acid content was determined after transmethylation using a GC-MS TurboMass instrument (Perkin-Elmer, Norwalk, USA). The derivatisation procedure was performed as published previously [19]. The amount of detected fatty acids in the samples was expressed as nmol per 10^6 cells.

Floating cells 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 whole cell number. Cell viability was determined by eosin (0.15 %) dye exclusion assay. The percentage of unstained (viable) cells was determined microscopically from a total number of 100 cells.

Flow cytometric (FCM) analysis of DNA

Floating and adherent cells were harvested together, washed twice in PBS, and fixed in 70 % ethanol at 4 °C. After washing with PBS, low-molecular-weight fragments of DNA were extracted for 10 min in citrate buffer (Na_2HPO_4, $C_6H_3O_7$, pH 7.8), RNA was removed by ribonuclease A (5 Kunitz U/ml), and DNA was stained with propidium iodide (20 µg/ml PBS) for 30 min in the dark. Fluorescence was measured using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA) equipped with an argon laser at 488 nm wavelength for excitation. A total of $2x10^4$ cells were analysed in each sample. The ModFit 2.0 (Verity Software House, Topsham, ME) and CellQuest (Becton Dickinson) softwares were used to generate DNA content frequency histograms and quantify the amount of the cells in the individual cell cycle phases including subG_0/G_1 population. Single cells were identified and gated by pulse-code processing of the area and width of the fluorescence signal. Cell debris was excluded by appropriate raising of the forward scatter threshold.

Fluorescence microscopy

After trypsinization, the cells were stained with 40 µl 4.6-diamidino-2-phenyl-indole (DAPI, 3 µg/ml of methanol, Fluka, Switzerland) at room temperature in the dark for 30 min. The cell suspension was then mixed with 30 µl of Mowiol 40–88 (Sigma-Aldrich Corp., St. Louis, MO, USA). The percentage of apoptotic cells (with chromatin condensation and fragmentation) was determined using a fluorescence microscope (Olympus IX-70) from a total number of 300 cells in each sample.

Production of reactive oxygen species (ROS)

The intracellular production of ROS was detected by FCM analysis using dihydrorhodamine-123 (DHR-123, Fluka, Switzerland), which reacts with intracellular hydrogen peroxide. The cells treated with the appropriate agent were harvested, washed twice in PBS, and resuspended in Hank's buffered saline solution (HBSS). DHR-123 was added in a final concentration of 0.2 µM. The samples were then incubated for 15 min at 37 °C in 5 % CO_2. Fluorescence was detected with a 530/30 (FL-1) optical filter. Forward and side scatters were used to gate the viable population of cells.

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). The cells were washed with HBSS, resuspended in 100 nM of TMRE in HBSS, and incubated for 20 min at room temperature in the dark. The cells were then washed twice with HBSS, resuspended in 500 µl of the total volume, and analysed ($2x10^4$ cells per sample). Fluorescence was detected with a 585/42 (FL-2) optical filter.

Lipid peroxidation

Lipid peroxidation products were measured by the spectrophotometric thiobarbituric acid (TBA, Sigma-Aldrich Corp., St. Louis, MO, USA) assay as described by Canuto et al. [20] with some modifications. Briefly: 0.75 ml of cell suspension was added to the TBA reagent (0.5 ml of 15 % trichloroacetic acid; 0.5 ml of 0.25 N hydrochloric acid; 0.5 ml of 0.6 % TBA). This mixture was incubated at 90 °C for 45 min, then cooled, extracted with 2.25 ml of N-butanol, and centrifuged (5 min, 1500 g). The absorbance of the upper phase was measured at 532 nm. The concentration of thiobarbituric acid reactive substances (TBARs) was calculated from a standard calibration curve generated with known amounts of 1,1,3,3-tetraethoxypropane (Sigma-Aldrich Corp., St. Louis, MO, USA).

Western blot analysis

The extracts of total proteins were obtained from cells cultured on 60x15 mm dishes and treated as described above. After cell lysation, determination of protein concentrations, and separation by SDS-PAGE, the immunoblotting procedure was performed as described previously [8, 21]. The membranes were probed with: rabbit anti-PARP (1:500, Santa Cruz Biotechnology, # SC-7150), mouse anti-Bax (1:500, Transduction Laboratories, # B73520), rabbit anti-Bak (1:4000, Pharmingen, # 556396), mouse anti-Mcl-1 (1:1000, Neomarkers, # MS683), mouse p27 (1:1000, Transduction Laboratories, # 610242), or rabbit p21 (1:500, Santa Cruz Biotechnology, # 397) antibodies. The recognised proteins were detected using horseradish peroxidase-labelled secondary antibodies: anti-mouse IgG (1:3000, NEB, # 7072–1), anti-rabbit IgG (1:3000, NEB, # 7071–7), and an enhanced chemiluminescence kit (ECL, Amersham Biosciences; Buckinghamshire, England). An equal loading was verified using non-specific amidoblack staining of proteins after immunoblotting.

Caspase-3 and caspase-9 activity

After washing with PBS, the cells were lysed in lysis buffer (250 mM HEPES, 25 mM CHAPS, 25 mM DTT, 40 μM protease inhibitor coctail, Sigma, Czech Republic) on ice for 20 min. The lysates were then centrifuged at 15000 g, 15 min in 4 °C. The protein concentration in supernatant was quantified using Bradford blue assay and the samples were diluted to an equal concentration of proteins. To measure caspase activity, samples were incubated with caspase-3 (Ac-DMQD-AMC, 50 μM, Alexis) or caspase-9 (Ac-LEHD-AMC, 50 μM, Alexis) substrates in assay buffer (40 mM HEPES, 20 % glycerol, 4 mM DTT). Fluorescence was measured (355/460 nm) using fluorometer Fluostar Galaxy (BMG Labtechnologies GmbH, Offenburg, Germany).

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 Tukey or LSD tests.

Results

The equieffective doses of AA (50 μM) or DHA (20 μM) for cell pre-treatment were chosen on the basis of a 48 h effect of their increasing concentrations on the total cell number, the percentage of floating cells, viability, and cell cycle parameters including subG_0/G_1 population (Table 1). AA or DHA caused significant 1.5–2.5 fold increase of the percentage of floating cells accompanied with a decrease of adherent cells. Thus the total cell number was not significantly different from control values. Also, subG_0/G_1 population and cell viability were not significantly influenced. The addition of the antioxidant Trolox together with PUFAs reestablished the percentage of floating cells at control values. At higher concentrations used, both PUFAs significantly decreased the amount of cells in the S phase and increased the amount of cells in either G_0/G_1 (DHA)or G_2/M (AA). Trolox in AA- but not in DHA-treated cells reversed this effect to control values. In concentrations used for cell pre-treatment, i. e. AA (50 μM) or DHA (20 μM) similar significantly increased ROS production (200–300 % of control value) and lipid peroxidation (about 5-fold compared to control) were observed. It was partially inhibited by the addition of Trolox (not shown). In these doses no significant changes of MMP were detected. An analysis of the

Table 1 The effect of arachidonic (AA) or docosahexaenoic (DHA) acid on HT-29 cell growth, cell death and cell cycle after 48 h of treatment. AA (50 μM) or DHA (20 μM) were also combined with Trolox (Tr, 50 μM)

Treatment (μM)	Total cell number (x10^5)	Floating cells (%)	Viable cells (%)	SubG_0/G_1 (%)	Cell cycle phase (%)		
					G_0/G_1	S	G_2/M
Control	4.67±0.64	7.03±1.10	85.25±4.82	3.07±2.17	59.45±1.72	28.16±1.57	12.38±0.85
AA 10	5.21±0.56	10.09±1.50	76.33±2.68	5.16±1.49	56.57±1.93	30.27±1.59	13.16±0.62
AA 20	4.34±0.38	12.04±2.81	81.75±1.72	4.89±1.98	54.40±2.38	28.81±1.64	16.79±1.25*
AA 50	3.96±0.30	17.98±2.93*	84.25±0.87	5.66±1.67	55.58±1.55	21.59±1.65*	22.83±1.25*
DHA 10	4.81±0.91	12.24±3.05	80.25±6.37	3.43±0.94	58.57±2.13	28.16±2.32	13.26±0.77
DHA 20	4.87±0.48	15.11±2.48*	81.25±5.53	4.71±0.59	63.95±2.28	19.74±2.41*	16.31±1.11*
DHA 50	4.27±0.45	18.79±3.69*	75.33±3.90	4.18±0.84	69.65±2.89*	16.26±2.19*	14.10±0.90
AA 50 + Tr	5.49±0.33	6.20±1.80^x	85.00±5.70	1.91±0.89	59.62±1.99	27.83±2.38^x	12.55±0.44^x
DHA 20 + Tr	5.34±0.16	7.90±1.20^x	71.00±3.70	2.52±1.51	68.22±2.26^x	20.54±3.06*	11.24±0.83^x

Values are means ± S. E. M.; n = 4, statistically signif. $P < 0.05$, (*) compared to control, (^{x}) compared to AA or DHA

European Journal of Nutrition (2005) Vol. 44, Number 1

fatty acid content in cellular lipids after 48 h of treatment showed a significant increase of the corresponding PUFA. Standardised as nmol per 10^6 cells, 56.6 nmol (after treatment with 20 μM DHA) versus 2.6 nmol (control) of DHA and 34 nmol (after treatment with 50 μM AA) versus 3.3 nmol (control) of AA were detected.

Cells either non-pre-treated or pre-treated 48 h with PUFAs were washed and further incubated in PUFA-free medium with or without NaBt (5 mM) as described in Material and methods. After additional 24 h of cultivation, the cell number, cell cycle parameters, and induction of cell death including parameters reflecting some proposed mechanisms involved in the effects observed were investigated.

Cell cycle

In the cells pre-treated for 48 h with AA or DHA and then cultivated 24 h in PUFA-free medium, decreased amount of cells in the S phase was still observed. However, when the cells were incubated with NaBt, pre-treatment with AA or DHA attenuated NaBt induced cell cycle changes which included an increased amount of cells in the G_0/G_1 and G_2/M phases accompanied by decrease of the S phase. This effect of NaBt was also attenuated by CHX (Fig. 1A, B) as well as by Trolox (not shown). We demonstrated that NaBt increased the expression of the cyclin kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1/WAF1}$, and that pre-treatment of the cells with both AA and DHA (which alone had no effects) apparently attenuated the expression of $p27^{Kip1}$ but not of $p21^{Cip1/WAF1}$ (Fig. 2).

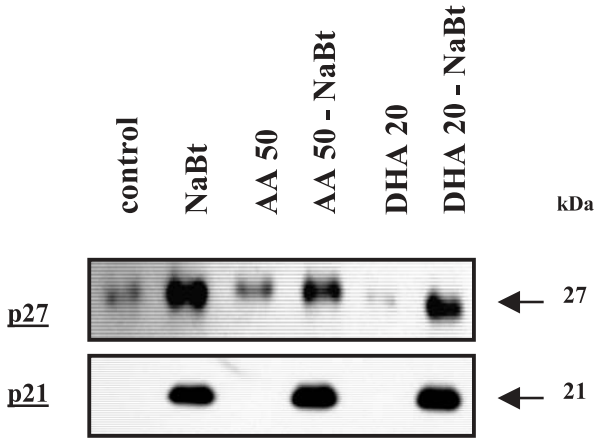

Fig. 2 Expression of cell cycle regulatory proteins $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ detected by Western blotting in HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA 50) or 20 μM of docosahexaenoic (DHA 20) acid and then incubated for 24 h in PUFA-free medium without or with 5mM sodium butyrate (AA50-NaBt, DHA20-NaBt). The figure shows a representative result of three independent experiments

Cell death

NaBt induced about 10–14% of floating cells or cells in subG_0/G_1 population, but these values were not significantly different from control. However, these effects were significantly increased by pre-treatment of cells with AA or DHA when compared to control and cells either only pre-treated with PUFAs or treated only with NaBt (left part of Fig. 3A, B). Pre-treatment with PUFAs increased also the amount of morphologically detected apoptosis after NaBt treatment. Although relatively low (10–12%), it was significantly higher compared to 2–4%

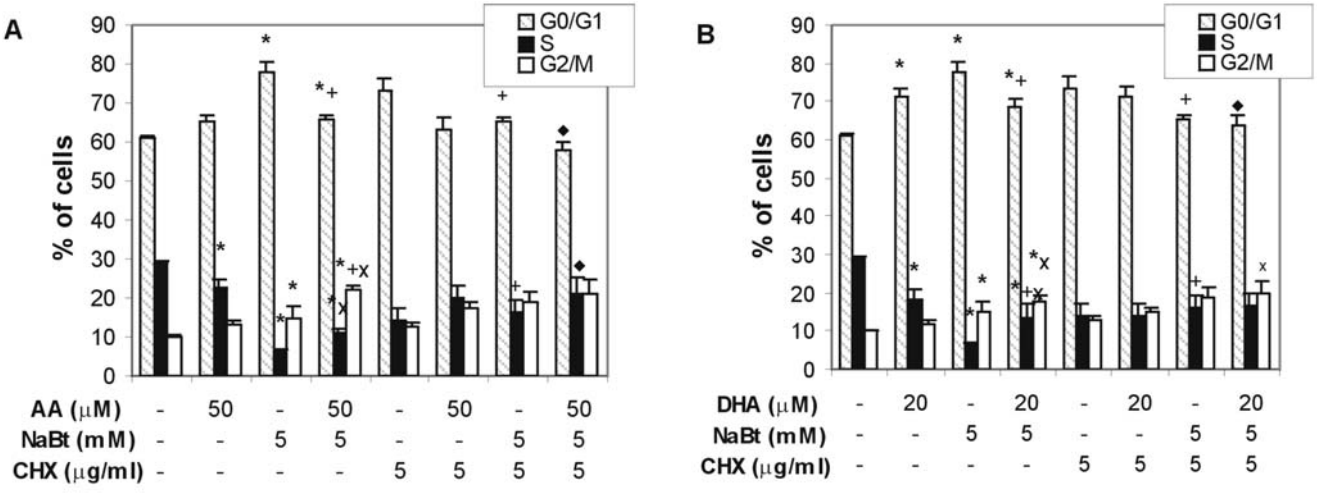

Fig. 1 Cell cycle analysis of HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA; **A**) or 20 μM of docosahexaenoic (DHA; **B**) acid and then incubated for 24 h in PUFA-free medium without or with sodium butyrate (NaBt, 5 mM). Experiments with cycloheximide (CHX, 5 μg/ml) added to the cells for the last 24 h are presented on the right parts of panels. Values are means + S. E. M.; n = 4; Statistical significance: $p < 0.05$ (*) compared to non-treated control; (+) compared to NaBt and/or (x) to AA or DHA as single factors; (◆) compared to cells pretreated with AA or DHA and then treated with NaBt

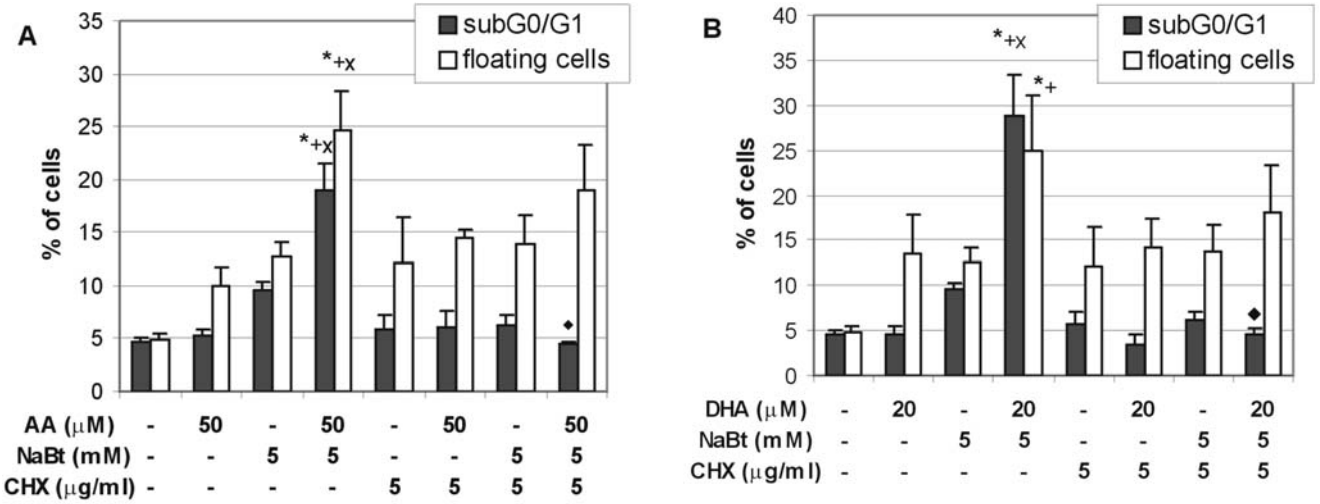

Fig. 3 The percentage of floating cells and/or subG$_0$/G$_1$ population of HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA; **A**) or 20 μM of docosahexaenoic (DHA; **B**) acid and then incubated for 24 h in PUFA-free medium without or with sodium butyrate (NaBt, 5 mM). Experiments with cycloheximide (CHX, 5 μg/ml) added to the cells for the last 24 h are presented on the right parts of panels. Values are means + S. E. M.; n = 4; Statistical significance: $p < 0.05$ (*) compared to non-treated control; (+) compared to NaBt and/or (x) to AA or DHA as single factors; (◆) compared to cells pretreated with AA or DHA and then treated with NaBt

in cells only pre-treated with PUFAs or treated only with NaBt (individual data not shown). At the same time, cell viability was not yet significantly different compared to control. The addition of CHX together with NaBt abolished the potentiating effects of AA or DHA pre-treatment (right part of Fig. 3A, B). The addition of Trolox partially decreased the percentage of floating cells induced by NaBt in cells pre-treated with AA, but not with DHA. It did not influence the amount of cells in subG$_0$/G$_1$ and cell cycle parameters (data not shown).

Lipid peroxidation and ROS production

In cells pre-treated with AA or DHA and cultivated for 24 h in PUFA-free medium, increased lipid peroxidation and ROS production were still detected. Treatment with NaBt for 24 h also slightly increased these two parameters in non-pre-treated cells, but pre-treatment of cells with both AA and DHA led to apparent potentiation of these effects (Fig. 4, Fig. 5A, B). Only in cells pre-treated with DHA was the effect on lipid peroxidation not statistically significant. Increased lipid peroxidation after AA pre-treatment was partially inhibited by the addition of Trolox (Fig. 4).

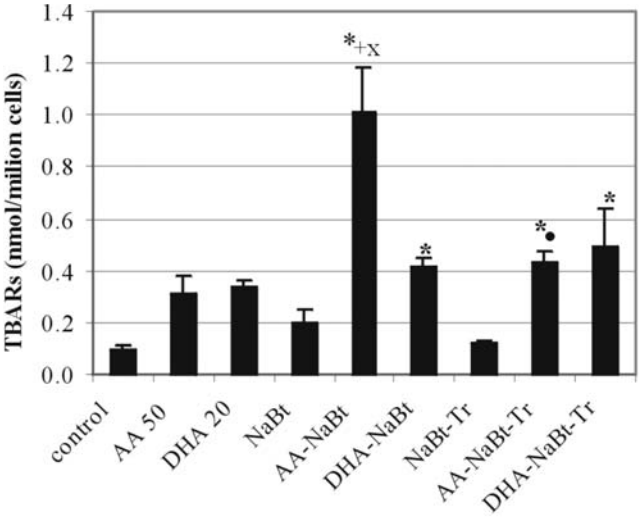

Fig. 4 Lipid peroxidation measured as thiobarbituric acid reactive substances (TBARs) production of HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA 50) or 20 μM of docosahexaenoic (DHA 20) acid and then incubated for 24 h in PUFA-free medium without or with 5 mM sodium butyrate (AA50-NaBt, DHA20-NaBt). The effect of Trolox (Tr, 50 μM) is presented in the right part of panel. Values are means + S. E. M.; n = 3; Statistical significance: $p < 0.05$ (*) compared to non-treated control; (+) compared to NaBt and/or (x) to AA or DHA as single factors; (●) compared to cells pretreated with AA or DHA and then treated with NaBt

Changes of membrane mitochondrial potential (MMP)

In cells pre-treated with AA or DHA and then cultivated in PUFA-free medium no changes in MMP were observed compared to the control. On the other hand, NaBt caused an apparent decrease of MMP already in PUFA non-pre-treated cells, but this effect was more pronounced in cells pre-treated with both AA and DHA (Fig. 6A, B). Particularly important is the region of dissipated (not collapsed) MMP marked as M2 in Fig. 6.

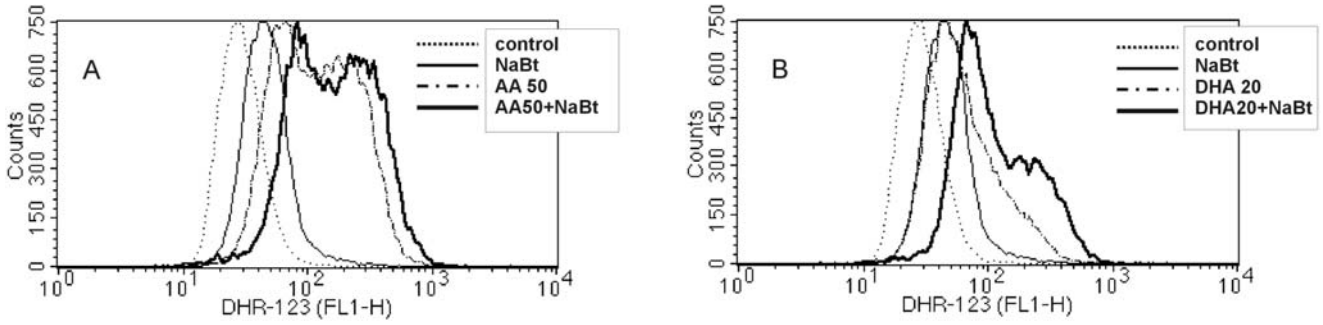

Fig. 5 Production of reactive oxygen species (ROS) in HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA 50; **A**) or 20 μM of docosahexaenoic (DHA 20; **B**) acid and then incubated for 24 h in PUFA-free medium without or with 5 mM sodium butyrate (AA50-NaBt, DHA20-NaBt). ROS were measured by FCM as dihydrorhodamine-123 (DHR-123) fluorescence (FL1-H). The figure shows a representative result of three independent experiments

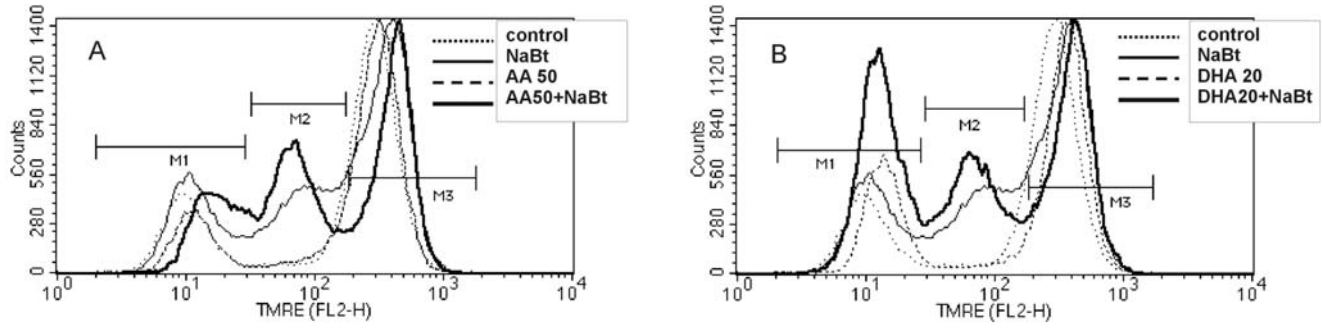

Fig. 6 Mitochondrial membrane potential (MMP) of HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA 50; **A**) or 20 μM of docosahexaenoic (DHA 20; **B**) acid and then incubated for 24 h in PUFA-free medium without or with 5 mM sodium butyrate (AA50-NaBt, DHA20-NaBt). MMP was measured by FCM as tetramethylrhodamine ethyl ester perchlorate (TMRE) fluorescence (FL2-H). *M1* region of collapsed MMP, *M2* region of dissipated MMP, *M3* region of normal MMP. The figure shows a representative result of three independent experiments

Caspase-3 and caspase-9 activity

NaBt significantly increased caspase-3 activity compared to control, which was enhanced by pre-treatment with PUFAs, particularly AA (Fig. 7A). In spite of the tendency to increased caspase-9 activity was similar to caspase-3, due to higher deviation of the values the significance was confirmed only after NaBt applied on the cells pre-treated with AA (Fig. 7B).

Cleavage of PARP

NaBt caused degradation and cleavage of PARP to its 89 kD product (detected by western blotting) already in PUFA non-pre-treated cells. However, this effect was markedly promoted in cells pre-treated both with AA and DHA (Fig. 8).

Expression of proteins involved in regulation of apoptosis

The expression of Bcl-2, Bax, Bak, and Mcl-1 proteins was studied after NaBt treatment of AA- or DHA-pre-treated or non-pre-treated cells. We found that HT-29 cells used in our experiments did not express any Bcl-2 protein, and the level of Bax protein remained unchanged after all types of treatment. NaBt increased the expression of Mcl-1 protein and marginally of Bak protein. PUFA pre-treatment did not additionally change Bak expression, but attenuated the expression of Mcl-1 protein (Fig. 8).

Discussion

It was proved that dietary intake of PUFAs represents a major source of lipid hydroperoxides in the intestinal lumen, which can contribute to oxidative stress induced in enterocytes and through this mechanism they may promote colon carcinogenesis [22]. On the other hand, anticancer activities of pure specific PUFAs or fish oil containing n-3 PUFAs were reported in systems *in vitro* [11, 23] as well as *in vivo* [24, 25]. As presented in our paper, PUFA induced processes could also promote the sensitivity of cancer cells to other regulatory factors operating in the colon such as NaBt. We observed that the treatment of HT-29 cells with n-6 AA as well as n-3 DHA for 48 h enhanced lipid peroxidation and ROS production,

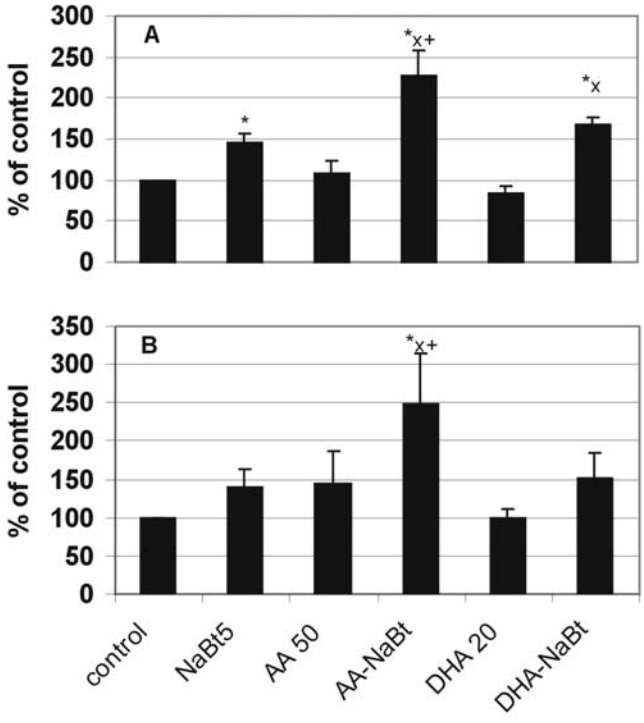

Fig. 7 Activity of caspase-3 (**A**) and caspase-9 (**B**) of HT-29 cells non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA 50) or 20 μM of docosahexaenoic (DHA 20) acid and then incubated for 24 h in PUFA-free medium without or with 5mM sodium butyrate (AA50-NaBt, DHA20-NaBt). Values are means + S. E. M.; n = 4; Statistical significance: $p < 0.05$ (*) compared to non-treated control; (+) compared to NaBt and/or (x) to AA or DHA as single factors

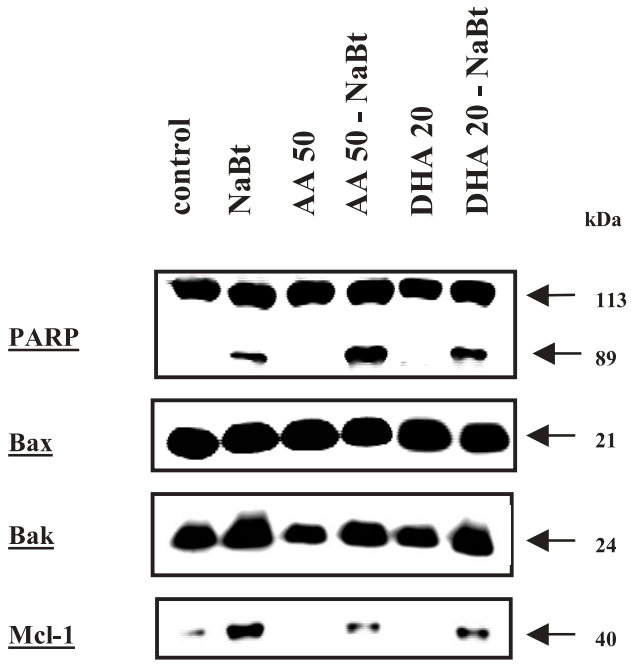

Fig. 8 Cleavage of poly(ADP-ribose) polymerase (PARP) to its 89 kD fragment, and expression of Bax, Bak and Mcl-1 proteins detected by Western blotting. HT-29 cells were non-pre-treated or pre-treated for 48 h with 50 μM of arachidonic (AA 50) or 20 μM of docosahexaenoic (DHA 20) acid and then incubated for 24 h in PUFA-free medium without or with 5mM sodium butyrate (AA50-NaBt, DHA20-NaBt). The figure shows a representative result of three independent experiments

processes which have been considered as the main mechanism of the PUFA cytostatic and cytotoxic action [26]. These events are mainly consequences of structural and functional changes in cell membranes [27] and conversion of PUFAs to eicosanoids [28]. Our own analysis of fatty acids in cellular lipids confirmed that supplementation with either AA or DHA significantly increased the content of corresponding PUFA in HT-29 cells.

Furthermore effects of AA and DHA presented in our paper were measured in cells washed and cultivated 24 h in PUFA-free media with or without NaBt. In these cells the effects on floating cells, subG_0/G_1, cell cycle and ROS production continued but there was no lipid peroxidation detected. However, the cells pre-treated with PUFAs were more sensitive to the effects of NaBt, although the effects of AA and DHA were rather different. It has been reported that AA and DHA are not incorporated into HT-29 cell phospholipids (PL) in the same way [29]. While AA was incorporated in total cell PL to a greater extent than DHA, DHA is most extensively incorporated into cardiolipin (CL), which is one of the essential components of the inner-mitochondrial membranes and has the most diet-responsive and changeable fatty acid composition among PL. The increase of its unsaturation is associated with changes of mitochondria structural integrity, electron transport chain, membrane potential, and ROS production. Mitochondrial lipid peroxidation resulted in a marked loss of both cytochrome c oxidase activity, CL content, and release of cytochrome c, which induces apoptosis. In the report of Watkins et al. [29], DHA increased ROS production by accumulating in CL, but DHA-enriched cells had even increased MMP. This may explain that despite of non-significant changes in total lipid peroxidation and no changes of MMP, there was high ROS production after DHA pre-treatment in our experiments observed. The different sites of incorporation and capacity to produce lipid peroxides could also be the reason for different efficiency of Trolox to reverse the effects caused by AA or DHA. Trolox is water soluble analogue of vitamin E and thus its ability to be incorporated into the cell is limited. Thus, we suggest that it may function namely on the level of the plasma membrane where the ROS and lipid peroxides after AA treatment were mainly produced, while it cannot be efficient within the cell on the level of mitochondrial membrane. In spite of described similarities between the acyl composition of HT-29 cell CL and CL isolated from animal tissues, the results of *in vivo* system were slightly different [27]. It was shown that fatty acid composition of individual mitochondrial PL classes of

colonocytes from rats fed with fish oil, corn oil or mix of n-3 fatty acid ethyl esters (FAEE) was affected by the diet and enrichment of individual PL with n-3 and n-6 PUFAs was different. The increased amount of EPA and DHA in mitochondrial PL resulted in the depletion of n-6 PUFAs, an elevation in ROS production, reduction of MMP and activation of caspase-3. However, in this *in vivo* study CL contained only modest levels of DHA following fish oil or FAEE consumption. Thus, the correlation between changes of total and mitochondrial PL, oxidative processes and cytokinetic changes after enrichment of cells with n-3 and n-6 PUFAs needs further investigation.

NaBt had antiproliferative effects on colon cancer cell lines *in vitro* due to G_0/G_1 arrest caused mainly by modulation of cell cycle-related proteins [30]. It also induced differentiation of HT-29 cells documented by increased ALP activity as we have confirmed previously [8, 31]. However, in agreement with other reports [6, 7, 31, 32] we observed a relatively low percentage of floating or $subG_0/G_1$ cells (about 10 %) as well as cells with morphologically detectable apoptosis (about 4 %) after 24 h treatment with physiologically relevant concentrations of NaBt alone. In this paper we demonstrate that typical NaBt induced G_0/G_1 arrest and decrease of the S phase were attenuated by pre-treatment of HT-29 cells with PUFAs. Similar effects incurred by addition of CHX or Trolox imply the role of new protein synthesis as well as ROS production. In agreement with other authors [33] our results showed a significant increase of the expression of cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ after NaBt treatment. In spite of reported activation of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ by DHA [34], the NaBt-induced expression of $p27^{Kip1}$ was markedly attenuated in cells pre-treated with both PUFAs, which was probably reflected by increased S phase values in these cells. It was reported that growth arrest and cell differentiation were associated with the induction of both $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ regulators, but with markedly different kinetics. The role of $p27^{Kip1}$ in the apoptosis of colon adenocarcinoma CaCo-2 cells was suggested [35]. However, our results showed that in spite of a potentiation of apoptosis the expression of $p27^{Kip1}$ in cells pre-treated with both AA and DHA and then incubated with NaBt is lower than in non-pre-treated cells. We suggest that the ability of NaBt to increase $p27^{Kip1}$ expression may be due to its effects on proteolysis of the key regulators [6, 36], and this effect may interfere with some yet unknown effects of PUFAs.

The most important result of the present paper is the potentiation of NaBt induced apoptosis in PUFA-pre-treated cells. We detected primarily the increased percentage of floating cells, which were primed for apoptosis as demonstrated by potentiation of caspase-3 and caspase-9 activity, cleavage of PARP, and increased amount of cells in $subG_0/G_1$ population. This mechanism may be analogous to "anoikis" (induction of apoptosis in response to loss of cell contact), typical of exfoliating epithelial cells. The results obtained by others with renal epithelial cells showed that caspase-mediated loss of adhesion caused by cleavage of focal adhesion kinase may be an early stage in the apoptosis of adherent cells committed to suicide [37]. All apoptotic parameters tested in our experiments after 24 h were parameters reflecting earlier stages of apoptosis while cells were still viable. Shedding of cells, caspase-3 activation, PARP cleavage with subsequent appearance of $subG_0/G_1$ population (decrease of DNA content) preceded morphologically detectable apoptosis (chromatin condensation and fragmentation), which was relatively low in this interval. Final stages of membrane disintegration and decrease of viability observable after a longer time period could not be detected in our experimental design.

Our important findings include those documenting interaction of PUFAs and NaBt on the level of mitochondria. Heerdt et al. [38] reported that intact MMP is required for the initiation and maintenance of the early p-53 independent $p21^{Cip1/WAF1}$ induction and growth arrest in G_0/G_1, while dissipation of the MMP is essential for the initiation and activation of downstream events that culminate in apoptosis of colon cancer cells after NaBt treatment. We showed that in HT-29 cells NaBt initiated processes whereby the MMP is markedly dissipated and associated with increased caspase-3 activity and subsequent cleavage of PARP similar to that reported in other human colon SW620 [38] and CaCo-2 cells [39]. Caspase-3 proteolysis is a critical element of the apoptotic process. In different cell lines it was demonstrated that caspase-3 proenzyme has a mitochondrial and cytosolic distribution in non-apoptotic cells [40]. After various apoptotic stimuli mitochondrial caspase-3 precursor staining is lost and caspase-3 proteolytic activity appears showing a temporal link between these processes. Participation of mitochondrial death pathway in the effects observed in our experiments is supported by enhanced activity of caspase-9 after PUFA pre-treatment. Caspase-9 is a part of multiprotein complex (cytochrome c, pro-caspase-3, Apaf-1, caspase-9) through which apoptotic Bcl-2 sensitive signal is transduced and that participate in cytochrome c-mediated processing of caspase-3. The relative values (compared to control) were similar to those of caspase-3, but due to higher deviation the significance was confirmed only after pre-treatment with AA. The non-significant effects of DHA can be associated with its lower concentration, cultivation of cells in PUFA-free medium, and different and more complex mechanisms of DHA action as discussed later.

Promotion of all the described NaBt effects by pre-treatment of cells with both AA and DHA in our experiments implies that modification of cellular and mitochondrial membrane composition associated with

increased ROS production and lipid peroxidation caused by PUFAs together with those caused subsequently by NaBt [41] contributes to the resulting more intensive response of HT-29 cells.

Recently, the role of fatty acid oxidation and signalling in apoptosis was well documented. It was reported that aldehydes derived from lipid peroxidation cause cell cycle arrest and alter apoptosis-associated signalling pathways including changes of cell adhesion [42]. Thus, we can speculate that changes of the cell cycle caused by lipid peroxides generated after PUFA treatment can contribute to shedding of the cells and apoptotic response. Increased production of ROS by NaBt was reported to contribute to the sensitisation of HT-29 cells to apoptosis induced by TNF-α or Fas [7]. Most of the colon cancer cells including HT-29 have mutated non-functional p53, which normally activates the expression of ROS-generating proteins. Thus increased ROS generation could compensate for their lower levels in this type of cells [38].

The results of *in vivo* experiments published by Hong et al. [43] supported our findings. They have shown that colonocytes from rats fed with fish oil or n-3 FAEE are primed for elevated butyrate-induced apoptosis by changes in mitochondrial phospholipids, increasing ROS production and initiating the apoptotic cascade. However, in contrast to other authors, who observed significant apoptotic effects *in vitro* or *in vivo* only with the n-3 type of PUFAs or fish oil [27, 29], we demonstrated similar significant potentiating effects also with n-6 AA. This is reasonable with regard to evidence that AA can cause cell death through mitochondrial permeability transition [44]. However, diverse mechanisms of action of AA and DHA can be supposed. Reported different incorporation of these PUFAs within the cell (cell vs. mitochondrial membranes, various types of PL) and production of different types of ROS (peroxide, superoxide, hydroxyl) as well as nitric radicals [45, 46] may be the reason for different level of lipid peroxidation, cell cycle changes and efficiency of Trolox in our experiments. While for AA more direct oxidative effects may be supposed [45], after DHA treatment other more complex mechanisms should be considered. This includes activation of specific intracellular receptors [47], altered expression of transcription factors, cell cycle and apoptosis regulating proteins as well as inactivation of prostaglandin family genes and lipoxygenases [34].

Since the inhibition of protein synthesis by CHX abolished the potentiating effect of PUFA pre-treatment on cell death, we suggest that new protein synthesis was involved in this effect. The most likely candidates associated with apoptosis are members of the bcl-2 gene family acting primarily at the mitochondrial level. Our results, in agreement with others [3, 48], showed no detectable level of Bcl-2 protein in HT-29 cells. In our experiments NaBt increased the expression of another antiapoptotic protein Mcl-1 that is reported to act as a rapidly deployable mediator increased by differentiation-inducing and cytotoxic agents. It is expressed early and transiently after the induction preventing cell death and thereby allowing cell differentiation [49]. Our results imply that the potentiating apoptotic effect of AA and DHA could be associated with attenuated expression of this protein. As to other apoptotic proteins, only marginally increased expression of Bak after NaBt treatment was observed in our experiments and it was not additionally modulated by pre-treatment with PUFAs. Even though the expression of Bax was also unchanged, its localisation remains to be detected, since Bax is translocated to the mitochondria in response to the apoptotic signal.

To determine further potential mechanisms for enhanced apoptosis, we detected the expression of the plasma membrane receptor Fas (CD95, APO-1) by FCM. HT-29 cells showed a certain basal level of Fas expression, but it was not changed by any type of treatment (not shown).

We can summarise that dietary PUFAs of both n-6 and n-3 series significantly alter the response of HT-29 colon cancer cells so that they become more sensitive to apoptosis induced by NaBt. We conclude that although lower concentrations of AA or DHA alone were not capable of inducing a wide range of apoptosis in colonic cells, our results and the reports of others imply that they may, through oxidative metabolism [42] and other mechanisms [34], [45], start a cascade of processes which a prepare permissive environment for more effective action of NaBt, thus promoting cancer cell sensitivity to this agent. Together our findings suggest the importance of the composition of diet in colon cancer prevention and adjuvant therapy.

Acknowledgments This work was supported by the Grant Agency of the Czech Republic (No. 525/01/0419). The authors thank Dr. R. Hyšpler, Dr. J. Netíková and Mrs. Lenka Vystrčilová for their willing technical assistance.

References

1. Chapkin RS, Fan Y, Lupton JR (2000) Effect of diet on colonic-programmed cell death: molecular mechanism of action. Toxicol Lett 113:411–414
2. Bedi B, Pasricha PJ, Akhtar AJ, et al. (1995) Inhibition of apoptosis during development of colorectal cancer. Cancer Res 55:1811–1816
3. Hague A, Singh B, Paraskeva C (1997) Butyrate acts as a survival factor for colonic epithelial cells: further fuel for the in vivo versus in vitro debate. Gastroenterology 112:1036–1040

4. Smith JG, Yokoyama WH, German JB (1998) Butyric acid from the diet: actions at the level of gene expression. Crit Rev Food Sci Nutr 38:259–297
5. Iacomino G, Tecce MF, Grimaldi C, Tosto M, Russo GL (2001) Transcriptional response of a human colon adenocarcinoma cell line to sodium butyrate. Biochem Biophys Res Commun 285:1280–1289
6. Tan S, Seow TK, Liang RCMY, et al. (2002) Proteome analysis of butyrate-treated human colon cancer cells (HT-29). Int J Cancer 98:523–531
7. Giardina C, Boulares H, Inan MS (1999) NSAIDs and butyrate sensitize a human colorectal cancer cell line to TNF-alpha and Fas ligation: the role of reactive oxygen species. Biochim Biophys Acta 1448:425–438
8. Kovaříková M, Pacherník J, Hofmanová J, Zadák Z, Kozubík A (2000) TNF-alpha modulates the differentiation induced by butyrate in the HT-29 human colon adenocarcinoma cell line. Eur J Cancer 36:1844–1852
9. Bonnotte B, Favre N, Reveneau S, et al. (1998) Cancer cell sensitization to fas-mediated apoptosis by sodium butyrate. Cell Death Differ 5:480–487
10. Bartsch H, Nair J, Owen RW (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. Carcinogenesis 20: 2209–2218
11. Diggle CP (2002) In vitro studies on the relationship between polyunsaturated fatty acids and cancer: tumour or tissue specific effects? Prog Lipid Res 41: 240–253
12. Petrik MB, McEntee MF, Johnson BT, Obukowicz MG, Whelan J (2000) Highly unsaturated (n-3) fatty acids, but not alpha-linolenic, conjugated linoleic or gamma-linolenic acids, reduce tumorigenesis in Apc(Min/+) mice. J Nutr 130: 2434–2443
13. Di Marzo V (1995) Arachidonic acid and eicosanoids as targets and effectors in second messenger interactions. Prostaglandins Leukot Essent Fatty Acids 53:239–254
14. Calder PC (2001) Polyunsaturated fatty acids, inflammation, and immunity. Lipids 36:1007–1024
15. Rudolph IL, Kelley DS, Klasing KC, Erickson KL (2001) Regulation of cellular differentiation and apoptosis by fatty acids and their metabolites. Nutr Res 21:381–393
16. Spector AA, Burns CP (1987) Biological and therapeutic potential of membrane lipid modification in tumors. Cancer Res 47:4529–4537
17. Bégin ME, Ells G, Horrobin DF (1988) Polyunsaturated fatty acid-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. J National Cancer Inst 80:188–194
18. Neoptolemos JP, Husband D, Imray C, Rowley S, Lawson N (1991) Arachidonic acid and docosahexaenoic acid are increased in human colorectal cancer. Gut 32:278–281
19. Liebich HM, Wirth C, Jakober B (1991) Analysis of polyunsaturated fatty acids in blood serum after fish oil administration. J Chromatogr 572:1–9
20. Canuto RA, Muzio G, Bassi AM, et al. (1995) Enrichment with arachidonic acid increases the sensitivity of hepatoma cells to the cytotoxic effects of oxidative stress. Free Radic Biol Med 18:287–293
21. Vaculová A, Hofmanová J, Souček K, Kovaříková M, Kozubík A (2002) Tumor necrosis factor-alpha induces apoptosis associated with poly(ADP-ribose) polymerase cleavage in HT-29 colon cancer cells. Anticancer Res 22:1635–1639
22. Aw TY (1999) Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. Am J Clin Nutr 70:557–565
23. Grammatikos SI, Subbaiah PV, Victor TA, Miller WM (1994) Diverse effects of essential (n-6 and n-3) fatty acids on cultured cells. Cytotechnology 15:31–50
24. Anti M, Armelao F, Marra G, et al. (1994) Effects of different doses of fish oil on rectal cell proliferation in patients with sporadic colonic adenomas. Gastroenterology 107:1709–1718
25. Chang WL, Chapkin RS, Lupton JR (1998) Fish oil blocks azoxymethane-induced rat colon tumorigenesis by increasing cell differentiation and apoptosis rather than decreasing cell proliferation. J Nutr 128:491–497
26. Das UN (1991) Tumoricidal action of cis-unsaturated fatty acids and their relationship to free radicals and lipid peroxidation. Cancer Lett 56:235–243
27. Chapkin RS, Hong MY, Fan YY, et al. (2002) Dietary n-3 PUFA alter colonocyte mitochondrial membrane composition and function. Lipids 37:193–199
28. Krause W, DuBois RN (2000) Eicosanoids and the large intestine. Prostaglandins Other Lipid Mediat 61: 145–161
29. Watkins SM, Carter LC, German JB (1998) Docosahexaenoic acid accumulates in cardiolipin and enhances HT-29 cell oxidant production. J Lipid Res 39: 1583–1588
30. Coradini D, Pellizzaro C, Marimpietri D, Abolafio G, Daidone MG (2000) Sodium butyrate modulates cell cycle-related proteins in HT29 human colonic adenocarcinoma cells. Cell Prolif 33: 139–146
31. Heerdt BG, Houston MA, Augenlicht LH (1994) Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. Cancer Res 54:3288–3293
32. Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D, Paraskeva C (1993) Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. Int J Cancer 55:498–505
33. Wang J, Friedman EA (1998) Short-chain fatty acids induce cell cycle inhibitors in colonocytes. Gastroenterology 114:940–946
34. Narayanan BA, Narayanan NK, Reddy BS (2001) Docosahexaenoic acid regulated genes and transcription factors inducing apoptosis in human colon cancer cells. Int J Oncol 19:1255–1262
35. Wachtershauser A, Stein J (2001) Butyrate-induced differentiation of Caco-2 cells occurs independently from p27. Biochem Biophys Res Commun 281: 295–299
36. Quaroni A, Tian JQ, Seth P, Rhys CA (2000) p27(Kip1) is an inducer of intestinal epithelial cell differentiation. Am J Physiol Cell Physiol 279: C1045–C1057
37. van de Water B, Nagelkerke JF, Stevens JL (1999) Dephosphorylation of focal adhesion kinase (FAK) and loss of focal contacts precede caspase-mediated cleavage of FAK during apoptosis in renal epithelial cells. J Biol Chem 274: 13328–13337
38. Heerdt BG, Houston MA, Anthony GM, Augenlicht LH (1998) Mitochondrial membrane potential (delta psi(mt)) in the coordination of p53-independent proliferation and apoptosis pathways in human colonic carcinoma cells. Cancer Res 58:2869–2875
39. Ruemmele FM, Dionne S, Qureshi I, Sarma DS, Levy E, Seidman EG (1999) Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP). Cell Death Differ 6: 729–735
40. Mancini M, Nicholson DW, Roy S, et al. (1998) The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. J Cell Biol 140:1485–1495
41. Horvath PJ, Awad AB, Andersen M (1993) Differential effect of butyrate on lipids of human colon cancer cells. Nutr Cancer 20:283–291
42. Tang DG, La E, Kern J, Kehrer JP (2002) Fatty acid oxidation and signaling in apoptosis. Biol Chem 383:425–442

43. Hong MY, Chapkin RS, Barhoumi R, et al. (2002) Fish oil increases mitochondrial phospholipid unsaturation, upregulating reactive oxygen species and apoptosis in rat colonocytes. Carcinogenesis 23:1919–1925
44. Scorrano L, Penzo D, Petronilli V, Pagano F, Bernardi P (2001) Arachidonic acid causes cell death through the mitochondrial permeability transition. Implications for tumor necrosis factor-alpha apoptotic signaling. J Biol Chem 276:12035–12040
45. Pompeia C, Lima T, Curi R (2003) Arachidonic acid cytotoxicity: can arachidonic acid be a physiological mediator of cell death? Cell Biochem Funct 21:97–104
46. Narayanan BA, Narayanan NK, Simi B, Reddy BS (2003) Modulation of inducible nitric oxide synthase and related proinflammatory genes by the omega-3 fatty acid docosahexaenoic acid in human colon cancer cells. Cancer Res 63:972–979
47. Fan YY, Spencer TE, Wang N, Moyer MP, Chapkin RS (2003) Chemopreventive n-3 fatty acids activate RXRalpha in colonocytes. Carcinogenesis 24: 1541–1548
48. Merchant AK, Loney TL, Maybaum J (1996) Expression of wild-type p53 stimulates an increase in both Bax and Bcl-xL protein content in HT29 cells. Oncogene 13:2631–2637
49. Yang T, Buchan HL, Townsend KJ, Craig RW (1996) MCL-1, a member of the BLC-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. J Cell Physiol 166:523–536