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The role of cyclooxygenase in n-6 and n-3 polyunsaturated fatty acid mediated effects on cell proliferation, PGE_2 synthesis and cytotoxicity in human colorectal carcinoma cell lines

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This study was conducted to investigate the role of the enzyme cyclooxygenase (COX) and its prostaglandin product PGE_2 in n-6 and n-3 polyunsaturated fatty acid (PUFA)-mediated effects on cellular proliferation of two human colorectal carcinoma cell lines. The long chain PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (AA; 20:4n-6) both inhibited cell proliferation of Caco-2 cells compared with the long chain fatty acids α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6). Neither incubation with PGE_2 nor reduction in PGE_2 synthesis by EPA compared with AA led to differential effects on cell proliferation in Caco-2 cells. This suggests that n-6 and n-3 PUFA-mediated cell proliferation in Caco-2 cells is not regulated via PGE_2 levels. AA and EPA had no effect on growth of HT-29 colon cancer cells with a low COX activity. However, stimulation of COX-2 activity by IL-1β resulted in a decrease in cell proliferation and an induction of cytotoxicity by AA as well as by EPA. Both inhibition of the COX pathway by indomethacin as well as inhibition of direct lipid peroxidation by antioxidants such as vitamin E and C diminished the anti-proliferative effects of AA as well as EPA. Also, malondialdehyde, a product of lipid peroxidation and COX-activity was decreased by addition of vitamin E and partially decreased by indomethacin. These data support the hypothesis that growth inhibitory and cytotoxic effects of PUFAs with methylene-interrupted double bonds such as AA and EPA are due to peroxidation products that are generated during lipid peroxidation and COX activity.

Introduction

Colorectal cancer is one of the leading causes of cancer deaths in both men and women in Western countries (1). Experimental animal studies (2–4) and epidemiological studies in humans (5,6) suggest that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can inhibit colorectal tumorigenesis. One potential mechanism for this chemopreventive effect is inhibition of the enzyme cyclooxygenase (COX). COX catalyses a key step in the conversion of arachidonic acid (AA; 20:4n-6) to prostaglandins, such as prostaglandin E_2 (PGE_2) (7). Also prostaglandin-independent mechanisms by which NSAIDs exert their anti-neoplastic effects are being considered because current studies reveal that NSAIDs inhibit the proliferation rate of colon cancer cell lines independent of their ability to inhibit PGE_2 synthesis (8,9).

Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 is responsible for 'housekeeping' prostaglandin biosynthesis and is constitutively expressed in most tissues in the body. COX-2 on the other hand is inducible by growth factors, cytokines and tumor promoters (7). Overexpression of COX-2 has been reported in 90% of colon tumors and premalignant colorectal adenomas (10), but the enzyme is not always detected in human colorectal carcinoma cell lines. COX-2 is not expressed in poorly differentiated colon cancer cell lines including HCT115 (8), HCT116 (11), HCT15, SW480, SW620, RKO, DLD-1 (12), SW1116, SW948 and SW48 cells (13). The well differentiated human colorectal carcinoma cells HCA-7, Moser, LS174(T), HT-29 (8), Caco-2 (11) and LoVo (14) do express COX-2 mRNA and protein (12,13).

NSAIDs can inhibit both COX-1 and COX-2 enzymes, which can cause adverse side effects and chemopreventive effects (7). A new class of specific COX-2 inhibitors such as celecoxib (15) and SC-58125 (16), which do not inhibit COX-1 at therapeutic doses, can serve as more effective chemopreventive agents. These selective COX-2 inhibitors inhibit the synthesis of prostaglandins that affect cell proliferation, tumor growth and immune responsiveness with fewer side effects (16,17).

N-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which are present in fish oil, have also been shown to inhibit COX mediated arachidonic acid metabolism (18–20). Singh *et al.* (21) further demonstrated that a high fat fish oil (HFFO) diet resulted in reduced expression of AOM-induced COX-2 expression in both colonic mucosa and colon tumors and decreased colon tumor outcome in rats, compared with a high fat corn oil (HFCO) diet with high levels of the n-6 PUFA linoleic acid (LA, 18:2n-6). No significant differences were observed in expression levels of COX-1. Other animal experiments have also shown that HFCO diets enhance colon tumorigenesis in rodents, whereas HFFO diets reduce colon carcinogenesis (22–25). It is hypothesized that n-6 PUFAs enhance colorectal carcinogenesis via increased production of prostaglandin E_2, which can stimulate cellular proliferation (26) in the colon (27,28). The mechanism responsible for the inhibitory effects of n-3 PUFAs on colorectal tumors may partly be related to inhibition of PGE_2 synthesis from AA (20).

This study was conducted to investigate the mechanisms responsible for the differential effects between n-6 and n-3

Abbreviations: AA, arachidonic acid; ALA, α-linoleic acid; BSA, bovine serum albumin; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FCS, fetal calf serum; HFCO, high fat corn oil; HFFO, high fat fish oil; IM, indomethacin drugs; LA, linoleic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; NSAIDs, non-steroidal anti-inflammatory; PGE_2, prostaglandin E_2; PUFAs, polyunsaturated fatty acids.

PUFAs on colon carcinogenesis at a molecular and cellular level. The role of cyclooxygenase and its prostaglandin product PGE_2 in n-6 and n-3 PUFA-mediated effects on cellular proliferation, an intermediate biomarker of colon cancer risk (29), was studied. Human colon adenocarcinoma cells a.o. Caco-2 and HT-29 were used in this study, because they express respectively high and lower levels of COX-2 (12,14) and so may represent different stages of colorectal carcinogenesis.

Materials and methods

Reagents

Bovine serum albumin (BSA; essential fatty acid-free), linoleic acid (LA), α-linoleic acid (ALA), arachidonic acid (AA), eicosapentaenoic acid (EPA), prostaglandin E_2 (PGE_2), α-tocopherol (vitamin E), indomethacin (IM), trichloroacetic acid, 2-thiobarbituric acid and 1,1,3,3-tetramethoxypropane (malondialdehyde) were obtained from Sigma Chemical Company (St Louis, MO, USA). 16,16-Dimethyl PGE_2 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Triton X-100 was obtained from Merck AG (Germany). Fetal calf serum (FCS), non-essential amino acids, penicillin-streptomycin and all growth media were obtained from Life Technologies (Breda, The Netherlands). Recombinant human IL-1β was purchased from ITK diagnostics (Uithoorn, The Netherlands).

Cells and cell culture

Caco-2 and HT-29 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). LIM1215 cells were a generous gift from Dr R.Whitehead (Ludwig Institute for Cancer Research, Australia) and HCA-7 Colony 29 cells were kindly provided by Dr S.Kirkland (University of London, UK).

All cells were maintained in an atmosphere of 5% CO_2/95% air at 37°C. Cells were sub-cultured at a ratio of 2:10, after they had reached 70–90% confluence in 75 cm^2 culture flasks (Costar, Cambridge, MA, USA).

Caco-2 cells were grown in DMEM, supplemented with 10% heat-inactivated FCS, 1% non-essential amino acids, 2% penicillin-streptomycin; HT-29 cells were maintained in 10% FCS-containing McCoy's 5a medium. LIM1215 cells and HCA-7 cells were grown in 10% FCS-containing DMEM supplemented with 0.11 g/l sodium pyruvate.

Cell proliferation

A colorimetric immunoassay kit (Cell proliferation ELISA, BrdU (colorimetric), Boehringer Mannheim GmbH, Mannheim, Germany) was used for quantification of cell proliferation. This assay is based on the measurement of BrdU incorporation during DNA synthesis. For these experiments, cells were plated at a concentration of 5×10^4 cells/ml in 200 µl of medium into 96-well tissue culture plates. The medium was removed after 24 h and 100 µl fresh serum-free medium, supplemented with the indicated concentrations of PGE_2 or 1 mg/ml fatty acid-free BSA and the indicated concentrations of fatty acids with or without IL-1β (10 ng/ml), vitamin E or indomethacin (IM) were added for 24 or 48 h. The antioxidant vitamin E was also pre-incubated before fatty acid treatment during the first 24 h of cell culture. Fatty acids were dissolved in ethanol up to a final concentration in the medium of 0.5%. Before addition to the cultures, the fatty acids and ethanol were pre-incubated in the BSA-containing medium for 30 min at 37°C. Control wells received serum-free medium supplemented with 1 mg/ml fatty acid-free BSA plus 0.5% ethanol. After incubation the medium was removed and BrdU was added to the cells for an additional 3 h. Subsequently the immunoassay was performed according to the protocol provided by the manufacturer. Cell proliferation is expressed as mean percentage of control values (set as 100%).

PGE_2 analysis

Cells were plated at a concentration of 3.54×10^5 cells/ml in 400 µl of medium into 24-well tissue culture plates. After 24 h, the medium was removed and fresh serum-free medium, supplemented with 1 mg/ml fatty acid-free BSA and the indicated concentrations of fatty acids with or without IL-1β (10 ng/ml) was added. The medium was harvested 1, 4, 10 or 24 h after incubation. PGE_2 levels were quantitated using a PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech UK, Buckinghamshire, UK) according to the high sensitivity enzyme immunoassay protocol 2, recommended by the manufacturer. The results are expressed as picograms of PGE_2 per ml medium (curve range 20–640 pg/ml). The program EZ-Fit™, version 5.03 for Windows (Perella Scientific, NH, USA) is used to determine whether EPA could reversibly inhibit COX activity in Caco-2 cells.

Cytotoxicity

Release of lactate dehydrogenase (LDH) in the culture medium through membrane leakage was measured as an indicator of cytotoxicity. Cells were plated and treated with fatty acids under the same conditions as described in the section cell proliferation. After treatment the medium was collected from each well and 100 µl 0.5% Triton X-100 in 50 mM potassium phosphate buffer was added to lyse the cells. LDH activity was measured spectrophotometrically at 340 nm, with pyruvate as a substrate (30). Results are expressed as LDH activity in the media as a percentage of the total LDH activity in both cells and media, corrected for background leakage from control cells.

Lipid peroxidation

Lipid peroxidation was measured in Caco-2 cells (6-well cell culture dishes; 17.18×10^4 cells/ml in 2 ml) and the culture medium, treated for 48 h with fatty acids with or without vitamin E or indomethacin. The thiobarbituric acid assay according to Buege and Aust (31) was used. This assay is based on the fact that malondialdehyde (MDA), which is a product of lipid peroxidation, reacts with thiobarbituric acid to give a red species absorbing at 535 nm. Absorbance was converted to nmol MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane. Lipid peroxidation was presented as nmol MDA per mg protein. Protein levels in cell cultures were measured according to Bradford (32).

Results

Effects of n-6 and n-3 PUFAs on human colon carcinoma cell proliferation

The influence of ALA (18:3n-3), LA (18:2n-6), EPA (20:5n-3) and AA (20:4n-6) on cell proliferation was measured to compare the differential effects of exogenous n-3 and n-6 PUFAs on growth of human colorectal carcinoma cells. ALA and LA had no effect on proliferation of Caco-2 cells up to exogenous concentrations of 80 µM. At a concentration of 160 µM, ALA and LA inhibited the proliferation of Caco-2 cells after 48 h of incubation (Figure 1A). The more PUFAs EPA and AA showed a dose-dependent decrease in cell proliferation after 48 h of incubation in Caco-2 cells (Figure 1A).

In comparison, ALA, LA, EPA and AA (0–160 µM) had no effect on cell proliferation of HT-29 cells (data not shown). Although, induction of prostaglandin synthesis by IL-1β treatment in HT-29 cells resulted in an AA and EPA induced dose-dependent decrease in cell proliferation (Figure 1B).

Effects of n-6 and n-3 PUFAs on PGE_2 production in human colon carcinoma cell lines

Caco-2 cells were treated with different fatty acids in order to determine the effects of exogenous n-6 and n-3 PUFAs on PGE_2 production. Figure 2 demonstrates clearly that AA (20:4n-6) increased PGE_2 production of Caco-2 cells after 24 h of treatment. Basal levels of PGE_2 in Caco-2 cells as well as effects of treatment with LA (18:2n-6), ALA (18:3n-3) or EPA (20:5n-3) on basal PGE_2 levels could not be detected with the PGE_2 enzyme immunoassay used.

Caco-2 cells were also incubated with 20 µM of AA and co-incubated with different concentrations of EPA for 1, 4, 10 and 24 h to investigate whether EPA could inhibit AA-induced PGE_2 production in these cells. As shown in Figure 3A, EPA inhibited COX activity in Caco-2 cells, i.e. PGE_2 production, already after 1 h of incubation. In order to get more insight into the type of reversible enzyme inhibition, different concentrations of AA were co-incubated with 0, 20 or 40 µM of EPA for 1 h. The results (see Figure 3B) can be described by the Lineweaver–Burk equation, which is a transformation of the Michaelis–Menten equation. Competitive kinetic constants were arbitrarily estimated in cells with the EZ-fit program. EPA inhibited COX competitively with a $K_{i\ app}$ of 31.3 ± 8 µM

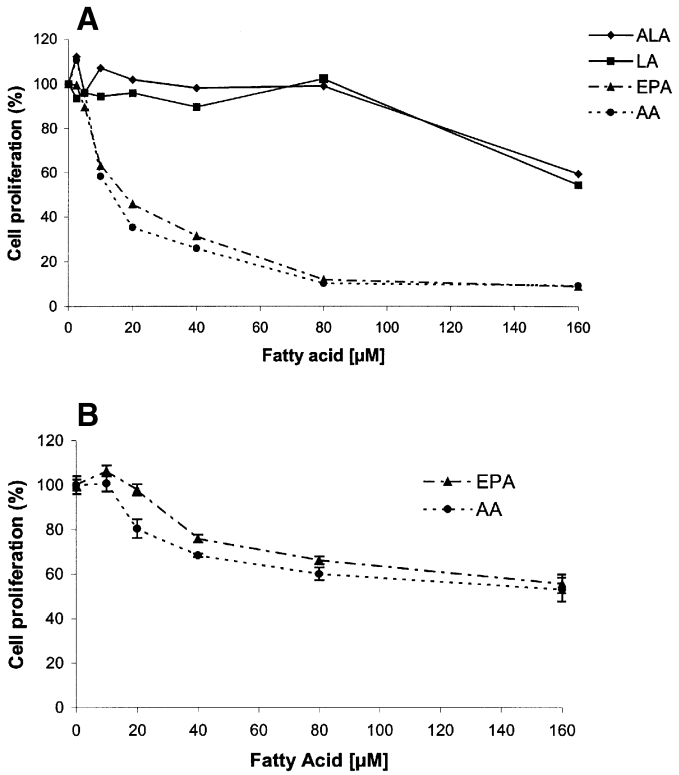


Fig. 1. (**A**) Effects of n-6 and n-3 PUFAs on cell proliferation of human colon carcinoma Caco-2 cells. Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid-free BSA and ALA (18:3n-3), LA (18:2n-6), EPA (20:5n-3) or AA (20:4n-6) at 0, 2.5, 5, 10, 20, 40, 80 and 160 μM. After 48 h, cell proliferation was measured by ELISA as described in Materials and methods. Results are the average of six replicate determinations of one of the representative experiments. SEM was <7%. (**B**) Effects of EPA and AA on cell proliferation of human colon carcinoma HT-29 cells treated with IL-1β. Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid-free BSA, 10 ng/ml IL-1β and EPA (20:5n-3) or AA (20:4n-6) at 0, 10, 20, 40, 80 and 160 μM. After 48 h, cell proliferation was measured by ELISA as described in Materials and methods. Results represent the means ± SEM of six replicate determinations of one of the representative experiments.

($K_{m\ app}$ of 7.9 ± 1.3 μM; $V_{max\ app}$ of 632.04 ± 24.2 pg per h per ml medium).

HT-29 human colon carcinoma cells showed less COX activity than Caco-2 cells. Only 160 μM of exogenous AA could stimulate PGE_2 production in these cells, but co-incubation of AA with the COX-2 inducer IL1-β resulted in a tremendous upregulation of PGE_2 synthesis in these cells (Figure 2). Addition of LA, ALA or EPA also had no measurable effect on basal PGE_2 production of HT-29 cells.

Effect of PGE_2 on human colon carcinoma cell proliferation

The responsiveness of human colorectal carcinoma cells to PGE_2 was tested to investigate whether PGE_2 itself could influence the proliferation of colon cancer cells. 16,16-Dimethyl PGE_2, a more stable analogue of PGE_2, was also used to test the effect on colonic cell proliferation because this PGE_2 analogue has a prolonged half-life in cell culture. Exogenous PGE_2 failed to stimulate proliferation of Caco-2 cells after 24 h of incubation. This was also the case for 16,16-dimethyl PGE_2. Concentrations >40 μg/ml PGE_2 inhibited proliferation. Also no stimulating effect was seen on HT-29, LIM1215 and HCA7 cells over a wide range of concentrations and incubation times (24–72 h) (data not shown).

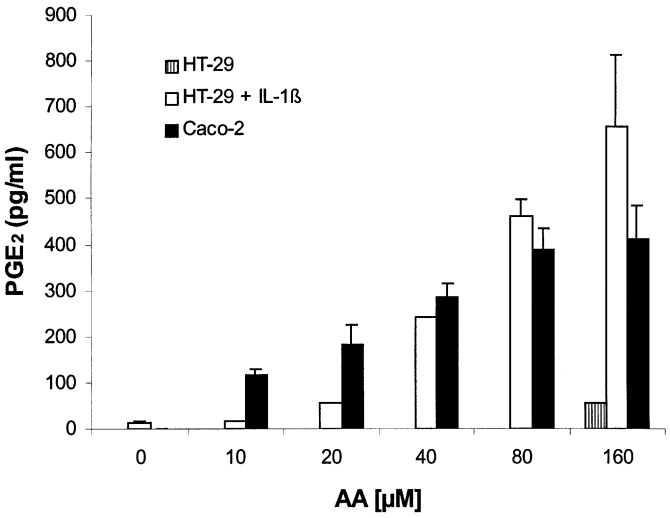


Fig. 2. Effect of exogenous AA on the production of PGE_2 in human colon carcinoma Caco-2 and HT-29 cells. Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid free BSA and AA at 0, 10, 20, 40, 80 and 160 μM ± 10 ng/ml IL-1β. After 24 h, PGE_2 production was measured in the medium by an enzyme immunoassay as described in Materials and Methods. Results represent the means ± SD of duplicate determinations of one of the representative experiments.

Cytotoxicity of EPA and AA in human colon carcinoma Caco-2 cells and HT-29 cells treated with IL-1β

LDH release in the medium was measured to determine whether the growth inhibitory effects of EPA and AA in Caco-2 cells and in HT-29 cells treated with IL-1β were due to cytotoxicity. In this assay, a LDH leakage through the cell membrane of 10% was considered as cytotoxicity. As shown in Figure 4A, concentrations of EPA and AA higher than 20 μM were cytotoxic for Caco-2 cells. EPA and AA were not cytotoxic for HT-29 cells. However, in combination with IL-1β treatment, EPA and AA were also cytotoxic for HT-29 cells. Addition of AA and EPA concentrations higher than 10 μM resulted in this situation in an increase in LDH leakage above 10% (Figure 4B).

Lipid peroxidation

Because oxidation products of PUFAs may play an important role in the EPA and AA-induced growth inhibition and cytotoxic effects, levels of MDA, which is an end-product of lipid peroxidation, were measured in Caco-2 cells and culture medium. After 48 h of incubation with different concentrations of AA or EPA, MDA formation increased in Caco-2 cells in comparison with untreated cells. This increase was dependent on PUFA concentration and especially obvious at high concentrations (80–160 μM) of AA and EPA (Figure 5). 40% of the total amount of MDA was found in the cells and 60% was found in the media. Only <12% of the total amount of MDA in the culture media was due to auto-oxidation of fatty acids. This was observed when the media with or without added fatty acids was incubated without cells. The radical scavenging antioxidant vitamin E could clearly diminish the AA and EPA-induced MDA production in Caco-2 cells (Figure 5). Because oxidation products such as MDA are also formed during the cyclooxygenase pathway (33), we investigated whether a cyclooxygenase inhibitor, IM, could also protect against MDA

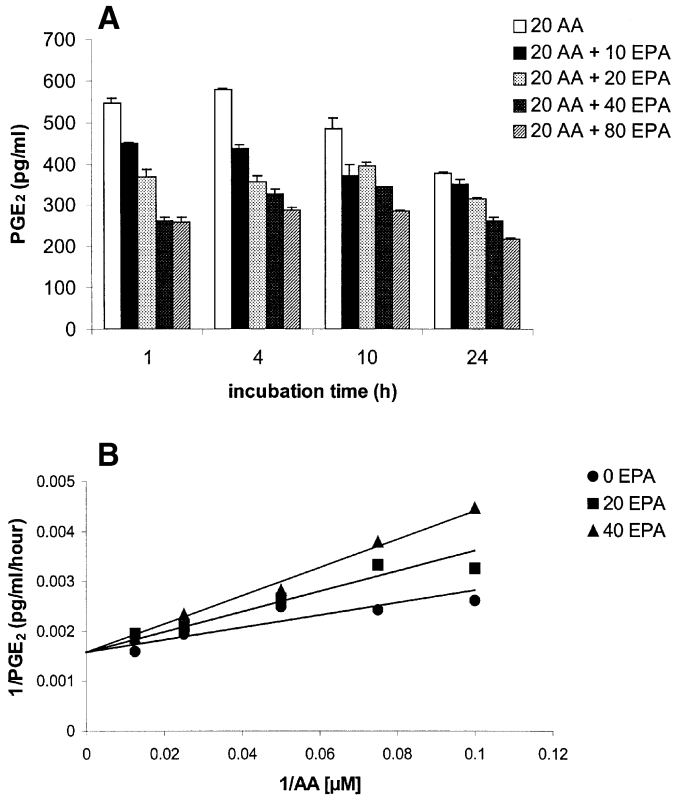


Fig. 3. (**A**) Effect of EPA on AA-induced PGE_2 production in human colon carcinoma Caco-2 cells. Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid free BSA, AA (20 µM) and co-incubated with EPA at 10, 20, 40 and 80 µM. After 1, 4, 10 and 24 h, PGE_2 production was measured in the medium by an enzyme immunoassay as described in Materials and methods. Results represent the means ± SD of duplicate determinations of one of the representative experiments. (**B**) A double-reciprocal (Lineweaver–Burk) plot of COX enzyme inhibition in Caco-2 cells by EPA. PGE_2 production was measured in the medium of Caco-2 cells, 1 h after addition of 10, 13.33, 20, 40 and 80 µM AA and 0, 20 and 40 µM EPA.

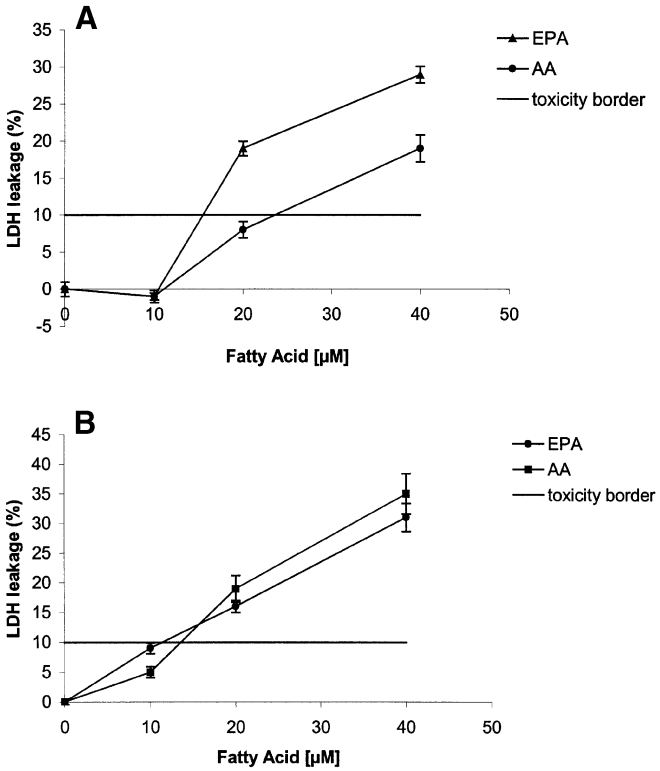


Fig. 4. Cytotoxicity of EPA and AA in human colon carcinoma cells Caco-2 (**A**) and HT-29 treated with IL-1β (**B**). Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid-free BSA and EPA or AA ± 10 ng/ml IL-1β at 0, 10, 20 and 40 µM. After 48 h, LDH leakage (%) was measured. Results represent the means ± SEM of four replicate determinations of one of the representative experiments.

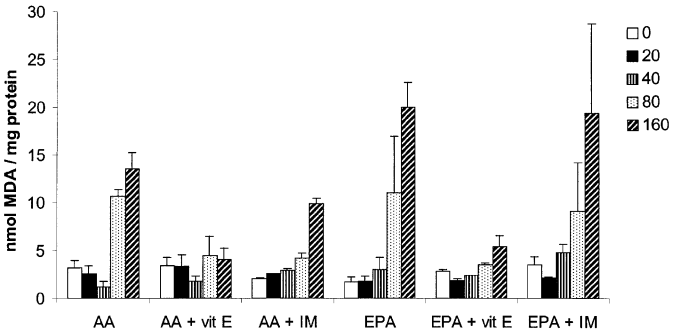


Fig. 5. Effect of AA and EPA on MDA formation in Caco-2 cells. Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid free BSA and AA or EPA at 0, 20, 40, 80 and 160 µM. The effect of 24 h pre-incubation and 48 h co-incubation with 10 µM vitamin E (vit E) and 48 h co-incubation with 20 µM IM on MDA formation of 0–160 µM AA or EPA was also investigated. MDA formation was measured in the cells by the thiobarbituric acid assay as described in the Materials and methods. Results represent the means ± SEM of duplicate determinations of one of the representative experiments.

formation in Caco-2 cells. From these experiments can be concluded that IM could partially inhibit MDA formation, especially from AA in Caco-2 cells.

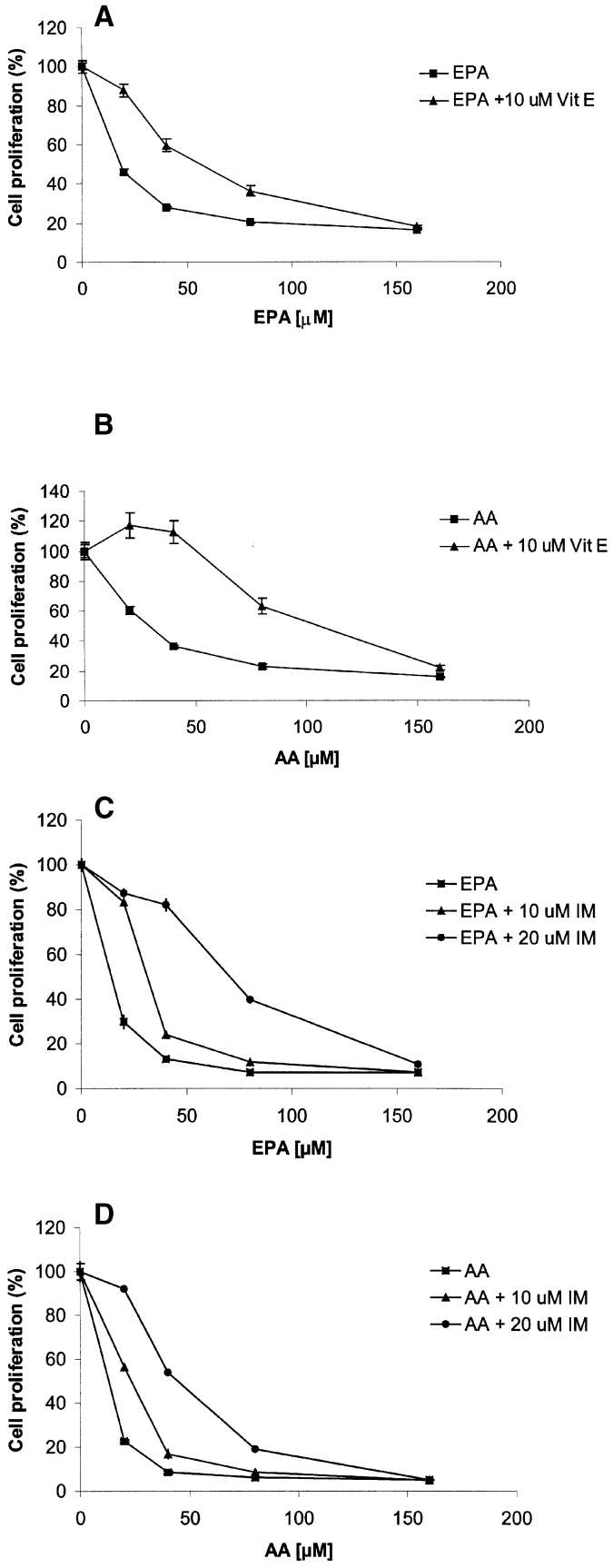
Effect of antioxidants and a cyclooxygenase inhibitor on EPA and AA-induced decrease in cell proliferation

To test whether vitamin E could also protect against the EPA and AA-induced growth inhibition and cytotoxic effects, cell proliferation of Caco-2 cells was measured after pre-incubation and co-incubation of EPA and AA with 10 µM of vitamin E. As shown in Figure 6 (A and B), vitamin E could partially reverse the AA and EPA-induced decrease in cell proliferation. Vitamin E (10 µM) alone had no effect on cell proliferation. Also pre- (2.5 h) and co-incubation (48 h) of vitamin C (100 µM) could partially reverse the AA and EPA-induced decrease in cell proliferation (data not shown).

Because IM could partially inhibit MDA formation in Caco-2 cells, we examined whether inhibition of cell proliferation could also be reversed by addition of a cyclooxygenase inhibitor. Therefore, EPA and AA were co-incubated with IM, a potent inhibitor of cyclooxygenase, for 48 h. Our studies showed that IM could also partially reverse the EPA and AA-induced decrease in cell proliferation of Caco-2 cells (Figure 6C and D). IM alone (10 and 20 µM) had no effect on Caco-2 cell proliferation (data not shown).

Effect of IM on PGE_2 production in human colon carcinoma Caco-2 cells

Addition of the non-selective cyclooxygenase inhibitor IM did not alter cellular proliferation of human colon carcinoma Caco-2 cells at exogenous concentrations of 10 and 20 µM, as mentioned above. These concentrations produced, on the other hand, significant decreases in AA-induced PGE_2 production in these cells already 1 h after incubation, as shown in Figure 7.



Discussion

Several *in vivo* studies hypothesize that corn oil with a high amount of n-6 PUFAs such as LA (18:2n-6) might enhance colorectal carcinogenesis via stimulation of colonic cell proliferation (24). Fish oil, on the other hand, with a high amount of n-3 PUFAs such as EPA (20:5n-3) is thought to have inhibitory effects on tumor cell growth (34,35). By contrast, fewer studies have been conducted in human colon cancer cell lines. In most *in vitro* studies performed, the n-3 fatty acid EPA has antitumoral effects through inhibition of cell proliferation (36,37) or induction of apoptosis (38). However, only a few studies have compared the potency of various n-3 and n-6 PUFAs in modulating cell growth in the same colon cell line (39,40).

Therefore, we studied the effect of ALA (18:3n-3), LA (18:2n-6), EPA (20:5n-3) and AA (20:4n-6) on cell proliferation in both Caco-2 as well as HT-29 cells to compare the differential effects of exogenous n-3 and n-6 PUFAs on growth of human colorectal carcinoma cell lines. From these studies it became clear that the long-chain n-3 and n-6 fatty acids ALA and LA had no effect on growth of Caco-2 and HT-29 cells. At a concentration of 160 μM, ALA and LA inhibited the proliferation of Caco-2 cells in comparison to HT-29 cells, indicating differences in sensitivities among different cell lines. We indeed found that the more polyunsaturated n-3 fatty acid EPA inhibited the growth of human colorectal carcinoma Caco-2 cells, but the n-6 fatty acid AA showed the same growth-inhibitory and cytotoxic effects. AA and EPA did not influence the proliferation rate in HT-29 cells, again indicating differences in sensitivities of different cell lines to n-6 and n-3 PUFAs. Overall, no differential effect between the long chain n-3 and n-6 fatty acids ALA and LA were observed in both Caco-2 as well as HT-29 cells, with regard to cell proliferation. Also, no differential effects were observed between the more polyunsaturated long chain n-3 and n-6 fatty acids EPA and AA, even at low non-toxic concentrations of exogenous PUFAs ($<$20 μM). On the other hand, the more polyunsaturated long chain fatty acids (20:5n-3 and 20:4n-6) did inhibit cell growth in Caco-2 cells compared with the long chain fatty acids (18:3n-3 and 18:2n-6). This suggests that the number of double bonds in the carbon atom chain of the fatty acid is more important for the anti-proliferative and cytotoxic effects of n-3 and n-6 fatty acids than the place of the double bond.

In vivo studies also suggest that n-6 PUFAs might enhance colorectal carcinogenesis via increased COX-2 expression and increased production of prostaglandins, such as PGE_2 (21,41). *N*-3 fatty acids might exert their antitumoral effect through inhibition of PGE_2 synthesis via the cyclooxygenase pathway (19,35). Therefore, the role of cyclooxygenase and its prostaglandin product PGE_2 in n-6 and n-3 PUFA-mediated effects on cellular proliferation was also investigated in this study. We demonstrated that human colon carcinoma Caco-2 cells,

Fig. 6. Effect of vitamin E (**A** and **B**) and indomethacin (**C** and **D**) on EPA and AA-induced decrease in cell proliferation in Caco-2 cells. Cells were plated in medium and pre-incubated with or without 10 μM vitamin E. After 24 h, the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid-free BSA and EPA or AA at 0, 20, 40, 80 and 160 μM with or without 10 μM vitamin E or IM (10 or 20 μM). After 48 h, cell proliferation was measured by ELISA as described in Materials and methods. Results represent the means ± SEM of six replicate determinations of one of the representative experiments.

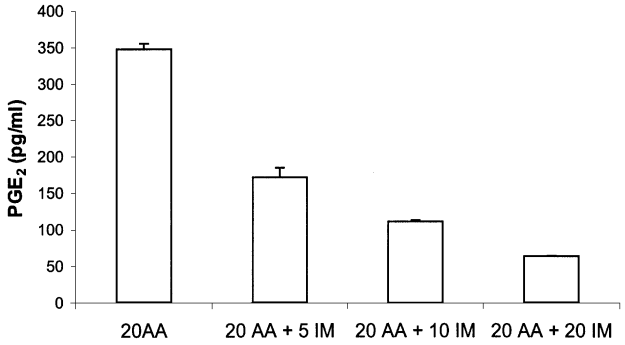


Fig. 7. Effect of exogenous IM on AA-induced PGE_2 production in human colon carcinoma Caco-2 cells. Cells were plated and after 24 h the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty acid free BSA, 20 μM of AA and IM at concentrations of 0, 5, 10 and 20 μM. One hour after incubation, PGE_2 production was measured in the medium by an enzyme immunoassay as described in Materials and methods. Results represent the means ± SD of duplicate determinations of one of the representative experiments.

which express high levels of COX-2 (14), produce PGE_2 upon addition of AA. HT-29 colon cells on the other hand did not produce high levels of PGE_2 upon addition of AA. PGE_2 synthesis in this cell line was rather inducible by IL-1β, a cytokine that can increase COX-2 expression (42,43). Thus, although COX-2 protein is expressed in Caco-2 as well as HT-29 cells, COX-2 enzyme activity seemed to be variable in the different cell lines. The observation that the n-6 fatty acid LA could not increase PGE_2 production is in agreement with the finding that the parent essential fatty acid LA is not converted to the prostaglandin precursor AA in Caco-2 (44) as well as HT-29 cells.

N-3 PUFAs are precursors of trienoic prostaglandins in stead of dienoic prostaglandins (45). EPA is converted by COX to the 3-series endoperoxide PGH_3, by way of PGG_3, which can be further metabolized to PGE_3 (46). Because n-3 and n-6 PUFAs are both substrates for prostaglandin synthesis, they compete for enzymes such as COX. EPA indeed competitively inhibited AA-induced PGE_2 synthesis in Caco-2 cells. This *in vitro* result is thus in agreement with the *in vivo s*tudies mentioned above that also show that n-3 PUFAs such as EPA inhibit COX activity and AA metabolism (19,35). However, regulation of cell proliferation is not directly mediated via PGE_2 levels in Caco-2 cells, since reductions in PGE_2 synthesis by EPA compared with AA did not lead to differential effects on cell proliferation in human colon carcinoma Caco-2 cells.

Moreover, the causal positive relationship between PGE_2 and proliferation of colon epithelial cells is also questioned through the contrasting results obtained by many laboratories (9,13,27,28,47). Addition of exogenous PGE_2 failed to stimulate cell proliferation of Caco-2, HT-29, LIM1215 and HCA-7 cells in our experiments. These findings confirm previous studies in which PGE_2 also failed to stimulate proliferation of human colon adenocarcinoma cells (9,13,47) and support the idea that PGE_2 does not play a direct role in the proliferation of human colon adenocarcinoma cells.

PUFAs with methylene-interrupted double bonds, such as EPA and AA, are highly susceptible to enzymatic and non-enzymatic peroxidation (48). Enzymatic peroxidation is brought about by the action of cell-derived peroxidizing enzymes such as cyclooxygenase (49). Non-enzymatic peroxidation is initiated by free-radical attack of membrane lipids, generating large amounts of reactive products (48). Peroxidation products of PUFAs may have played an important role in the EPA and AA-induced growth inhibition and cytotoxic effects. MDA is a product of both spontaneous lipid peroxidation (48) and of prostaglandin biosynthesis (33,50). Therefore, levels of MDA were measured in Caco-2 cells to determine the extent of peroxidation reactions in Caco-2 cells after treatment with either AA or EPA. MDA was indeed increased after incubation with AA or EPA in Caco-2 cells. Incubation of AA or EPA with vitamin E, an antioxidant that prevents lipid peroxidation, almost completely abolished MDA formation in Caco-2 cells. Because Gavino *et al.* (50) showed that cellular uptake of antioxidants is not rapid enough for inhibition of MDA formation at short incubation time intervals, both the lipid-soluble vitamin E as well as the water-soluble antioxidant vitamin C were pre- and co-incubated in the medium. Addition of both vitamin E as well as vitamin C also partially reversed cell proliferation of Caco-2 cells, especially at low concentrations of AA or EPA. Overall, these results support the potential role of lipid peroxidation in the AA and EPA-induced growth inhibition and cytotoxic effects. Also, other studies (36,50–52) have demonstrated the protective role of vitamin E and C against lipid peroxidation and PUFA-induced inhibition of cell proliferation.

In addition, we are the first to show that IM, a COX inhibitor that clearly inhibited AA-induced PGE_2 synthesis in Caco-2 cells, could also partially reverse the antiproliferative effects of both AA and EPA. Furthermore, induction of COX-2 activity in HT-29 cells by IL-1β did not increase cell proliferation by AA, but rather caused a decrease in cell proliferation and an induction of cytotoxicity by AA as well as EPA. Both results suggest that in human colon cancer cells, n-6 and n-3 PUFA induced growth-inhibition is also related to COX activity. Because cell-derived peroxidizing enzymes such as COX can also lead to oxidation of a wide range of compounds and to production of MDA via enzymatic and non-enzymatic breakdown of PGH_2 and PGH_3 (49,53), we measured whether inhibition of COX-activity by IM could also inhibit AA and EPA-induced MDA formation. IM partially inhibited AA-induced MDA formation in Caco-2 cells, with minor effects on EPA. These results suggest though that the inhibitory effects of PUFAs on cell proliferation could also partially be due to peroxidation products that are generated during COX activity. Also other COX-derived products (PUFA metabolites) may explain the effect that PUFAs have on the extent of cell proliferation.

These effects are intriguing and paradoxical to our current understanding of the role of COX-2 in cancer cells. However, previous studies only focused on the 'single' effects of non-specific NSAIDs such as IM, on the proliferation of intestinal epithelial cells. These studies indicate that high-dose IM reduces the proliferation rate of colon cancer cells (54,55). Low concentrations of IM (10^{-8} to 10^{-4} M) do not inhibit cell growth, which is in agreement with our result that IM alone (10 and 20 μM) had no effect on Caco-2 cell proliferation. On the other hand, we demonstrate that IM in combination with the PUFAs AA and EPA thus supports the growth of colon carcinoma Caco-2 cells, partially through inhibition of peroxidation products that are generated during COX activity.

Overall our data suggest that the number of double bonds in the carbon atom chain of the fatty acid is more important for the anti-proliferative and cytotoxic effects of n-3 and n-6 fatty acids in our *in vitro* system than the place of the double bond. Non-enzymatic lipid peroxidation products as well as

COX-derived (peroxidation) products were probably responsible for the growth-inhibitory effects of the highly PUFAs such as AA and EPA. The extent to which these results are relevant for the *in vivo* effects of n-6 and n-3 PUFAs on colorectal carcinogenesis need to be evaluated.

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