Effects of n-6 and n-3 polyunsaturated fatty acids on colorectal carcinogenesis

Yvonne Dommels

Promotor:

Prof. dr. P.J van Bladeren
Hoogleraar in de Toxicokinetiek en Biotransformatie
Wageningen Universiteit

Copromotoren:

Dr. G.M Alink
Universitair Hoofddocent, Sectie Toxicologie
Wageningen Universiteit
Dr. ir. B van Ommen
Senior research fellow nutritional systems biology
TNO Nutrition and Food Research

Promotiecommissie:

Prof. dr. M Müller
Wageningen Universiteit
Prof. dr. ir. I.M.C.M Rietjens
Wageningen Universiteit
Prof. dr. ir. G.J Schaafsma
Wageningen Universiteit / TNO Nutrition and Food Research
Prof. dr. F.J van Schooten
Universiteit Maastricht

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Yvonne Elisabeth Maria Dommels

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Author Yvonne Elisabeth Maria Dommels
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'The best research comes from asking Nature simple questions, one at a time'

Pennie *et al.*, Toxicological sciences, 2000

Abstract

In vivo studies have demonstrated that high fat fish oil (HFFO) diets with high levels of n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5n-3) can inhibit the formation of chemically-induced colon tumors during both the initiation and post-initiation phases of colorectal carcinogenesis compared with high fat corn oil (HFCO) diets which are rich in n-6 PUFAs such as linoleic acid (LA; 18:2n-6).

Studies described in this thesis show that HFFO diets also protect against the initiation of aberrant crypt foci (ACF; precursor lesions of colon cancer) in F344 rats compared to HFCO diets. Furthermore, EPA also inhibited the proliferation of human colon adenocarcinoma Caco-2 cells compared to LA. The mechanism responsible for the inhibitory effects of n-3 PUFAs such as EPA on colorectal tumors may partly be related to inhibition of PGE_2 synthesis from arachidonic acid (AA; 20:4n-6). Plasma levels of PGE_2 were indeed lower in HFFO fed rats compared to HFCO fed rats. However, reductions in PGE_2 synthesis by EPA compared to AA in Caco-2 cells did not lead to differential effects on cell proliferation, which suggests that PGE_2 is not directly involved in regulation of cell proliferation in colon cancer cells by n-6 and n-3 PUFAs.

Our results suggest that lipid peroxidation-induced oxidative stress might be an important mechanism by which n-3 PUFAs possess anticarcinogenic effects. This is supported by the fact that HFFO diets with a high amount of EPA increased the amount of lipid peroxidation in F344 rats compared to HFCO diets with a high amount of LA. Levels of malondialdehyde, which is an end product of lipid peroxidation were also increased after incubation of Caco-2 cells with EPA. Furthermore, transcription of genes involved in oxidative stress is increased in HFFO fed rats, whereas addition of antioxidants diminishes the anticancer effects of n-3 PUFAs in Caco-2 cells, which also suggests that oxidation of n-3 PUFAs underlies their anticancer effects. Overall, the results might imply that n-3 PUFAs protect against colon carcinogenesis via increased oxidative stress that can ultimately lead to inhibition of cell proliferation to prevent clonal expansion or induction of apoptosis to stimulate disposal of damaged colonic epithelial cells. Diets enriched with high levels of n-3 PUFAs may thus have beneficial colon cancer inhibiting effects, which may be reduced by high levels of dietary antioxidants.

Contents

Chapter 1

General introduction

based on: Yvonne EM Dommels, Gerrit M Alink, Peter J van Bladeren and Ben van Ommen. (2002) Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies. *Environmental Toxicology and Pharmacology,* **12(4)**, 233-244

1.1 Background

Colorectal cancer is one of the most common causes of cancer deaths in the industrialized Western countries. It is the most prevalent cancer form for men after lung- and prostate cancer. For females, colorectal cancer is the second form after breast cancer (1). In the Netherlands, the colon cancer incidence is around 8600 new cases per year (2). In 1998, 4400 people died of colorectal cancer, which is around 12% of the total cancer deaths (Health Council of the Netherlands, 2001).

Many factors can be responsible for the development of colon cancer. Genetic predisposition is considered as an important risk factor. There are two separate genetic syndromes that markedly increase the risk of colorectal cancer; Hereditary Non-Polyposis Colorectal Cancer (HNPCC) and Familial Adenomatous Polyposis (FAP) (3). HNPCC is characterized by mutational inactivation of mismatch repair genes at an early age. Patients with FAP may develop hundreds of polyps, also at an early age, due to an inactivation mutation of the Apc gene (4,5).

In addition to genetic predisposition, diet is also an important risk factor for colorectal carcinogenesis (3). Already in the early 1980s Doll and Peto (6) estimated that 90% of deaths from colorectal cancer would be avoidable by dietary means.

Accumulating evidence suggests an association between dietary fat intake and colorectal carcinogenesis (6). Migration studies have shown that the low mortality rates from colon cancer in Japan increase when Japanese migrate to the US and adapt to a Western diet, which contains for example higher levels of n-6 polyunsaturated fatty acids (PUFAs) such as linoleic acid (LA; 18:2n-6). Like Japanese, also Eskimos have a relatively low incidence of colon cancer (1). Both populations consume large quantities of fish (1000-3000 mg per day), which is rich in n-3 PUFAs, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (7). Also experimental animal studies indicate that high fat fish oil (HFFO) diets with a high amount of n-3 PUFAs can reduce colorectal carcinogenesis compared to high fat corn oil (HFCO) diets with a high amount of n-6 PUFAs (8,9).

So overall, epidemiological and experimental studies reveal that not only the amount of fat consumed, but also the type of fat consumed plays an important role in the development of colon cancer.

1.2 Nomenclature, dietary sources and metabolism of n-6 and n-3 PUFAs

N-6 and n-3 fatty acids are polyunsaturated fatty acids (PUFAs) with two or more double bonds in the carbon atom chain. N-6 and n-3 fatty acids are named after the position of the first double bond from the methyl end of the molecule. For example, linoleic acid

(LA; 18:2n-6) has 18 C-atoms and two double bonds, with the first double bond at the 6th carbon atom counted from the methyl end. Linoleic acid is the parent compound of the n-6 family, whereas α-linolenic acid (ALA; 18:3n-3) is the parent compound of the n-3 fatty acid family. These two PUFA families are considered as essential and must be derived from the diet (10). Linoleic acid is mostly found in vegetable seeds and oils such as safflower, soybeans, corn and sunflower oil. Perilla oil from the Asian beefsteak plant (*Perilla frutescens*), linseed oil, rapeseed, walnuts and blackcurrant oil are rich in α-linolenic acid. α-Linolenic acid is also present in dark green leafy plants (11).

Linoleic acid and α-linolenic acid can be metabolized to more polyunsaturated fatty acids by the combined action of desaturation and elongation enzymes (10). Desaturation enzymes introduce a new double bond in the carbon atom chain and elongation enzymes introduce two new C-atoms. Arachidonic acid (AA; 20:4n-6) is the major long-chain n-6 PUFA. Eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are the major PUFAs of the n-3 family.

The marine food chain is based on n-3 fatty acids, which are present in plankton and algae on which fish feed (11). Fatty fish such as salmon, tuna, herring, mackerel and anchovy are rich sources of EPA and DHA (12). Although lean meats and meat fat are direct dietary AA sources, dietary LA is considered to be the main source of tissue AA (12).

Production of AA and EPA from LA and ALA, respectively, is thought to proceed preferentially by Δ^6-desaturation followed by a two-carbon atom chain elongation and Δ^5-desaturation (13) (see Figure 1.1). Metabolism of n-6 and n-3 fatty acids follows a series of competitive elongation and desaturation steps, which is limited by the activity of Δ^6–desaturase, with the n-3 fatty acids having greater affinities for the enzyme. In consequence, increasing dietary intake of n-3 fatty acids reduces the desaturation of LA and so, the production of AA (12).

AA and EPA also show substrate competition for cyclooxygenase (COX) enzyme activities. Cyclooxygenase 1 and -2 (COX-1, COX-2 respectively) introduce two atoms of oxygen into AA to form the hydroperoxy endoperoxide PGG_2, which is then reduced by the endoperoxidase moiety of the enzyme to the hydroxy endoperoxide PGH_2. PGH_2 can be a substrate for several enzymes including PGE synthase, which forms prostaglandin E_2 (PGE_2) (14) (see Figure 1.2).

EPA is a precursor of the prostaglandins of the 3-series, with three double bonds. The n-3 fatty acid is converted by the same COX enzyme to the 3-series endoperoxide PGH_3, by way of PGG_3, which can be further metabolized to PGE_3 (12) (see Figure 1.2).

Family	*First member*		*Δ6-Desaturation*		*Elongation*		*Δ5-Desaturation*		
n-6	18:2n-6 **LA**	→	18:3n-6	→	20:3n-6	→	20:4n- 6 **AA**		
n-3	18:3n-3 **ALA**	→	18:4n-3	→	20:4n-3	→	20:5n-3 **EPA**		

Figure 1.1: Metabolism of n-6 and n-3 polyunsaturated fatty acids

EPA supplementation can thus lead to competitive inhibition of arachidonic acid metabolism as well as the production of other metabolites such as PGE_3. These metabolites are biologically less active than the corresponding arachidonic acid metabolites like PGE_2 (14). AA and EPA can besides the cyclooxygenase pathway, also be metabolized by the lipoxygenase pathway and the cytochrome P450 monooxygenase pathway (15) to prostaglandins, thromboxanes and leukotrienes, collectively referred to eicosanoids. In general, the potency of eicosanoids derived from the n-3 PUFAs is less than of those derived from the n-6 PUFAs (10).

	Cyclooxygenase		*Endoperoxidase*		*PGE synthase*	
20:4n-6 **AA**	→	PGG_2	→	PGH_2	→	PGE_2
20:5n-3 **EPA**	→	PGG_3	→	PGH_3	→	PGE_3

Figure 1.2: Formation of prostaglandin E from arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3)

1.3 Polyunsaturated fatty acids and lipid peroxidation

PUFAs with methylene-interrupted double bonds are highly susceptible to oxidative decomposition or what is commonly known as lipid peroxidation. Lipid peroxidation can take place in the absence or presence of enzymes. Enzymatic lipid peroxidation is brought about by the action of cell-derived peroxidizing enzymes such as cyclooxygenase

(COX). Non-enzymatic lipid peroxidation is initiated by free radicals, requires oxygen and has the characteristics of a chain reaction (13). The process begins when a free radical abstracts a hydrogen atom from a PUFA, thus forming a lipid radical (see Figure 1.3). The lipid radical ($R^{\bullet}$) quickly reacts with oxygen to form a lipid peroxyl radical ($ROO^{\bullet}$), which in turn either forms a lipid endoperoxide or abstracts a hydrogen atom from another PUFA to form a new lipid radical and a lipid hydroperoxide (ROOH) (13). Decomposition of lipid hydroperoxides and endoperoxides eventually gives rise to end products including alcohols, ketones, ethers and aldehydes. Some end products, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal are biologically very active and possess cytotoxic potential (13).

A method commonly used to assess lipid peroxidation is the determination of MDA or a derivative of MDA, by its reaction with thiobarbituric acid (TBA) (16). Ito *et al.* (17) measured serum concentrations of fatty acids and lipid peroxides among Japanese in Japan and Japanese and Caucasians in the US. They concluded that serum thiobarbituric acid-reactive substances (TBARS) were highest among Japanese in Japan, followed by Japanese in the US and Caucasians in the US. A significant positive correlation was observed between serum values of TBARS and n-3 PUFAs among Japanese both in Japan and in the US. These findings suggest that high serum TBARS values among Japanese might depend in part on the induction of lipid peroxidation of n-3 PUFAs due to the Japanese diet which is rich in fish oil (17).

Peroxidation products, such as MDA, can however damage the cell membrane and DNA resulting in cytotoxic effects (18). MDA is found to be mutagenic in bacterial and mammalian cells and carcinogenic in rats (19). MDA can also react with DNA, predominantly deoxyguanosine, to form pyrimidopurinone-deoxyguanosine adducts (M_1G) (19). M_1G seems to be present in human colorectal tissue. The relationship between MDA-DNA damage and the risk of adenomatous polyps in the colon is however still unclear (20).

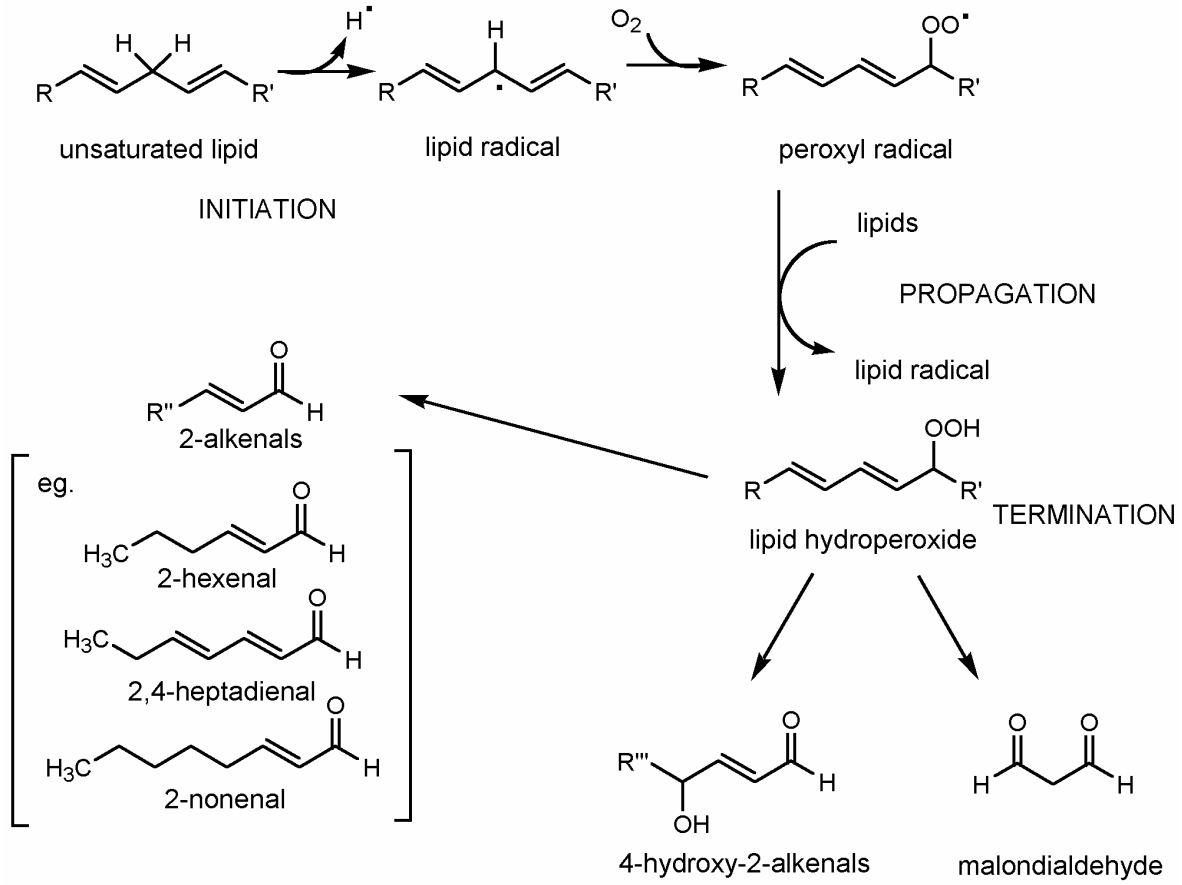

Figure 1.3: Schematic view of lipid peroxidation

1.4 Intermediate biomarkers of colorectal carcinogenesis used in this thesis

1.4.1 Colonic cell proliferation, differentiation and cell death

The colon is a self-renewing tissue with a high cellular turnover rate (48-72 hour) (21). In the colonic crypt, cell proliferation is confined to the lower two third of the crypt. As cells migrate upwards in the crypt, the cells differentiate into absorptive enterocytes, mucous secreting goblet cells or enteroendocrine cells (22). This migration of cells in the colonic crypt is tightly coupled to differentiation. Exfoliation at the top of the crypt could be due to passive sloughing off of cells, expulsion by mechanical forces from neighboring cells or due to programmed cell death (apoptosis) (22).

Tissue homeostasis in the colonic crypt relies on a balance between proliferation, differentiation and apoptosis. Disruption of the balance between proliferation, differentiation and apoptosis, for example by dietary factors can lead to colorectal

carcinogenesis. Hyperproliferation of the intestinal mucosa is regarded as an intermediate biomarker of colorectal cancer (23) (see Figure 1.4).

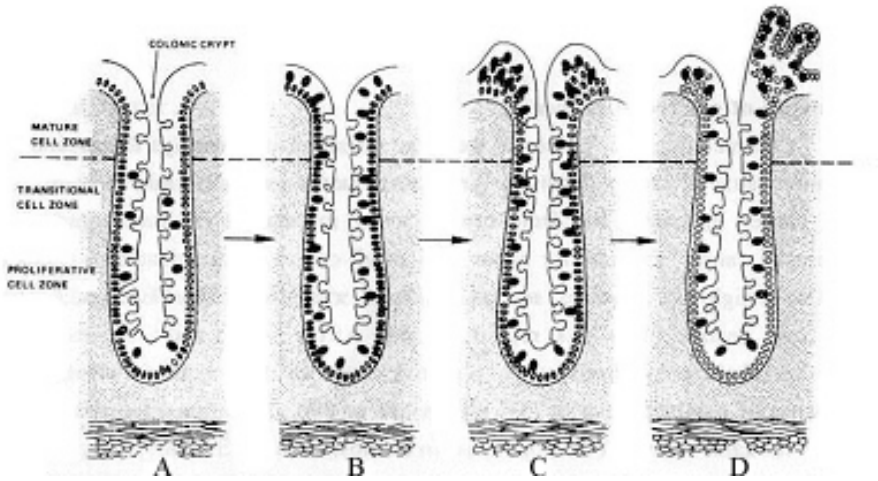

Figure 1.4: The role of cell proliferation in the development of colorectal cancer according to Lipkin (24). A) Normal colonic crypts. B) Proliferative zone expands to higher one-third of the crypt. C) The zone of maximal proliferative activity shifts to the top of the crypt. Cells accumulate in the mucosa. D) Adenomas appear

Measurement of cell proliferation is possible by use of certain physiological assays such as 5-bromo-2'-deoxyuridine incorporation (BrdU), ^{3}H-thymidine labeling, proliferating cell nuclear antigen (PCNA), ornithine decarboxylase (ODC) activity, Ki67 and polyamine levels (25). In addition to the measurement of overall colonic cell proliferation, also the displacement of proliferation towards higher sections of the colonic crypts is considered a marker for colon cancer risk in animals and humans (26).
Dolichos biflorus agglutinin (DBA) binding, which is a marker of differentiation, has been shown to have a great prognostic value for detecting dietary effects on tumor incidence. Lectins, such as DBA are glycoproteins that can bind to specific carbohydrate residues. DBA, for example, has a carbohydrate specificity for α-N-acetylgalactosamine. This carbohydrate residue is thought to increase with normal differentiation of colonic epithelial cells *in vivo*; the more differentiated colonic epithelial cells are, the greater the DBA binding is (27). A significant reduction in DBA labeling was found in microscopic polyps of patients with familial adenomatous polyposis and in rectal biopsies obtained from patients at risk for hereditary non-polyposis colon cancer (27).

Cell death can occur by two distinct mechanisms, necrosis or apoptosis. Necrosis ("accidental" cell death) is the pathological process, which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis on the other hand is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes such as colonic crypt cell homeostasis.
In addition, certain chemical or dietary compounds are said to be cytotoxic to cells, what means, that they can cause their death. Cytotoxicity is thus the cell-killing property of a compound (such as food, cosmetic, or pharmaceutical). In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism. Most current assays for measuring cytotoxicity are based on alterations of membrane permeability and the consequent release (leakage) of components into the supernatant (28). An example of such a cell leakage assay is the lactate dehydrogenase (LDH) assay. LDH is a cytoplasmic enzyme present in all cells, which is rapidly released into the cell culture supernatant when the cell membrane is damaged. Another example is alkaline phosphatase (ALP). ALP is an enzyme that is located on the apical membrane of colonic epithelial cells. After disruption of the epithelial cell membrane, ALP will be excreted into the lumen and can be measured in the faeces as a marker of colonic epithelial cell lysis (29).

1.4.2 Gap junctional intercellular communication

Gap junctional intercellular communication (GJIC) is also involved in cellular homeostasis via regulation of cellular proliferation, differentiation and apoptosis via gap junctions. A gap junction is a junctional complex, which is formed between adjacent cells and consists of aggregated channels that directly link the interiors of neighboring cells (30). Each gap junction channel is comprised of two hemi channels or connexons and each connexon is formed by the aggregation of six protein subunits known as connexins (see Figure 1.5). The gap junction channel-forming connexins comprise a multi-gene family with at least 13 different mammalian connexins discovered so far (30). Connexins are expressed in a cell-, tissue-, and development-specific manner and the number associated with each connexin indicates its molecular mass. Gap junction channels have a diameter of approximately 1.5-2 nm depending upon the type of junction-forming protein and are large enough to permit the direct diffusion of small (< 2000 Da) molecules between cells (30). Substances that are small enough to move between cells through gap junction channels include ions, water, sugars, nucleotides, amino acids, fatty acids, small peptides, drugs and carcinogens. Channel passage does not require ATP and appears to result from passive diffusion (30). In addition to regulation of cell growth,

many other physiological roles have been proposed for GJIC, for example homeostasis of nutrients, ions and fluids between cells; electrical coupling; tissue response to hormones and regulation of embryonic development.
Once formed, gap junction channels can open and close and this 'gating' is controlled by several mechanisms including connexin phosphorylation. Other mechanisms regulating channel gating include intracellular levels of hydrogen and calcium ions, transjunctional voltage and free radicals (30). Decreased pH or pCa induce channel closure in a cell- and connexin-specific manner. Excessive intracellular levels of free radicals can also decrease gap junction channel permeability. The radicals may directly attack connexins or other plasma membrane components (e.g. fatty acids) or may induce changes in

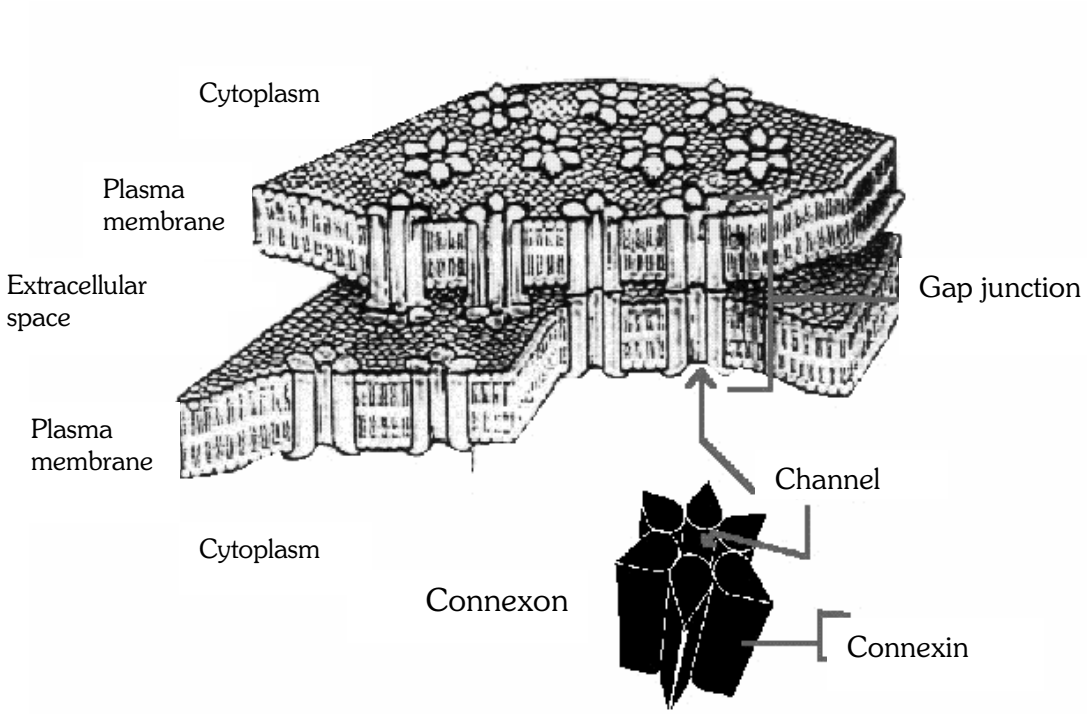

Figure 1.5: Schematic view of gap junctions, connexons and connexins (31)

intracellular calcium levels or redox status. GJIC can be seen as another intermediate biomarker of colorectal carcinogenesis because cancer cells, which do not contact inhibit, do not have growth control, do not terminally differentiate and usually have an abnormal apoptosis response, do not appear to have functional GJIC (30). The vast majority of tumor cells have fewer and smaller gap junctions, express less connexins and have

reduced GJIC compared to their non-neoplastic counterparts (30). Moreover, tumor promoters, such as dietary fat and TPA have been shown to inhibit GJIC (30,32,33).

1.5 *In vitro* effects of n-6 and n-3 PUFAs on human colorectal carcinoma cell lines

Several *in vitro* studies have been carried out to investigate the effects of essential fatty acids on various cells in culture (13,18). Intermediate biomarkers of carcinogenesis, which are mostly determined *in vitro* are cell proliferation, apoptosis and GJIC. So far, no studies have been performed on the differential effects of n-6 and n-3 PUFAs on GJIC in colon cell lines. Recent studies that focused on the effects of individual n-6 and n-3 PUFAs on cell proliferation and apoptosis in human colorectal carcinoma cell lines are summarized in Table 1.1. From the responses of the different cell lines, it can be concluded that there is no obvious differential effect between n-6 and n-3 PUFAs on colon cancer cell lines. In most studies, LA (18:2n-6) and ALA (18:3n-3) showed no effect on cell proliferation (34,35,36,37). Other polyunsaturated fatty acids with more double bonds, such as AA (20:4n-6), EPA (20:5n-3) and DHA (22:6n-3) caused an overall decrease in cell proliferation (37,38,39,40,41) or increase in apoptosis (39). These fatty acids appear to act directly because indomethacin (IM), an inhibitor of prostaglandin synthesis, did not modify these effects. The decrease in cell proliferation and increase in apoptosis were however highly related to lipid peroxidation, as antioxidants such as vitamin E (38), BHT (35) and β-carotene (41) diminished the effects.

To better compare the differential effects between n-6 and n-3 PUFAs, the effects of fatty acids with comparable chain lengths and double bonds such as LA versus ALA and AA versus EPA, should be determined together in one colon cell line. Only Awad *et al*. (34), Tsai *et al.* (37) and Chen *et al.* (35) performed these kinds of experiments. This limited number of studies also reveals that there is no differential effect between LA (18:2n-6) and ALA (18:3n-3) and between the more polyunsaturated fatty acids AA (20:4n-6) and EPA (20:5n-3).

Table 1.1 a: Effects of n-6 and n-3 PUFAs on human colorectal carcinoma cell lines as shown by some recent studies

References	Colon Cell Lines	Fatty Acids	Concentration	Exposure Time	Parameters	Response
Mengeaud *et al.* 1995	HT-29 HRT-18 Caco-2	GLA EPA	0-100 μg/ml	48 hours	Cell proliferation	GLA, EPA: decrease (HRT18>HT29>Caco-2)
					Lipid peroxidation	GLA, EPA: increase in MDA. Vitamin E reduced MDA, IM did not
Awad *et al.* 1995	HT-29 LS174T	LA ALA	30 μM	0-9 days	Cell proliferation	HT-29: LA, ALA: better growth than 18:0, no difference between LA and ALA LS174T: no effect on growth
Tsai *et al.* 1998	SIC oncogene transformant cell line	LA GLA AA ALA EPA DHA	1-50 μg/ml	48 hours	Cell proliferation	AA: no effect till 20 μg/ml, inhibitory at higher concentrations EPA, DHA: 5-50 μg/ml inhibitory LA, ALA, GLA: no significant inhibition
Clarke *et al.* 1999	HT-29	EPA	0-15 μg/ml	0-8 days	Cell proliferation	EPA: reduction of adherent cells and increase in floating cells
					Apoptosis	EPA: increase in floating cells

Table 1.1 b: Effects of n-6 and n-3 PUFAs on human colorectal carcinoma cell lines as shown by some recent studies

References	Colon Cell Lines	Fatty Acids	Concentration	Exposure Time	Parameters	Response
Chen and Istfan, 2000	HT-29	LA ALA EPA AA DHA	0-200 μM	24 hours	Cell number Apoptosis	LA, ALA: no effect AA, EPA: no significant decrease DHA: inhibition DHA: induction BHT reversed apoptosis, IM did not
Collett *et al*. 2001	YAMC-*ras*	DHA LA	50 μM	72 hours	Cell proliferation Cell viability Apoptosis Ras membrane localization	LA: no effect DHA: inhibition No effect No effect No effect on total Ras protein LA increased Ras membrane-to-cytosol ratio
Kim *et al.* 2000	Caco-2	LA EPA DHA	100 μM	0-14 days	Cell proliferation	LA: increase till day 14 EPA, DHA: increase till day 4, decrease day 4-14
Palozza *et al.* 2000	WiDr	EPA	0-25-50-100-250 μM	12 and 24 hours	Cell proliferation Lipid peroxidation	EPA: dose-dependently decrease β-carotene diminished EPA-induced MDA release

1.6 Effects of n-6 and n-3 PUFAs in animal models of colorectal carcinogenesis

Many *in vivo* studies have been performed to evaluate the effects of n-6 and n-3 PUFAs on colorectal carcinogenesis. In a review of 1992, Reddy (42) concluded that the chemically-induced colon tumor incidence was increased in rats fed diets containing 23% corn oil as compared to those fed 5% corn oil diets. Diets containing high levels of dietary fish oil (FO) inhibited the colon tumor incidence, compared to high fat corn oil diets, during both the initiation and postinitiation phases of colorectal carcinogenesis. This was mediated by an effect of FO on ornithine decarboxylase activity (ODC), colonic secondary bile acids and/or prostaglandin synthesis. In the present paragraph the recent state of the art knowledge of n-6 and n-3 PUFAs on colorectal carcinogenesis in animal models will be described.

In Table 1.2 several recent experimental animal studies on the effects of n-6 and n-3 PUFAs on colorectal carcinogenesis are summarized. From these studies it became indeed clear that corn oil (CO) enhances colon tumorigenesis during the postinitiation phase, whereas FO inhibits colon tumorigenesis during both the initiation and postinitiation phases of colorectal carcinogenesis (9). Moreover, Singh *et al.* (43) revealed that animals fed a high fat fish oil (HFFO) diet have a lower colon tumor incidence and multiplicity compared to those fed high fat corn oil (HFCO) or low fat corn oil (LFCO) diets. Not only FO with a high amount of EPA and DHA but also perilla oil (44) with high levels of ALA and individual n-3 fatty acids such as DHA (45,46) can inhibit colorectal carcinogenesis.

Reddy and Sugie (8) performed a study to investigate the modulating effects of varying levels of n-6 and n-3 fatty acids during the promotional phase of colon carcinogenesis in order to determine the optimum dietary levels of these fatty acids that elicit maximum inhibition of colon tumors. Inhibition of colon tumor incidence by decreasing the level of dietary corn oil in the high fat diets or increasing the ratio of n-3 to n-6 fatty acids in the diet was however not dose-dependent. A 23.5% high fat diet with only 5.9% Menhaden fish oil and 17.6% corn oil had no enhancing effect on the incidence of total colon tumors and colon adenocarcinomas as compared to a low fat 5% corn oil diet, whereas the incidence of total colon tumors and adenocarcinomas was increased in animals fed a high fat diet containing only 23.5% corn oil compared to a low fat 5% corn oil diet. These results indicate that a high fat intake is a necessary but not a sufficient condition for colon tumor promotion, and that the relative proportions of n-3 and n-6 fatty acids in the diet are determinants of the high fat effects (8).

Besides chemically-induced colon tumors, effects of n-6 and n-3 PUFAs have been investigated on the development of chemically-induced aberrant crypt foci (ACF) and in

transgenic mice. Oshima *et al.* (46) have investigated the effects of DHA on mouse intestinal polyposis using Apc gene knockout mice. Dietary DHA decreased tumor number in female, but not in male mice. This was the first study that demonstrated that DHA also inhibits intestinal polyposis induced by an Apc mutation. According to the authors, this may open a possibility for chemopreventive intervention of familial adenomatous polyposis (FAP) by dietary supplementation with DHA. In addition, Petrik *et al.* (47) were the first to report that dietary EPA also has anti-tumorigenic properties in the $Apc^{Min/+}$ mice.

ACF have been used as intermediate biomarkers of colon cancer development in animal studies (5). Aberrant crypts are crypts, which appear to be larger, thicker and darker than normal crypts and cluster in aggregates, foci. There are relatively few studies on effects of n-6 and n-3 PUFAs on ACF formation. Those that have been performed show a decrease in total ACF incidence and multiplicity by perilla oil (44), DHA (45) and HFFO (48). These studies only focused on the protecting effect of n-3 PUFAs during the postinitiation phase of colorectal carcinogenesis. However, the experimental design of these studies superimposes effects on initiation such as carcinogen metabolism.

It has been demonstrated that fish oil (FO) reduced azoxymethane (AOM)-induced K-ras mutations and decreased membrane ras expression (49) when given for and after initiation. These results suggest that FO may protect against colon carcinogenesis by either decreasing DNA adduct formation and/or enhancing DNA repair. Hong *et al.* (7) determined the ability of FO and corn oil (CO) to simultaneously modulate O^6-methylguanine DNA adduct formation (DNA damage), removal by O^6-methylguanine-DNA-methyltransferase (repair) and deletion (apoptosis). No main effect of diet on O^6-methylguanine-DNA-methyltransferase was found. However, FO enhanced apoptosis combined with a reduction in adduct formation. This may account, in part, for the observed protective effect of n-3 PUFAs against experimentally-induced colon cancer during the initiation phase (9). The protective effect could also be explained by modulation of biotransformation enzymes related to carcinogen activation, thereby altering the amounts and activities of oxidative (phase I) and conjugative (phase II) xenobiotic metabolizing enzymes (7,42).

Various mechanisms have been postulated to explain the enhancing effect of a high fat corn oil diet and the protecting effect of a high fat fish oil diet during the promotion phase of carcinogenesis. These mechanisms include as stated at the beginning of this paragraph, modulation of colonic mucosal ODC activity, colonic secondary bile acids and/or PGE_2 synthesis. Secondary bile acids can increase ODC activity and cell proliferation and act as tumor promoters (50). It has been shown that arachidonic acid metabolites are involved in increased secondary bile acid production and the induction

of tissue ODC activity (51,52). It is possible that diets rich in n-3 fatty acids result in decreased levels of arachidonic acid and its metabolites and thereby inhibits tissue ODC activity and cell proliferation.

N-3 PUFAs have indeed been reported to inhibit the production of the type-2 series of eicosanoids, including PGE_2, from arachidonic acid (44). Endogenous PGE_2 has been shown to promote rat colon tumors and COX-inhibitors that prevent prostaglandin production such as indomethacin (IM) can block the development of colon carcinomas (53). Therefore, the mechanism responsible for the inhibitory effects of n-3 PUFAs on colorectal tumors may also partly be related to inhibition of PGE_2 synthesis from AA and reduction of the AA levels itself (45). Overexpression of COX-2 has been reported in 90% of colon tumors and premalignant colorectal adenomas. Singh *et al.* (43) observed that n-3 PUFAs inhibit AOM-induced expression of COX-2, whereas n-6 PUFAs enhance levels of AOM-induced COX-2 expression. Also Rao *et al.* (48) found that n-3 PUFAs in a high fat fish oil (HFFO) diet inhibited the levels of COX-2 and AA metabolites (eicosanoids). They suggested that HFFO diets enhance apoptosis via modulation of AA metabolism and inhibition of COX-2 expression, since overexpression of COX-2 in intestinal epithelial cells can result in inhibition of apoptosis (54).

Chang *et al.* (27) investigated whether the protective effect of dietary fish oil during the promotion phase of tumorigenesis is mediated through changes in proliferation, differentiation or apoptosis, all intermediate biomarkers for colon tumor development. *Dolichos biflorus* agglutinin (DBA) binding (marker of differentiation) was higher for fish oil versus corn oil fed animals in both proximal and distal colon. There were also a greater number of apoptotic cells per crypt column in both proximal and distal colon after feeding with fish oil compared with corn oil, as determined by a combination of the TUNEL assay and apoptosis morphological identification. However, changes in cell proliferation (BrdU incorporation) did not explain the beneficial effect of fish oil versus corn oil. Though, Onogi *et al.* (44) found that perilla oil significantly reduced silver-stained argyrophilic nucleolar organizer regions (AgNORs), suggesting that perilla oil decreased the number of cells in S-phase and thus decreased cell proliferation.

Another inconsistent relationship exists between fish oil and farnesyl protein transferase (FPTase) expression. FPTase is an enzyme which catalyses the biological activation of ras proteins. The first step in this process is the transfer of a 15-carbon isoprene, farnesyl, to the cysteine residue of the C-terminal tetrapeptide sequence, -CAAX, of ras precursors, which is catalyzed by FPTase. Farnesylation of ras precursors is a critical step during post-translational modification of ras oncoproteins, thereby enabling their anchorage to the plasma membrane. Singh *et al.* (55) demonstrated that consumption of high amounts of fish oil reduced the levels of FPTase comparing to high corn oil levels, thus inhibiting

post-translational processing of ras precursors resulting in decreased ras functioning. Davidson *et al.* (49) found indeed that expression of ras in the mucosal membrane was 13% lower for animals fed fish oil compared with corn oil feeding. They reported however that perturbation in the farnesylation of ras is not a decisive factor regulating membrane localization during malignant transformation in the colon, because no differences in FPTase activity and prenylation state of ras were found between tumors and uninvolved mucosa.

Overall results from the animal models suggest that n-3 fatty acids protect against colorectal carcinogenesis during both the initiation and promotion phase and that n-6 fatty acids enhance the risk of colorectal carcinogenesis during the promotion phase. The mechanisms behind the molecular, cellular and biochemical effects of these fatty acid families on the complex and multistage process of carcinogenesis are however not clear and cannot easily be explained by one mechanism.

Table 1.2a: Effects of n-6 and n-3 PUFAs in experimental animal models of colorectal carcinogenesis as shown by some recent studies

References	Animals Sex and Age	Control Diet	Experimental Diet	AOM	Experimental Diet Supplementation: (post)initiation	Sacrificed	Parameters	Results
Reddy and Sugie, 1988	F344 rats Male 5 week weanlings	4 weeks modified AIN-76A (5% LFCO)	5%CO 23%CO 1%CO+4%MO 17.6%CO+5.9%MO 11.8%CO+11.8%MO 5.9%CO+17.6%MO	once weekly for 2 weeks	4 days after last injection	38 weeks post AOM treatment	Tumor incidence Multiplicity ODC activity	5.9%MO inhibited tumor incidence Multiplicity was only decreased with 17.6%MO 23.5%CO highest activity
Reddy *et al.* 1991	F344 rats Male 5 week weanlings	Modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	2 weeks before injection, 3 weeks after injection subgroups changed: LFCO→HFCO HFCO→HFFO HFFO→HFCO	36 weeks post AOM treatment	Tumor incidence Multiplicity	HFCO increased incidence + multiplicity during postinitiation period HFFO reduced incidence + multiplicity during the post+ initiation period
Rao and Reddy, 1993	F344 rats Male 5 week weanlings	Modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	after 1 week control diet, 2 weeks before injection	5 days post AOM treatment	ODC activity TPK activity PGE_2, 6-ketoPGF1	HFCO: increase HFFO: reduction HFFO: slightly reduction

Table 1.2b: Effects of n-6 and n-3 PUFAs in experimental animal models of colorectal carcinogenesis as shown by some recent studies

References	Animals Sex and Age	Control Diet	Experimental Diet	AOM	Experimental Diet Supplementation: (post)initiation	Sacrificed	Parameters	Results
Oshima *et al.* 1995	$Apc^{\Delta 716}$ mice Male+ Female 3 weeks	AIN-76A	3% DHA as ethyl ester + AIN-76A		7 weeks	after 7 weeks diet	Polyp number	DHA: no effect in males, decrease in females
Onogi *et al.* 1996	F344 rats Male 5-6 weeks	basal laboratory diet	O12: 12% olive oil S12: 12% safflower oil P12: 12% perilla oil P6O6: 6% perilla oil + 6% olive oil P3O9: 3% perilla oil + 9% olive oil	once weekly for 3 weeks	1 week before first injection	4 weeks after first injection	ACF PGE_2 Cell proliferation C-H-ras Expression	Perilla oil: less ACF (dose-dependant) P12: less PGE_2 reduced by perilla oil reduced by perilla oil
Reddy *et al.* 1996	F344 rats Male Weanlings	2 weeks modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	1 day after last injection	1, 12 and 36 weeks post AOM treatment	Bacterial 7α-dehydroxylase PI-PLC Fecal bile acids	LFCO, HFFO: decrease HFFO: decrease HFFO: increase in primary and decrease in secondary bile acids

Table 1.2c: Effects of n-6 and n-3 PUFAs in experimental animal models of colorectal carcinogenesis as shown by some recent studies

References	Animals Sex and Age	Control Diet	Experimental Diet	AOM	Experimental Diet Supplementation: (post)initiation	Sacrificed	Parameters	Results
Rao *et al.* 1996	F344 rats Male 5 week weanlings	Modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	1 day after last injection	1, 12 and 36 weeks post AOM treatment	PLA2 + PI-PLC activity COX activity	PLA2: no difference PI-PLC: decrease by LFCO, HFFO HFFO: decrease
Chang *et al.* 1997	Sprague-Dawley rats Male 21 days weanlings	Standard rat chow	CO FO	once weekly for 2 weeks	1 week before injection	16 and 34 weeks post AOM treatment	Cell proliferation Cell differentiation Apoptosis Adenocarcinoma incidence	CO: higher FO: higher FO: higher CO: 70.3% FO: 56.1%
Singh *et al.* 1997	F344 rats Male 4 week weanlings	2 weeks modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	1 day after last injection	1, 12 and 36 weeks post AOM treatment	Tumor incidence Multiplicity COX-1 and COX-2 protein expression	LFCO, HFCO, HFFO: 57,76,40% 0.73 ± 0.78, 1.38 ± 1.24, 0.45 ± 0.6 COX-1: no differences COX-2: higher in HFCO

Table 1.2d: Effects of n-6 and n-3 PUFAs in experimental animal models of colorectal carcinogenesis as shown by some recent studies

References	Animals Sex and Age	Control Diet	Experimental Diet	AOM	Experimental Diet Supplementation: (post)initiation	Sacrificed	Parameters	Results
Singh *et al.* 1997	F344 rats Male 4 week weanlings	2 weeks modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	1 day after last injection	1, 12 and 36 weeks post AOM treatment	Tumor incidence	LFCO, HFCO, HFFO: 57,76,40%
							Multiplicity	0.73±0.78, 1.38±1.24, 0.45±0.6
							Ras-p21 protein expression	HFCO: increase ras-p21 HFFO: increased cytosolic ras
Takahashi *et al.* 1997	F344 rats Male 6 weeks	AIN-76A	1 ml DHA as ethyl ester / water 5x a week by gastric incubation	once weekly for 2 weeks	1 day before injection	4, 12 and 36 weeks post AOM treatment	ACF	DHA: at 4 and 12 weeks; 76 and 62% of control values
							PGE_2, PUFAs	DHA: lower PGE_2 + AA
							Cancer incidence	No difference
							Multiplicity	AOM+water: 3.65±2.18 AOM+DHA: 2.41±1.58

Table 1.2e: Effects of n-6 and n-3 PUFAs in experimental animal models of colorectal carcinogenesis as shown by some recent studies

References	Animals Sex and Age	Control Diet	Experimental Diet	AOM	Experimental Diet Supplementation: (post)initiation	Sacrificed	Parameters	Results
Singh *et al.* 1998	F344 rats Male 4 week weanlings	2 weeks modified AIN-76A (LFCO)	LFCO HFCO HFFO	once weekly for 2 weeks	1 day after last injection	1, 12 and 36 weeks post AOM treatment	Tumor incidence	LFCO, HFCO, HFFO: 57,76,40%
							Multiplicity	0.73±0.78, 1.38±1.24, 0.45±0.6
							FPTase protein expression	HFFO: reduced
Rao *et al.* 2001	F344 rats Male 5 week weanlings	Modified AIN-76A (LFCO)	LFCO HFML HFFO	once weekly for 2 weeks	one day after last injection	8, 23 and 38 weeks post AOM treatment	ACF	2 to 3 fold increase in multiplicity ($\geq$ 4) for HFML
							Tumor number	HFML: higher incidence and multiplicity
							Apoptosis	HFML: less
							COX-2 activity	HFML: increase
							COX-2 protein	HFML: increase

1.7 Possible mechanisms of the (anti)-carcinogenic effects of n-3 and n-6 PUFAs on colorectal carcinogenesis; the role of PGE_2 and oxidative stress

PUFAs (n-6 and n-3) are released from phospholipids in the cell membrane by phospholipase A2 and can be further metabolized via cyclooxygenase (COX) to form prostaglandins (PG). 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 (56).

Prostaglandins act in an autocrine or paracrine fashion at nanomolar levels via binding to G-protein-coupled nuclear or cell-surface receptors (57). Although eight prostanoid receptor subtypes have been identified, four of them (the EP receptors EP1, EP2, EP3 and EP4) are specific for PGE_2 signaling (56,58). Activation of the EP-receptors by PGE_2 leads to changes in intracellular cAMP or calcium. These changes serve as second messengers that can activate signaling mechanisms that have profound effects on transcription factor function and attribute to changes in gene expression (see Figure 1.6).

A few reports have documented a pro-carcinogenic effect of PGE_2 in cultured colorectal cancer cells. Sheng *et al.* (59) reported that PGE_2 might stimulate an increase in proliferation and motility of colorectal carcinoma cells via activation of the PI3/Akt pathway, an effect likely due to activation of the PGE_2 receptor subtype EP4. Increased levels of PGE_2 have been found in colon tumors (60) and moreover overexpression of COX-2 has been reported in 90% of colon tumors and premalignant colorectal adenomas (61).

The n-6 fatty acid arachidonic acid (AA; 20:4n-6) is the precursor of the 2-series of prostaglandins, including PGE_2. The n-3 fatty acid eicosapentaenoic acid (EPA; 20:5n-3) can compete with AA, both at the level of incorporation into the cell membrane phospholipids as well as at the level of substrate for the COX pathway generating the 3-series of prostaglandins, including PGE_3. Although similar in structure and stability, PGE_2 is thought to be more mitogenic compared with PGE_3. It is hypothesized that AA could enhance colorectal carcinogenesis via increased production of PGE_2. The mechanism responsible for the inhibitory effects of n-3 PUFAs such as EPA on colorectal tumors may partly be related to inhibition of PGE_2 synthesis from AA. However, the exact role of PGE_2 in the colon cancer modulating effects of AA and EPA is not known. Moreover, studies directly comparing the effects of AA and EPA on colon tumor cell growth have not been reported.

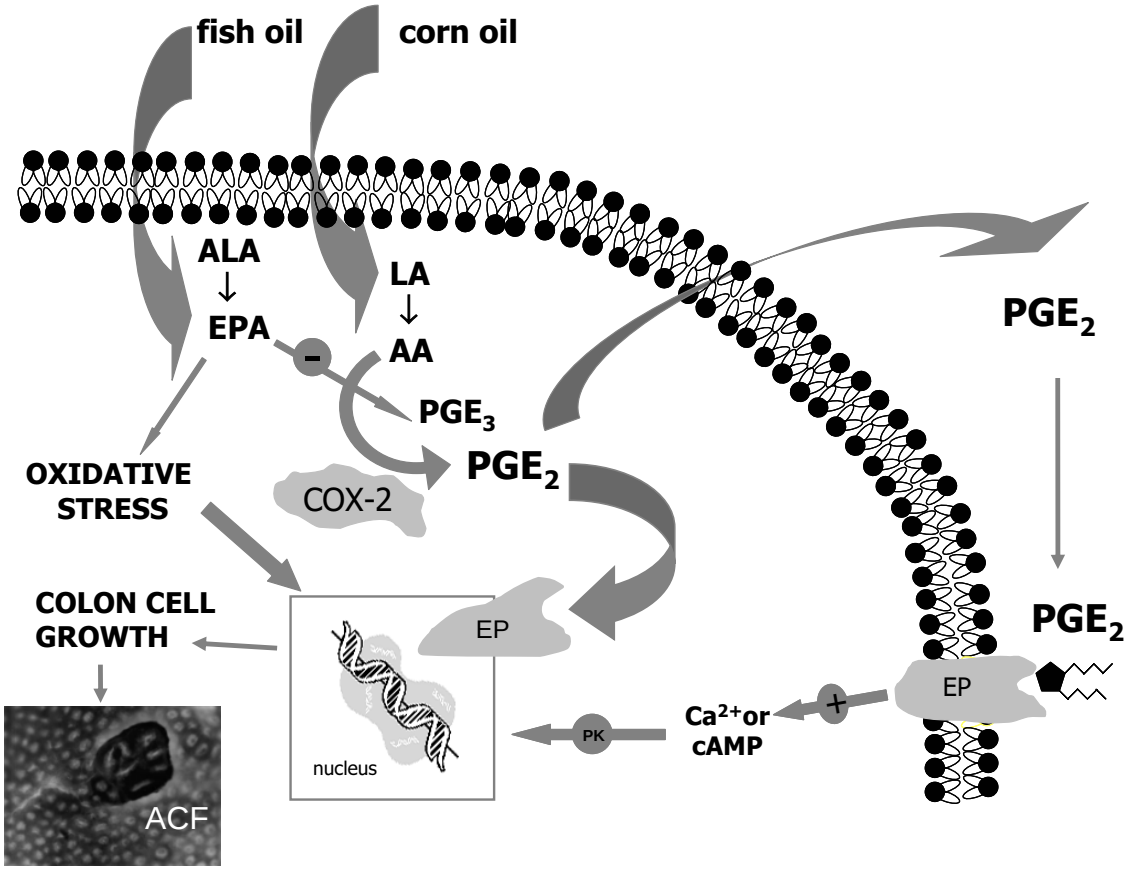

Figure 1.6: Prostaglandin and oxidative stress related hypothesis regarding the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs

In addition to the place of the double bond in PUFAs, which determines the type of prostaglandin formation, also the number of double bonds in the carbon atom chain of PUFAs may play an important role in the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs. The number of double bonds will affect the susceptibility to lipid peroxidation. Increased lipid peroxidation can lead to oxidative stress, which is an imbalance between the production of oxidizing molecular species (oxidants) and the presence of cellular antioxidants (62). This imbalance can lead to potential cell damage, including oxidative DNA damage, that can lead to mutations and eventually tumors (63).

More recently newer concepts about oxidative stress have gained broad acceptance. These concepts imply that oxidative stress in a cell not only modifies target macromolecules but also alerts a number of 'redox sensors' that can alter cell signaling which ultimately leads to specific gene activation.

Lipid peroxidation may, in this way, also play a role in the modulation of colonic cell growth and cell death. The intestinal mucosa is constantly challenged by diet-derived oxidants as well as by endogenously generated reactive oxygen species (ROS). To preserve cellular integrity and tissue homeostasis, the intestine possesses several defense mechanisms such as the ability to maintain high antioxidant concentrations (glutathione, vitamin E and vitamin C), to upregulate antioxidant enzyme systems (glutathione peroxidase, glutathione reductase and superoxide dismutase) and to induce cell death by apoptosis to dispose of damaged enterocytes (21).

Aw (21) proposed an interesting working hypothesis of lipid peroxide-induced cell proliferation and apoptosis in the intestine (see Figure 1.7). Low levels of oxidative stress are required for normal tissue homeostasis (e.g. is involved in cell proliferation). Intermediate levels of oxidative stress might cause selective gene activation and protein expression, which can be considered as an adaptive response to this increased oxidative stress (e.g. antioxidants, stress proteins). If the oxidant dose is sufficiently large enough, the cells may be pushed beyond the maximal point of cell division into the apoptotic phase. At high cytotoxic levels of oxidative stress cells can die by necrosis (21,62).

Several lines of evidence suggest that high fat fish oil (HFFO) diets with a high amount of n-3 PUFAs can enhance apoptosis in colonic epithelial cells (27). Apoptosis is an essential component of cell number regulation in the colonic epithelium and provides a crucial protective mechanism against colon carcinogenesis by removing damaged or mutated cells from the colonic epithelium before they can undergo clonal expansion (64). There is also increasing evidence that both reactive oxygen species and the intracellular redox potential play a role in the regulation of apoptosis (65,66,67). The oxidative stress related mechanism might thus also account for the anti-colon carcinogenic effects of HFFO diets with a high amount of EPA (20:5n-3), because of the higher susceptibility of HFFO diets to lipid peroxidation compared to high fat corn oil (HFCO) diets with high amounts of linoleic acid (LA; 18:2n-6) (see Figure 1.6).

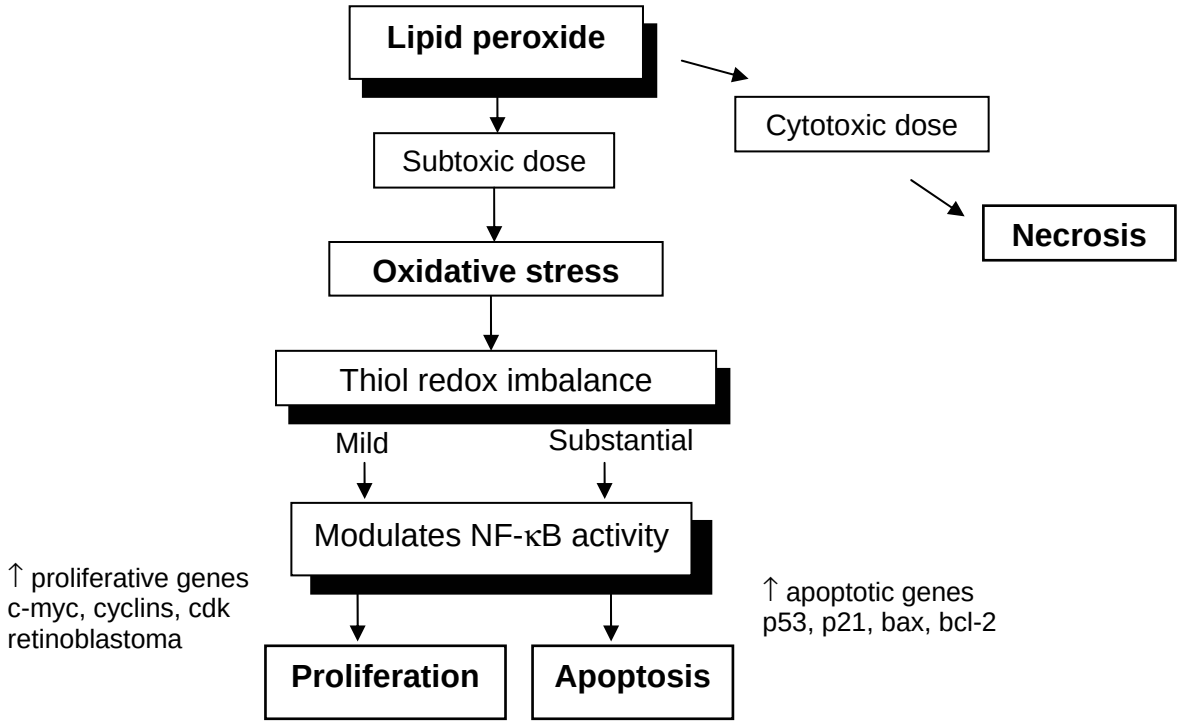

Figure 1.7: Working hypothesis of Aw (21) of lipid peroxide-induced cell proliferation or apoptosis in the intestine

1.8 Aim, approach and outline of the thesis

Previous studies (as summarized in this introduction) led us to suggest that both modulation of COX-mediated PGE_2 signaling as well as lipid peroxidation-induced oxidative stress may play a role in the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs. The exact mechanism by which n-3 PUFAs prevent or inhibit colorectal carcinogenesis compared with n-6 PUFAs is however currently poorly understood. The aim of this thesis therefore is to get more insight into the cellular, biochemical and molecular mechanisms of the adverse and beneficial effects of n-6 and n-3 PUFAs on colorectal carcinogenesis, with special emphasis on PGE_2 and the oxidative stress response.

Most experimental animal studies focused on the enhancing effect of high fat corn oil diets with high amounts of linoleic acid (LA; 18:2n-6) and the protecting effect of high fat fish oil diets with high amounts of eicosapentaenoic acid (EPA; 20:5n-3) during the promotion phase of colorectal carcinogenesis. Little attention has been paid to the chemopreventive effects of n-3 PUFAs during the initiation phase of colorectal

carcinogenesis, such as carcinogen metabolism and initiation of aberrant crypt foci (ACF). Therefore we studied the differential effects of n-3 and n-6 PUFAs and the role of PGE_2 and oxidative stress in the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs in different stages (tumor, ACF, normal) of colorectal carcinogenesis:

- human colon adenocarcinoma cells (*in vitro*)
- AOM-induced aberrant crypt foci (ACF; precursor lesions of colon cancer) in rat
- normal rat colonic epithelium

Different intermediate biomarkers of colorectal carcinogenesis such as GJIC, cellular proliferation, cellular differentiation and cell death or cytotoxicity may be involved in the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs. The role of GJIC in the carcinogenic and anticarcinogenic effects of n-6 and n-3 PUFAs during spontaneous proliferation and differentiation of the human colon adenocarcinoma cell line Caco-2 is described in chapter 2. Chapter 3 describes the role of enzymatic and spontaneous lipid peroxidation and the role of PGE_2 in n-6 and n-3 PUFA-mediated effects on cellular proliferation and cell death. The experiments described in this chapter have been performed *in vitro* in human colon adenocarcinoma cell lines.
Chapter 4 deals with an animal experiment that has been carried out to investigate the role of lipid peroxidation and PGE_2 in high fat fish oil (HFFO) and high fat corn oil (HFCO)-mediated effects on rat colon carcinogenesis. Because so far little attention has been paid to the chemopreventive effects of n-3 PUFAs, differential effects of HFFO and HFCO diets on AOM-induced aberrant crypt foci (ACF), precursor lesions of colon cancer were studied. In order to investigate the modulating effects of these diets on the initiation of ACF, differential effects on metabolic activation and detoxification of AOM were also analyzed in this chapter.
In chapter 5 and 6 transcriptomics experiments are described that were carried out to get more insight into the molecular mechanisms behind the physiological effects described in this thesis and to provide new mechanistic insights underlying the differential effects of n-6 and n-3 PUFAs on colorectal carcinogenesis (chapter 5) and tumor cell growth (chapter 6). In chapter 7, main findings are summarized and discussed.

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Chapter 2

Effects of n-6 and n-3 polyunsaturated fatty acids on gap junctional intercellular communication during spontaneous differentiation of the human colon adenocarcinoma cell line Caco-2

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Yvonne E.M. Dommels[1,2]

Gerrit M. Alink[2]

Jozef P.H. Linssen[3]

Ben van Ommen[4]

[1] WUR/TNO Centre for Food Toxicology

[2] Division of Toxicology, Wageningen University, Wageningen, The Netherlands

[3] Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands

[4]TNO Nutrition and Food Research, Department of Biomolecular Sciences, Zeist, The Netherlands

Abstract

Gap junctional intercellular communication (GJIC) which modulates cell growth and differentiation may play an important role in tumor growth. Cancer cells have dysfunctional GJIC, but it is not known whether GJIC is mechanistically involved in the carcinogenic and anticarcinogenic effects of n-6 and n-3 polyunsaturated fatty acids (PUFAs) on colon tumor cells. Caco-2 cells were used as an *in vitro* model to study the effects of PUFAs on differentiated as well as undifferentiated human colon cells. The GJIC capacity of this cell line increased during spontaneous differentiation. However, no differential effects between n-6 and n-3 PUFAs on GJIC were observed. Short-term incubation with linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) did not influence GJIC, while long-term incubation (> 10 days) with linoleic acid and α-linolenic acid inhibited GJIC of these colon cells. Long-chain metabolites such as AA and EPA were not formed after incubation with linoleic and α-linolenic acid, thus excluding the involvement of prostaglandins in the observed effects. Although the exact mechanism of GJIC inhibition is unclear, cytotoxicity probably mediated by lipid peroxidation products seems to be related because incubation with more polyunsaturated fatty acids (AA and EPA) completely abolished GJIC.

Introduction

Human colon adenocarcinoma Caco-2 cells spontaneously differentiate into polarized intestinal epithelial cells during approximately three weeks of cell culture. Characteristic markers of differentiation, including brush border membrane alkaline phosphatase (ALP) activity and dome formation in cell monolayers, increase after cells reach confluency (1). Hara *et al. (2)* proposed that the Caco-2 cell line could be used as an *in vitro* model to study molecular alterations during spontaneous differentiation of colon cancer cells. This can provide more insight into the multistep process from adenoma to carcinoma in the colon (i.e. de-differentiation). Hara *et al.* (2) found that Caco-2 cells spontaneously switch from a proliferative state with a high c-myc oncogene expression to a differentiated phenotype with a low c-myc expression. We determined proteome changes during Caco-2 differentiation to identify novel proteins involved in differentiation and possibly colon carcinogenesis (R.H. Stierum *et al*. in press).

Gap junctional intercellular communication (GJIC) is important in the control of colonic cell proliferation and differentiation. Low molecular weight growth regulatory molecules stay in equilibrium among GJIC-connected cells (3). Because GJIC is considered to play an essential role in homeostasis maintenance, it is assumed that its disruption will result in a variety of diseases. Several studies have shown that aberrant GJIC is often found in tumors and that many tumor-promoting agents inhibit GJIC (4).

Accumulating evidence exists that a high dietary fat intake is associated with an increased risk of colorectal cancer. Moreover, epidemiological studies and studies with animal models have demonstrated that not only the amount but also the type of fat consumed is important (5). Polyunsaturated fatty acids can be divided into two families of essential fatty acids, namely the n-6 and n-3 PUFAs. N-6 PUFAs in corn oil, such as linoleic acid, enhance colon tumorigenesis in rodents, whereas n-3 PUFAs like eicosapentaenoic acid, present in fish oil, can reduce colorectal carcinogenesis (6,7). Several related mechanisms have been proposed for the effects of dietary n-6 and n-3 PUFAs on colorectal carcinogenesis, such as differences in prostaglandin production, COX-2 expression, cell proliferation, apoptosis and bile acid formation (6,8,9). However, the precise mechanisms by which n-6 and n-3 fatty acids exert their carcinogenic and anti-carcinogenic action are unknown (10,11).

PUFAs have been shown to be strong modulators of GJIC (12-15). De Haan *et al.* (12) reported that linoleic acid was the most potent inhibitor of GJIC in human smooth muscle cells. However, it is not known whether GJIC is mechanistically involved in the differential effects of n-6 and n-3 PUFAs on colon tumor cells. Mesnil *et al.* (16) demonstrated the GJIC capacity of a panel of human colorectal adenocarcinoma cell lines isolated from different stages of tumor progression. The aim of our study was to

investigate the effects of two long-chain PUFAs that naturally exist in the colon lumen (17), linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) and two more polyunsaturated fatty acids, arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3), on GJIC of human colon adenocarcinoma Caco-2 cells during proliferation and differentiation.

Materials and methods

Chemicals

Bovine serum albumin (BSA; essential fatty acid-free), Lucifer Yellow, linoleic acid, α-linolenic acid, arachidonic acid and eicosapentaenoic acid were obtained from Sigma Chemical Company (St Louis, MO, USA). Triton X-100 was obtained from Merck AG (Germany). Fetal calf serum (FCS), non-essential amino acids, penicillin-streptomycin, Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS) and Dulbecco's modified eagle medium (DMEM) were obtained from Life Technologies (Breda, The Netherlands).

Cell culture

Caco-2 cells, obtained from the American Type Culture Collection (Rockville, MD, USA) were grown in DMEM, supplemented with 10% heat-inactivated FCS, 1% non-essential amino acids, 2% penicillin-streptomycin, 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). Cells at passages between 32 and 45 were used for the experiments.

Treatment of cells with PUFAs

Short-term exposure

Caco-2 cells were plated at a concentration of 30 x 10^4 cells/ml in 35 mm petridishes in a medium volume of 2 ml. Cells were used for experiments at day 3 or 20 after plating, representing respectively undifferentiated and differentiated cells. Medium was refreshed three times a week. At day 3 or day 20, medium was replaced by serum-free DMEM, supplemented with 1 mg/ml fatty acid-free BSA and the different fatty acids. Cells were cultured in this medium for 24 hours.

For the GJIC experiments linoleic acid, α-linolenic acid, AA and EPA were used at a concentration of 300 μM, while 100 μM linoleic and α-linolenic acid were used for the fatty acid uptake studies. Fatty acids were dissolved in ethanol with a final concentration in the medium of 0.5%. Control wells received serum-free medium supplemented with 1 mg/ml fatty acid-free BSA plus 0.5% ethanol. Before addition to the cultures, the fatty

acids and ethanol were preincubated in the BSA-containing medium for 30 minutes at 37 °C.

Long-term exposure

Caco-2 cells were plated at a concentration of 30 x 10^4 cells/ml in 35 mm petridishes in a medium volume of 2 ml. After 24 hours, the medium was replaced by fresh medium with serum, supplemented with 1 mg/ml fatty acid-free BSA and 100 µM fatty acids. Control wells received medium with serum, supplemented with 1 mg/ml fatty acid-free BSA plus 0.5% ethanol. The incubation medium with the various fatty acids or ethanol was replaced every 72 hours. Cells were grown until 4, 10, 16 and 22 days after seeding.

Gap junctional intercellular communication assay

Gap junctional intercellular communication was determined after injection of a 10% Lucifer Yellow CH solution (in 0.33 M lithium chloride) in about 20 single Caco-2 cells per 35 mm petridish. Cells were microinjected using a vertical injection system (Narishige, Tokyo, Japan) with a dye-filled capillary glass tip (Clark, Pangbourne, UK). The glass capillary tip with a diameter of 1 µm was prepared by an automatic magnetic puller (Narishige, Tokyo, Japan). The number of communicating cells was determined using fluorescence microscopy between 10-15 minutes after the first injection.

Cell growth

During the long-term exposure experiments, Caco-2 cells were washed with 2 ml PBS supplemented with 0.022% EDTA, harvested using trypsin and counted with a hematocytometer (Bürker-Türk, Emergo, Landsmeer, The Netherlands) at day 4, 10 16 and 22 after seeding.

Enzyme and protein assays

Caco-2 cells were washed with 2 ml PBS supplemented with 0.022% EDTA and scraped into 1 ml of PBS. Cell homogenates were sonicated for 1 minute (3 x 20 seconds) in ice-cold PBS. Activity of alkaline phosphatase was determined according to Tietz (18), on the BM/Hitachi 911 using *p*-nitrophenol as a substrate. Protein was measured according to Bradford (19).

Cytotoxicity assay

Caco-2 cells were plated at a concentration of 3.54 x 10^5 cells/ml in 0.5 ml of medium into 24 well tissue culture plates. After treatment the media was collected from each well and centrifuged for 10 minutes at 8000 rpm. Cells were scraped into 500 µl 0.5% Triton

X-100 in 0.1 M potassium phosphate buffer (pH 7.5). After sonification (Vibra Cell, Sonics & Materials Inc, Danbury, USA) of the cells for 1 min (3 x 20 seconds) and centrifugation (10 minutes, 8000 rpm), lactate dehydrogenase activity was measured spectrophotometrically at 340 nm in both medium and cell supernatant, with pyruvate as a substrate (20).

Analysis of fatty acid composition

After 24 hours exposure to 100 µM linoleic acid or α-linolenic acid on day 3 and day 20 after seeding, medium was collected. The cells were washed with an ice-cold solution of 1% BSA in DMEM for 5 minutes to remove extracellular free fatty acids (20). Cell monolayers were then washed twice with 2 ml PBS supplemented with 0.022% EDTA and scraped into 1 ml PBS with a rubber policeman. Cell suspensions were centrifuged for 5 minutes at 400 g. The cell pellet was resuspended in 200 µl PBS and stored under nitrogen at -80 °C until analysis of fatty acid composition. Total cellular lipids were extracted from the cell pellet by the method of Bligh and Dyer (21). Total extracted lipids were methylated according to Morrison and Smith (22). The methyl esters of the fatty acids were analyzed on a Carlo Erba 5160 megaseries gas chromatograph (Interscience, Breda, The Netherlands) using cold-on-column-injection and flame ionization detection (temperature detection 260 °C). Temperature program starts at 80 °C, followed by a temperature increase of 20 °C/min until 180 °C and 5 °C/min until 240 °C with a final hold for 2 minutes. Samples were analyzed on an OMEGAWAX™ 320 fused silica capillary column, 30 m x 0.32 mm; 0.25 µm film thickness (Supelco, Bellefonte, USA). Individual fatty acids were identified by comparison with two standard fatty acid mixtures, PUFA nr. 2 Animal source and PUFA nr. 3 Menhaden oil (Supelco, Bellefonte, USA) and reported as a percentage of the total fatty acids identified.

Statistics

Correlation between ALP, GJIC, cell number and days in culture for control cells were tested by Pearson R correlation coefficients. Statistical differences between group means were determined with one-way analysis of variance. A probability level of $p < 0.05$ was considered significant. The differences in fatty acid composition were analyzed by the Student's t-test.

Results

Gap junctional intercellular communication capacity of Caco-2 cells

The GJIC capacity of Caco-2 cells during spontaneous differentiation is shown in Figure 2.1. At day 4 of cell culture, the microinjected cells communicated with about 9 of their neighboring cells. The Lucifer Yellow dye transfer increased up to about 26 surrounding cells at day 22 of cell culture, which is a significant ($R^2 = 0.86$, $p < 0.001$) increase of intercellular communication during Caco-2 cell differentiation.

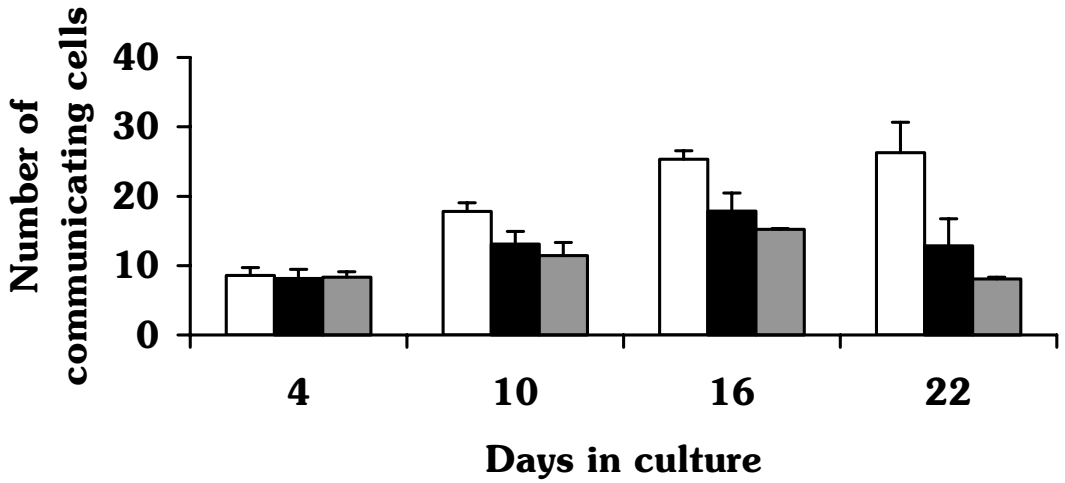

Figure 2.1: Effect of long-term incubation of Caco-2 cells with linoleic acid and α-linolenic acid on GJIC. Caco-2 cells are treated with 0.5% ethanol as a control (white bars), 100 μM linoleic acid (black bars) or 100 μM α-linolenic acid (gray bars) for 4, 10, 16 and 22 days. Results are expressed as means ± SD of one of the representative experiments

Effect of n-6 and n-3 PUFAs on GJIC

Undifferentiated (day 3) and differentiated (day 20) Caco-2 cells were exposed to serum-free medium supplemented with 1 mg/ml BSA and 300 μM linoleic acid, α-linolenic acid, AA or EPA for 24 hours to determine the effect of n-6 and n-3 fatty acids on GJIC in colon cells. In Figure 2.2, it is shown that short-term treatment with all different PUFAs did not influence GJIC of these colon cells. To elucidate the effect of long-term incubation of fatty acids on GJIC of Caco-2 cells during differentiation, GJIC assays were performed after cells were exposed to medium supplemented with serum, 1 mg/ml BSA and 100 μM linoleic acid, α-linolenic acid, AA or EPA for 4, 10, 16 and 22 days. After 4 days of exposure, also no effect on GJIC could be observed. However, linoleic acid and α-linolenic acid both inhibited GJIC ($p < 0.05$) in Caco-2 cells treated for 10, 16 and 22

days (Figure 2.1). Inhibition of GJIC was most evident after 22 days, but there was no differential effect between the n-6 and n-3 PUFAs. AA and EPA inhibited GJIC even more than linoleic acid and α-linolenic acid. GJIC was completely abolished in Caco-2 cells treated for 10 days with AA and EPA and could not be measured anymore (data not shown).

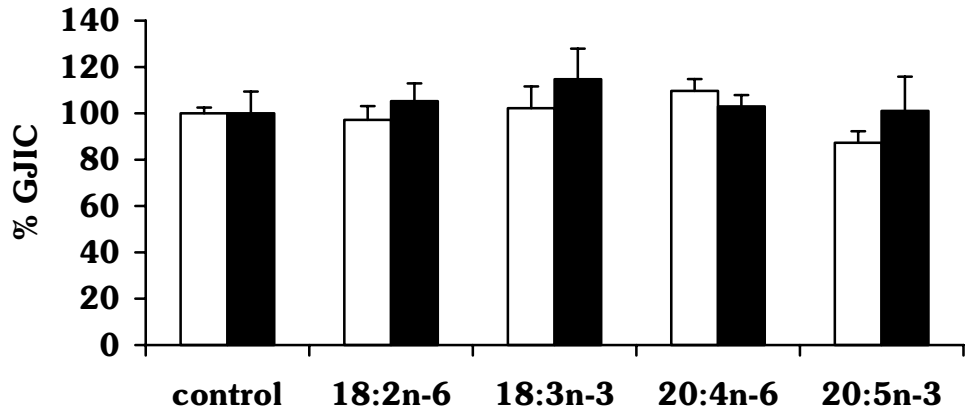

Figure 2.2: Gap junctional intercellular communication capacity of Caco-2 cells after 24 hour treatment with n-3 and n-6 PUFAs. Undifferentiated (day 3, white bars) and differentiated Caco-2 cells (day 20, black bars) are treated for 24 hours with 0.5% ethanol (control), 300 μM linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), AA (20:4n-6) or EPA (20:5n-3). Results are expressed as mean percentage of control (set as 100 %) ± SEM of three independent tests

Effect of n-6 and n-3 PUFAs on proliferation and differentiation of Caco-2 cells

Human colon adenocarcinoma Caco-2 cells reached confluence on the 4th day of cell culture. The cells proliferated up to the 10th day of cell culture (Figure 2.3). Caco-2 cells spontaneously exhibited differentiation phenotypes. Around day 16, large domes (columnar cells) appeared in the cell monolayer. The differentiation marker alkaline phosphatase gradually increased in activity up to the 22nd day of cell culture (Figure 2.4). ALP activity is positively correlated with days in culture ($R^2 = 0.93$, $p < 0.001$).

To determine whether long-term incubation with 100 μM linoleic, α-linolenic acid, AA and EPA also modulated the growth of Caco-2 cells, cell count assays were performed at several days during Caco-2 cell growth. As shown in Figure 2.3, linoleic acid and α-linolenic acid both suppressed cell growth at day 4. This inhibition tended to result in a delayed growth curve of Caco-2 cells. The tendency in growth retardation was accompanied by a decrease in cell differentiation for α-linolenic acid (Figure 2.4). The effects of AA and EPA on cell number were also even more obvious (Figure 2.5). The

abolishment of GJIC by AA and EPA after 10 days in culture was accompanied by a strong inhibition of cell number.

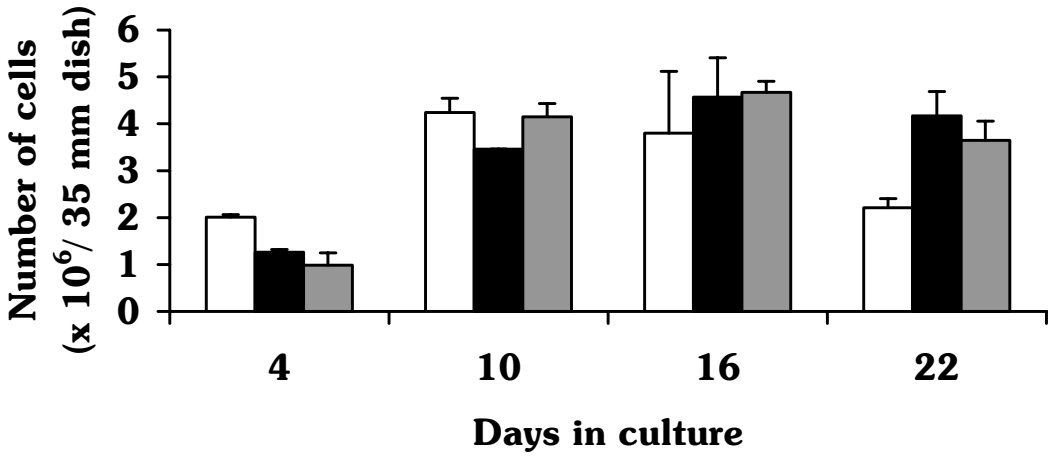

Figure 2.3: Effect of long-term incubation with linoleic acid and α-linolenic acid on cell proliferation of Caco-2 cells. Caco-2 cells are treated with 0.5% ethanol as a control (white bars), 100 μM linoleic acid (black bars) or 100 μM α-linolenic acid (gray bars) for 4, 10, 16 and 22 days. Results are expressed as means ± SD of one of the representative experiments

Determination of cytotoxicity

Release of lactate dehydrogenase in the culture medium was measured as an indicator of cytotoxicity. Both short-term incubation (24 hours) with concentrations up to 300 μM and long-term incubation (4, 10, 16 and 22 days) up to 100 μM linoleic acid and α-linolenic acid showed no increase in LDH release in the medium of Caco-2 cells compared to control wells (data not shown). However, AA and EPA were highly cytotoxic during long-term incubation. Lactate dehydrogenase release in the medium of Caco-2 cells treated for 10 days with 100 μM AA (32.6%) or EPA (20.8%) was significantly higher than lactate dehydrogenase release in control cells (13.7%).

Fatty acid uptake in undifferentiated and differentiated Caco-2 cells

As shown in Table 2.1, treatment with linoleic acid and α-linolenic acid changed the fatty acid composition of Caco-2 cells. Linoleic acid as well as α-linolenic acid increased the levels of both respective fatty acids ($p < 0.05$). However, the uptake of both types of fatty acids was lower in differentiated cells compared to the undifferentiated cells. Incubation with linoleic acid (18:2n-6) as well as α-linolenic acid (18:3n-3) did not

increase elongation and desaturation products such as 18:3n-6, AA (20:4n-6), 18:4n-3 and EPA (20:5n-3).

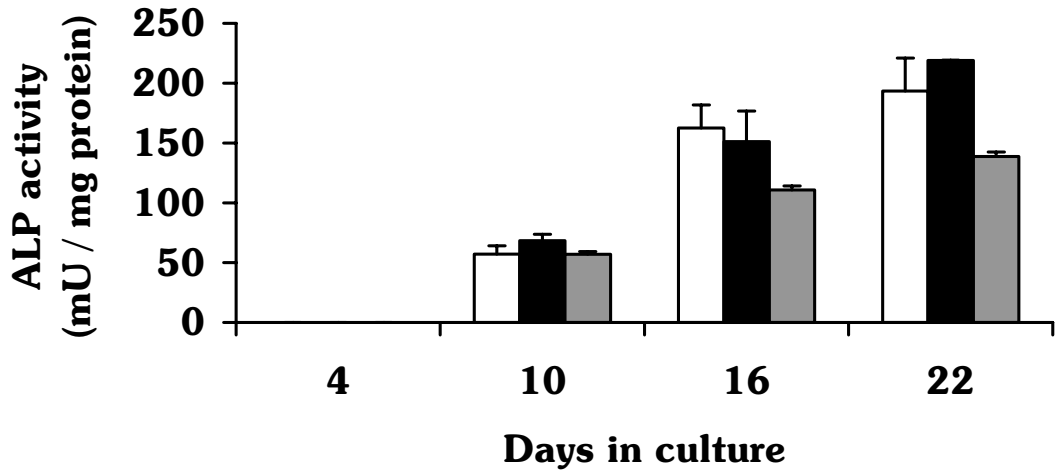

Figure 2.4: Effect of long-term incubation with linoleic acid and α-linolenic acid on differentiation of Caco-2 cells. Caco-2 cells are treated with 0.5% ethanol as a control (white bars), 100 μM linoleic acid (black bars) or 100 μM α-linolenic acid (gray bars) for 4, 10, 16 and 22 days. Differentiation is expressed as activity of alkaline phosphatase (ALP). Results are expressed as means ± SD of one of the representative experiments

Table 2.1: Fatty acid uptake of undifferentiated (U) and differentiated (D) Caco-2 cells. Caco-2 cells were incubated for 24 hours with serum-free DMEM with 1 mg/ml fatty acid-free BSA and 0.5% ethanol (ethanol), 100 μM linoleic acid (18:2n-6) or 100 μM α-linolenic acid (18:3n-3). Data are expressed as means ± SD in % of total fatty acids

	ethanol	ethanol	18:2n-6	18:2n-6	18:3n-3	18:3n-3
	U	D	U	D	U	D
Fatty acids						
18:2n-6	3.40 ± 2.29	0.87 ± 0.16	13.59 ± 0.01	8.32 ± 0.22	1.19 ± 0.15	0.80 ± 0.04
18:3n-6	0.10 ± 0.13	0.01 ± 0.00	0.02 ± 0.02	0.00 ± 0.00	0.05 ± 0.03	0.00 ± 0.00
20:4n-6	1.60 ± 0.05	1.44 ± 0.11	2.08 ± 0.08	1.35 ± 0.04	1.68 ± 0.06	1.21 ± 0.13
18:3n-3	0.41 ± 0.40	0.03 ± 0.01	0.17 ± 0.07	0.04 ± 0.04	11.30 ± 1.64	6.47 ± 0.35
18:4n-3	0.02 ± 0.02	0.05 ± 0.01	0.27 ± 0.38	0.06 ± 0.02	0.22 ± 0.02	0.04 ± 0.01
20:5n-3	1.73 ± 1.48	0.47 ± 0.13	1.45 ± 0.34	0.50 ± 0.02	1.00 ± 0.18	0.42 ± 0.13

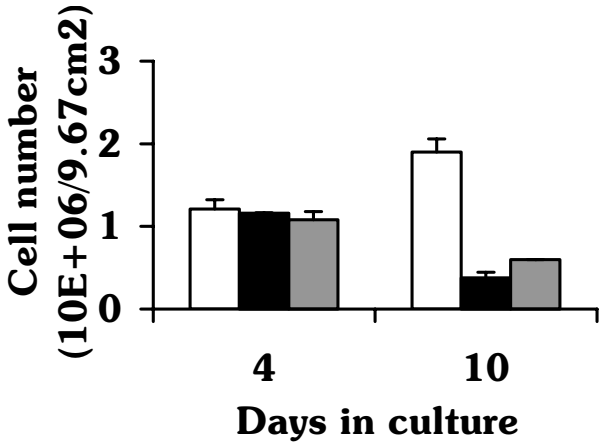

Figure 2.5: Effect of long-term incubation with arachidonic acid and eicosapentaenoic acid on cell proliferation of Caco-2 cells. Caco-2 cells are treated with 0.5% ethanol as a control (white bars), 100 µM arachidonic acid (black bars) or 100 µM eicosapentaenoic acid (gray bars) for 4 and 10 days. Results are expressed as means $\pm$ SD of one of the representative experiments

Discussion

Caco-2 colon cells were used as an *in vitro* model to study the effects of PUFAs on GJIC during spontaneous proliferation and differentiation. The degree of Caco-2 differentiation, as measured by expression of ALP activity was in the same order of magnitude as in the literature (2,23). Mesnil *et al*. (16) reported the low GJIC capacity of human colon adenocarcinoma cell lines, including Caco-2 cells. Our results show for the first time that the GJIC capacity of Caco-2 cells changed during spontaneous differentiation: an increase from about 9 communicating cells at confluence (which resembles colon tumor cells) to about 26 communicating cells after 22 days of *in vitro* cell culture (which resembles more 'normal' cells). These results confirm the data of Hara *et al*. (24) which also suggest that the communication ability of Caco-2 cells appear to be correlated with the degree of cellular differentiation. However, Hara *et al*. induced differentiation state of Caco-2 cells by FCS and we investigated the GJIC capacity of spontaneously differentiated Caco-2 cells. Statuto *et al*. (25) reported that restoration of connexin 32 (Cx32)-mediated GJIC capacity of thyroid epithelial cells, by stable expression of Cx32, caused a decrease in cell proliferation and an increase in cellular differentiation. This might indicate that the number of communicating Caco-2 cells increase during differentiation because of an increasing amount of Cx32 mRNA or protein expression, which is present in Caco-2 cells (16).

We examined the effect of short-term as well as long-term exposure of PUFAs on GJIC of Caco-2 cells. After 24 hours of exposure, no effect of linoleic acid, α-linolenic acid, AA and EPA on GJIC in both undifferentiated and differentiated Caco-2 cells was observed. This clearly indicates that PUFAs at a concentration of 300 μM do not affect the short-term modulation of GJIC in Caco-2 cells. In addition, de Haan *et al*. (12) found that both linoleic acid and α-linolenic acid, in the same concentration and exposure time inhibited GJIC in human smooth muscle cells. Also others have demonstrated the ability of α-linolenic acid (15) and AA (26) to down regulate GJIC after short-term incubation. So there appears to be a tissue-specific sensitivity to inhibition of GJIC by n-6 and n-3 PUFAs.

Distinct from our short-term incubation results, we measured an effect of long-term incubation of n-6 and n-3 PUFAs on GJIC. Linoleic acid and α-linolenic acid inhibited GJIC in Caco-2 cells treated for 10, 16 and 22 days. AA and EPA completely abolished GJIC after less than 10 days of incubation. However, no differential effect between n-6 and n-3 PUFAs was observed, thus excluding the possibility that GJIC would be mechanistically involved in the carcinogenic and anticarcinogenic effects of n-6 and n-3 PUFAs on colon tumor cells.

Because Chen and Nilsson (27) and Huang *et al.* (20) found different results in the Δ-6 desaturase ability of Caco-2 cells, we also tested the ability of Caco-2 cells to metabolize linoleic acid and α-linolenic acid into AA and EPA. Our results clearly demonstrated that Caco-2 cells could readily absorb linoleic acid and α-linolenic acid and that no increase in Δ6-desaturation products was observed. This is in agreement with Huang *et al*. (20), who also suggested that Caco-2 cells possess a low Δ6-desaturation activity. Inhibition of GJIC after long-term incubation with linoleic acid and α-linolenic acid is thus caused by these long-chain fatty acids and not by metabolites such as AA, EPA or even prostaglandins.

Hayashi *et al*. (28) found that linoleic acid inhibited GJIC in WB-F344 cells treated for 6 days in a dose-dependent manner. Long-term treatment with linoleic acid caused hyperphosphorylation of Cx43 protein, which correlated with loss of gap junction plaques from the plasma membrane and apparent internalization of Cx43. Cx43 mRNA levels also decreased following long-term incubation. The inhibition of GJIC in Caco-2 cells after long-term exposure to linoleic acid and α-linolenic acid, as observed in this study, can probably also be explained by long-term modulation of gap junctions, such as connexin transcription, phosphorylation and internalization.

Besides an inhibition in GJIC capacity of Caco-2 cells after incubation with linoleic acid and α-linolenic acid, we also noticed a tendency in growth retardation for both fatty acids. Abolishment of GJIC by AA and EPA after 10 days of culture was also

accompanied by a strong inhibition of cell number. Inhibition of GJIC can thus probably better be explained by a general effect on cell growth and differentiation.
Rozenkranz *et al. (29)* observed that inhibitors of GJIC are significantly related to toxicity. Lipid peroxidation products may have played an important role in mediating the inhibitory effects of linoleic acid and α-linolenic acid on GJIC. Lipid peroxidation products of unsaturated fatty acids can cause dysfunction of gap junction structures, thereby reducing GJIC (14). This might also explain why long-term incubation with AA and EPA completely inhibited GJIC of Caco-2 cells. AA and EPA became highly cytotoxic, because these polyunsaturated fatty acids are even more prone to lipid peroxidation. De Haan *et al*. (12) indeed showed that 4-hydroxynonenal, which is a product of lipid peroxidation, inhibited GJIC in a dose-dependant way, also at non-cytotoxic concentrations.
In conclusion, our results show that the GJIC capacity of human colon adenocarcinoma Caco-2 cells increased during spontaneous differentiation. Short-term incubation of linoleic acid, α-linolenic acid, AA and EPA had no effect on GJIC. After 10 to 22 days of treatment, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) inhibited GJIC of Caco-2 cells. More polyunsaturated fatty acids, AA (20:4n-6) and EPA (20:5n-3) completely inhibited GJIC. However, no differential effects between n-6 and n-3 PUFAs were observed. We suggest that inhibition of GJIC was caused by a general cytotoxic effect due to the production of intracellular lipid peroxides.

Acknowledgements
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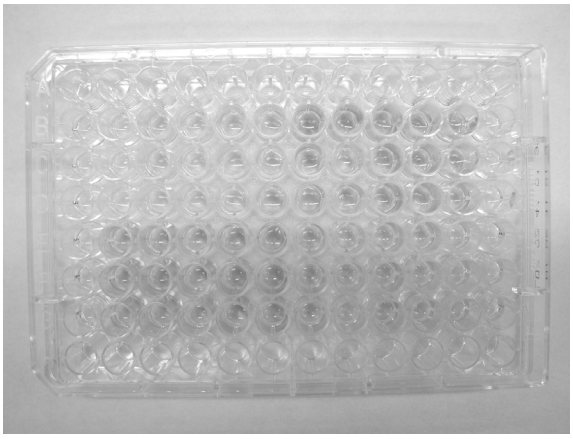
Chapter 3

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

Carcinogenesis, **24(3)**, 385-392, 2003



Yvonne E.M. Dommels[1,2]

Merel M.G. Haring[2]

Nynke G.M. Keestra[2]

Gerrit M. Alink[2]

Peter J. van Bladeren[2,3]

Ben van Ommen[4]

[1]WUR/TNO Centre for Food Toxicology

[2]Division of Toxicology, Wageningen University, Wageningen, The Netherlands

[3]Present address: Nestlé Research Center, Vers-chez-le-blanc, 1000 Lausanne 26, Switzerland

[4]TNO Nutrition and Food Research, Department of Biomolecular Sciences, Zeist, The Netherlands

Abstract

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 comparing to the long chain fatty acids α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6). Neither incubations with PGE_2 nor reductions in PGE_2 synthesis by EPA comparing to AA did lead 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 nonsteroidal 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, comparing to 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 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.

Material and methods

Reagents

Bovine serum albumin (BSA; essential fatty acid-free), linoleic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic acid, prostaglandin E_2, α-tocopherol (vitamin E), indomethacin, trichloroacetic acid, 2-thiobarbituric acid and 1,1,3,3-tetramethoxy-propane (malondialdehyde) were obtained from Sigma Chemical Company (St Louis, MO, USA). 16,16-dimethyl PGE_2 was purchased from Cayman Chemical Company (Ann Arbor, Michigan, 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, England).
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 McCoys 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 x 10^4 cells/ml in 200 µl of medium into 96 well tissue culture plates. The medium was removed after 24 hours 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 hours. The antioxidant vitamin E was also pre-incubated before fatty acid treatment during the first 24 hours 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 minutes 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 hours. 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 x 10^5 cells/ml in 400 µl of medium into 24 well tissue culture plates. After 24 hours, 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 hours after incubation. Prostaglandin E_2 (PGE_2) levels were quantitated using a PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech UK limited, Buckinghamshire, England) according to the high sensitivity EIA 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(tm), version 5.03 for Windows (Perella Scientific Inc., 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. Lactate dehydrogenase 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 x 10^4 cells/ml in 2 ml) and the culture medium, treated for 48 hours 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 α-linolenic acid (ALA; 18:3n-3), linoleic acid (LA; 18:2n-6), eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (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 hours of incubation (Figure 3.1A). The more polyunsaturated fatty acids EPA and AA showed a dose-dependent decrease in cell proliferation after 48 hours of incubation in Caco-2 cells (Figure 3.1A).

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 3.1B).

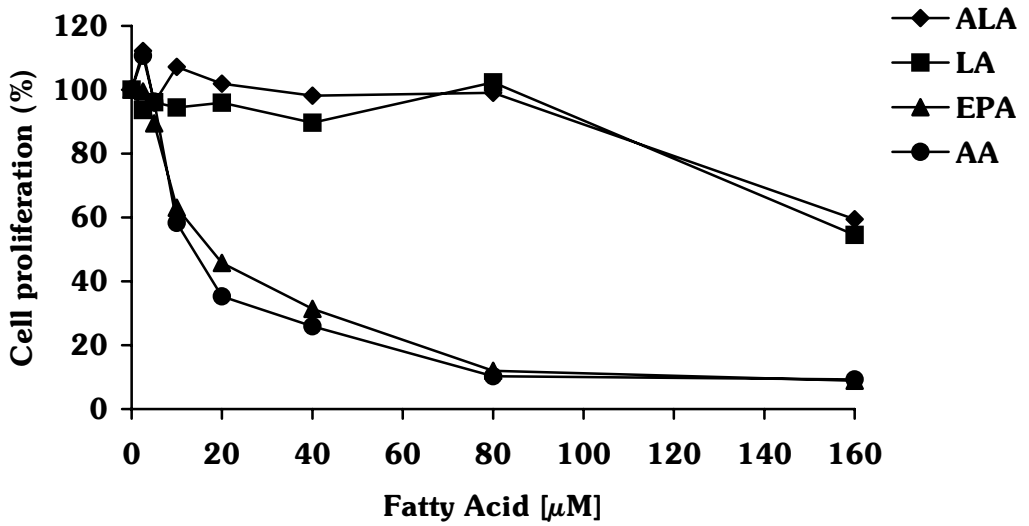


Figure 3.1A: Effects of n-6 and n-3 PUFAs on cell proliferation of human colon adenocarcinoma Caco-2 cells. Cells were treated for 48 hours with α-linolenic acid (ALA; 18:3n-3), linoleic acid (LA; 18:2n-6), eicosapentaenoic acid (EPA; 20:5n-3) or arachidonic acid (AA; 20:4n-6) at 0, 2.5, 5, 10, 20, 40, 80 and 160 μM. Results are the average of 6 replicate determinations of one of the representative experiments. The SEM was < 7%

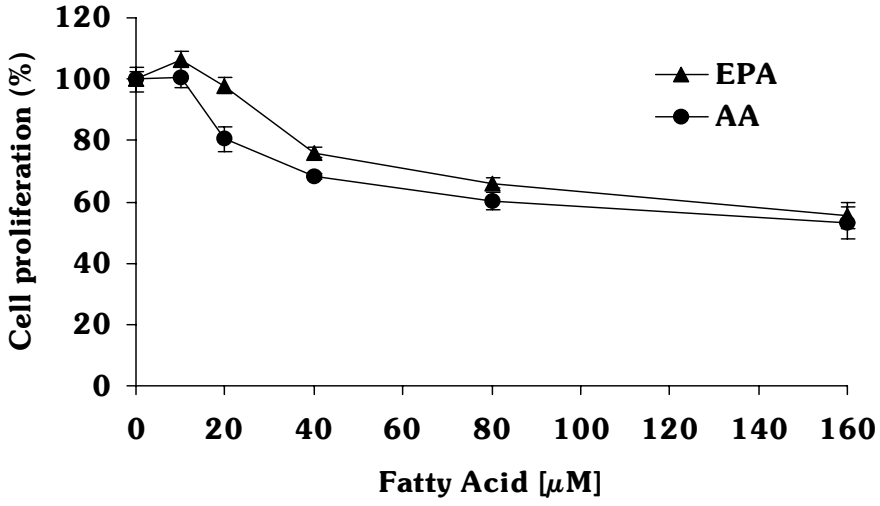


Figure 3.1B: Effects of EPA and AA on cell proliferation of human colon carcinoma HT-29 cells treated with IL-1β. Cells were treated for 48 hours with 10 ng/ml IL-1β and eicosapentaenoic acid (EPA; 20:5n-3) or arachidonic acid (AA; 20:4n-6) at 0, 10, 20, 40, 80 and 160 μM. Results represent the means $\pm$ SEM of 6 replicate determinations of one of the representative experiments

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 3.2 demonstrates clearly that arachidonic acid (AA; 20:4n-6) increased PGE_2 production in the medium of Caco-2 cells after 24 hours of treatment. Basal levels of PGE_2 in Caco-2 cells as well as effects of treatment with linoleic acid (18:2n-6), α-linolenic acid (18:3n-3) or eicosapentaenoic acid (EPA; 20:5n-3) on basal PGE_2 levels could not be detected with the PGE_2 EIA used.

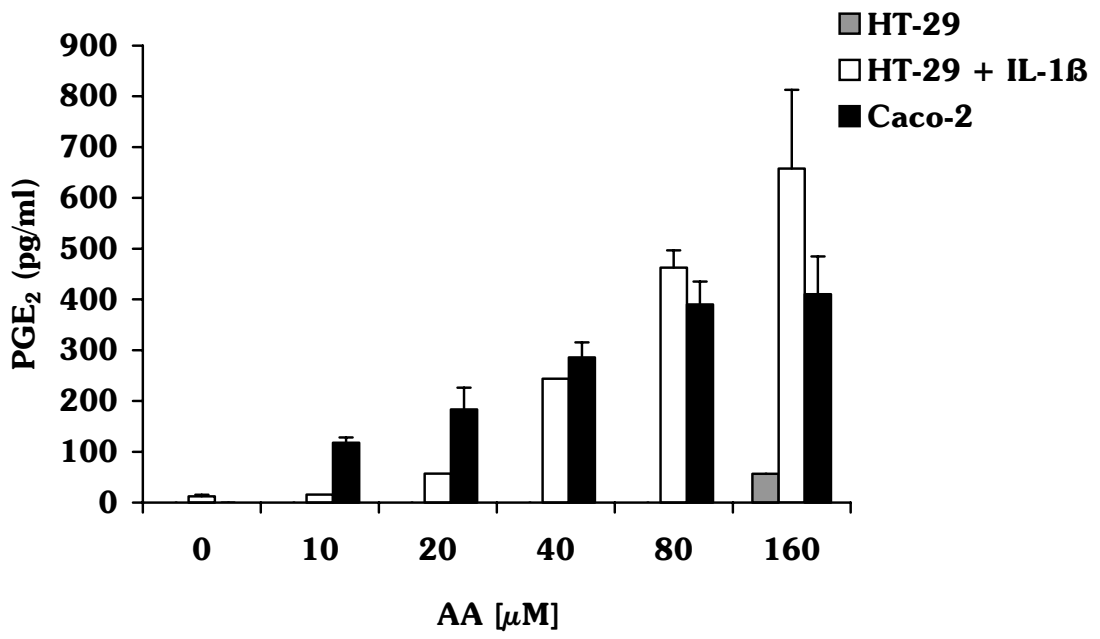


Figure 3.2: Effect of exogenous AA on the production of PGE_2 in human colon carcinoma Caco-2 and HT-29 cells. Cells were treated for 24 hours with AA at 0, 10, 20, 40, 80 and 160 μM +/- 10 ng/ml IL-1ß. Results represent the means ± SD of duplicate determinations of one of the representative experiments

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 hours to investigate whether EPA could inhibit AA-induced PGE_2 production in these cells. As shown in Figure 3.3A, EPA inhibited COX activity in Caco-2 cells, i.e. PGE_2 production, already after 1 hour 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 hour. The results (see Figure 3.3B) 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 $\pm$ 8 μM ($K_{m\ app}$ of 7.9 $\pm$ 1.3 μM; $Vmax_{\ app}$ of 632.04 $\pm$ 24.2 pg x $hour^{-1}$ 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 3.2). Addition of linoleic acid, α-linolenic acid or EPA also had no measurable effect on basal PGE_2 production in the medium of HT-29 cells.

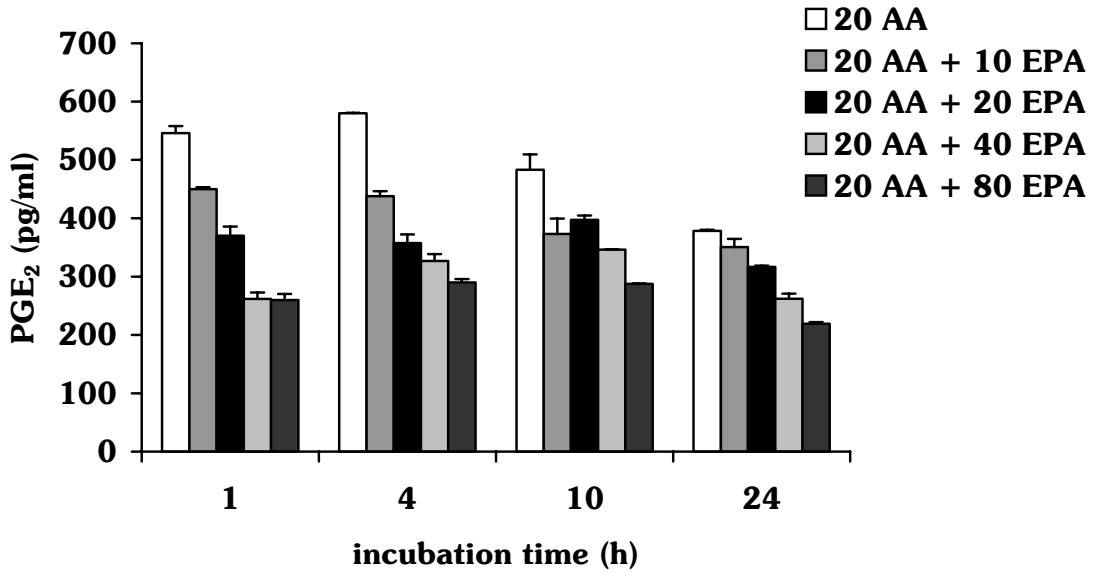


Figure 3.3A: Effect of EPA on AA-induced PGE_2 production in human colon carcinoma Caco-2 cells. Cells were treated for 1, 4, 10 and 24 hours with AA (20 µM) with co-incubation of EPA at 0, 10, 20, 40 and 80 µM. Results represent the means $\pm$ SD of duplicate determinations of one of the representative experiments

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 hours 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 hours) (data not shown).

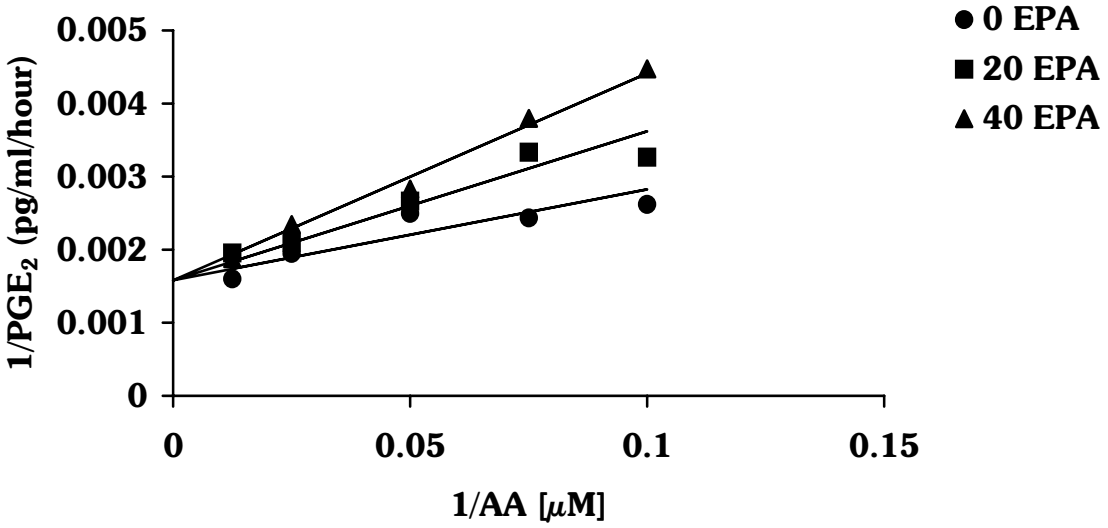


Figure 3.3B: A double-reciprocal (Lineweaver-Burk) plot of COX enzyme inhibition in Caco-2 cells by EPA. PGE_2 production was measured in the medium of Caco-2 cells, 1 hour after addition of 10, 13.33, 20, 40 and 80 μM AA and 0, 20 and 40 μM EPA

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 3.4A, 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-1ß 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 3.4B).

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 malondialdehyde (MDA), which is an end product of lipid peroxidation, were measured in Caco-2 cells and culture medium. After 48 hours of incubation with different concentrations of AA or EPA, MDA formation increased in Caco-2 cells in comparison to untreated cells.

Figure 3.4A

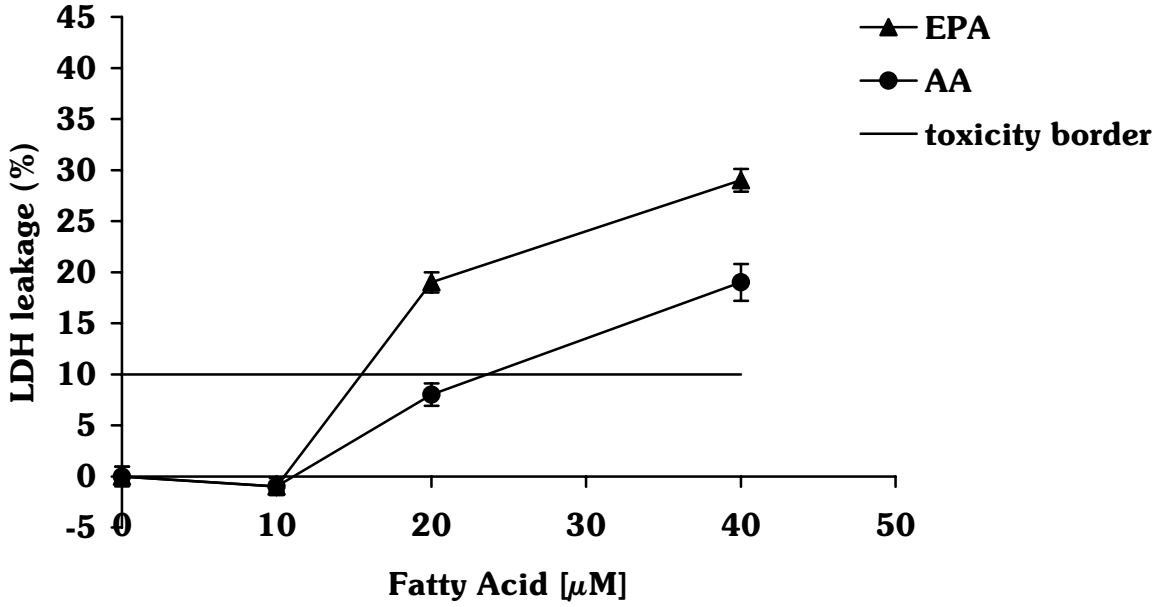


Figure 3.4B

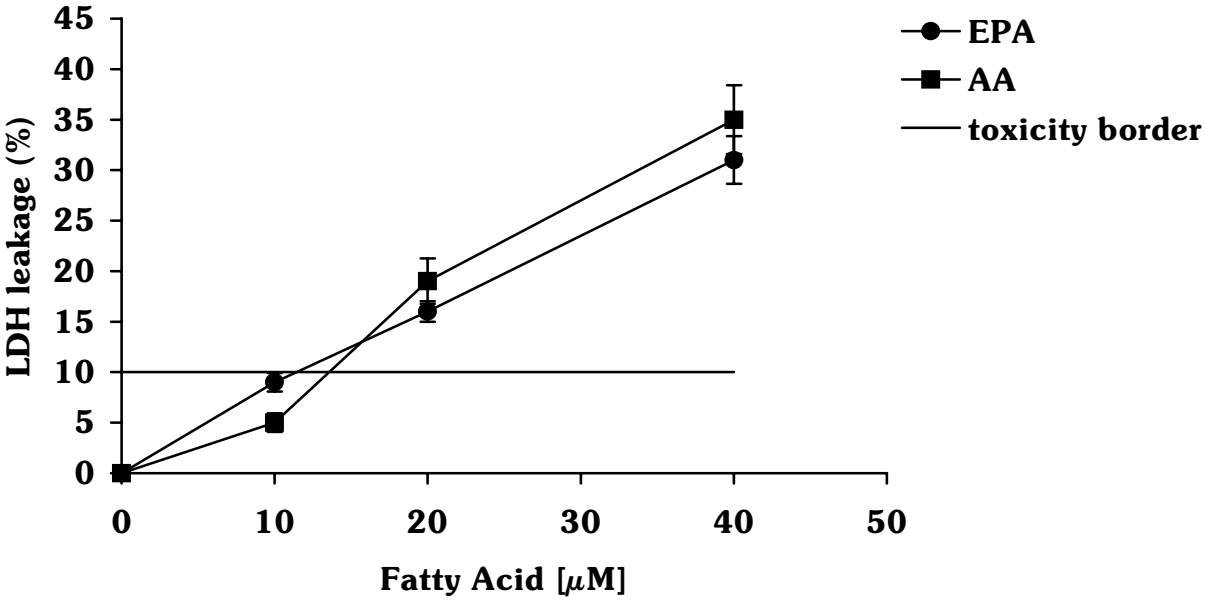


Figure 3.4: Cytotoxicity of EPA and AA in human colon carcinoma cells Caco-2 (3.4A) and HT-29 treated with IL-1β (3.4B). Cells were treated for 48 hours with EPA or AA +/- 10 ng/ml IL-1β at 0, 10, 20 and 40 μM. Results represent the means $\pm$ SEM of 4 replicate determinations of one of the representative experiments

This increase was dependent on PUFA concentration and especially obvious at high concentrations (80-160 μM) of AA and EPA (Figure 3.5). 40% of the total amount of MDA was found in the cells and 60% was found in the media. Only less than 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 3.5). Because oxidation products such as MDA are also formed during the cyclooxygenase pathway (33), we investigated whether a cyclooxygenase inhibitor, indomethacin (IM), could also protect against MDA formation in Caco-2 cells. From these experiments can be concluded that indomethacin could partially inhibit MDA formation, especially from AA in Caco-2 cells.

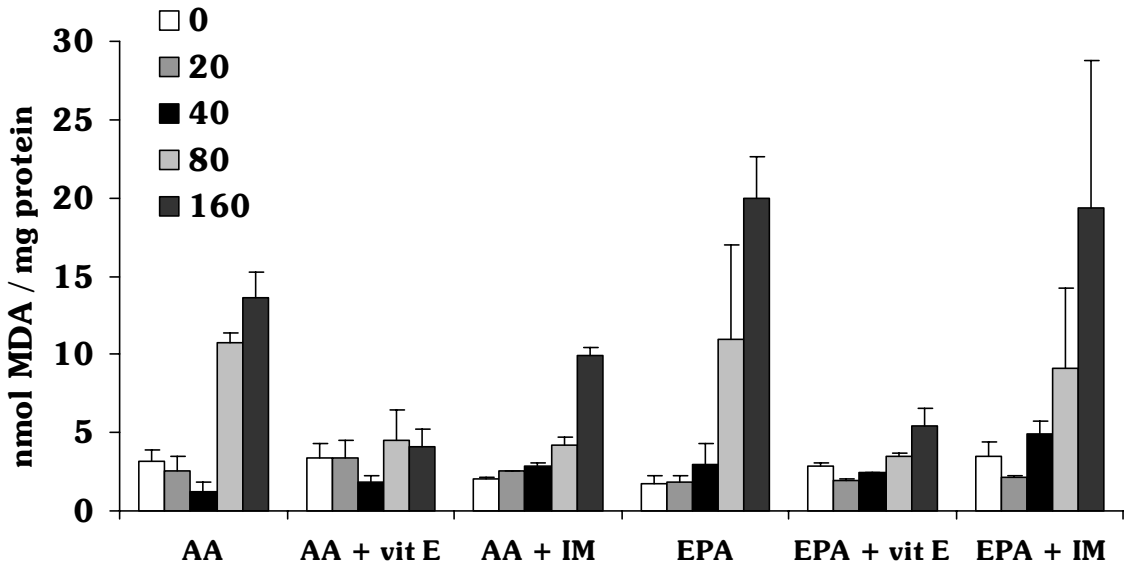


Figure 3.5: Effect of AA and EPA on MDA-formation in Caco-2 cells. Cells were plated and after 24 hours the medium was replaced with fresh serum-free medium supplemented with 1 mg/ml fatty-acid free BSA and AA or EPA at 0, 20, 40, 80 and 160 μM. The effect of 24 hours pre-incubation and 48 hours co-incubation with 10 μM vitamin E (vit E) and 48 hours co-incubation with 20 μM indomethacin (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 $\pm$ SEM of duplicate determinations of one of the representative experiments

Figure 3.6A

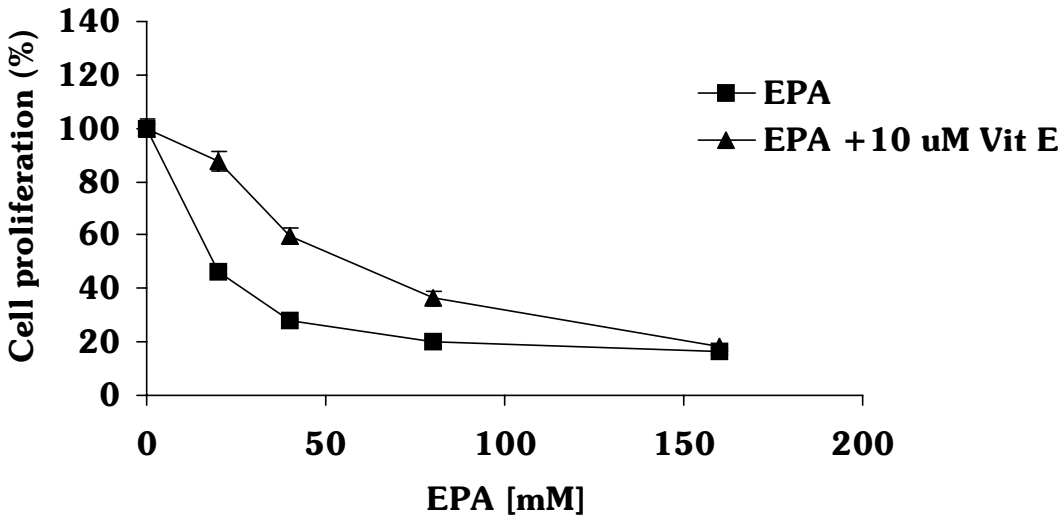


Figure 3.6B

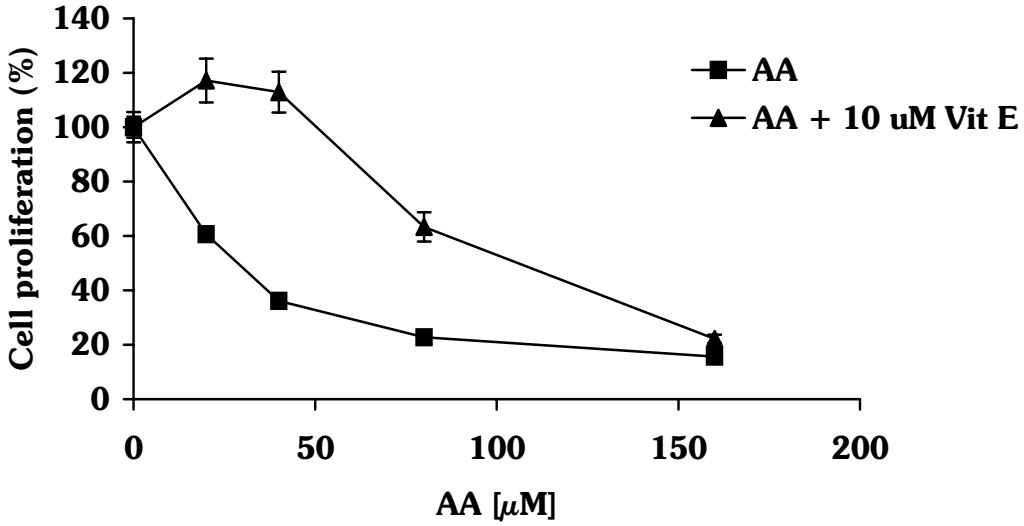


Figure 3.6: Effect of vitamin E (3.6A&B) and indomethacin (3.6C&D) on EPA and AA-induced decrease in cell proliferation in Caco-2 cells. Cells were plated in medium and pre-incubated with or without 10 μM vitamin E. After 24 hours, the medium was replaced with fresh serum-free medium supplemented with 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 indomethacin (IM) (10 or 20 μM). After 48 hours, cell proliferation was measured by ELISA as described in Materials and methods. Results represent the means $\pm$ SEM of 6 replicate determinations of one of the representative experiments

Figure 3.6C

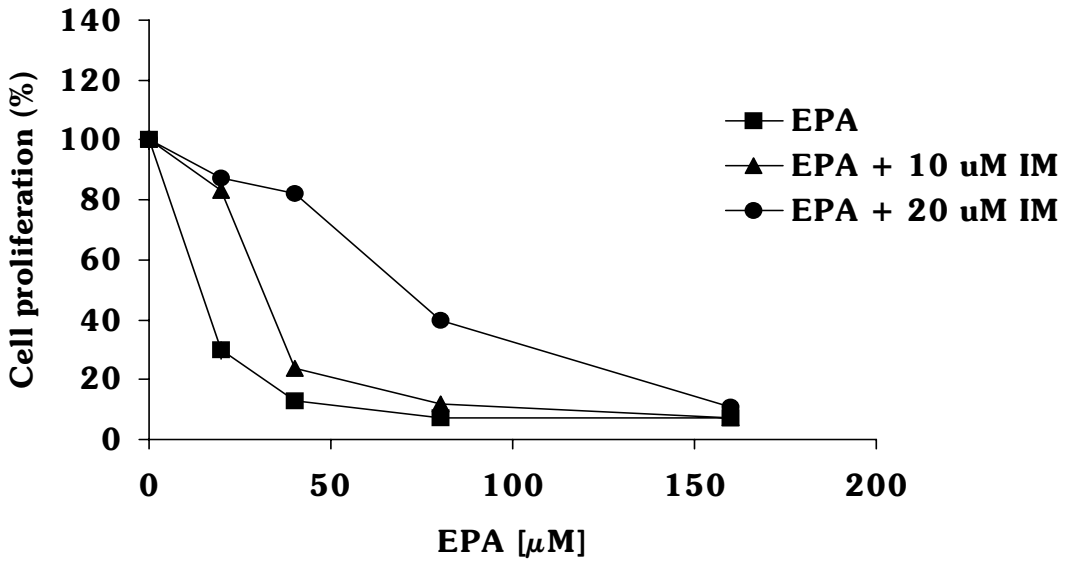
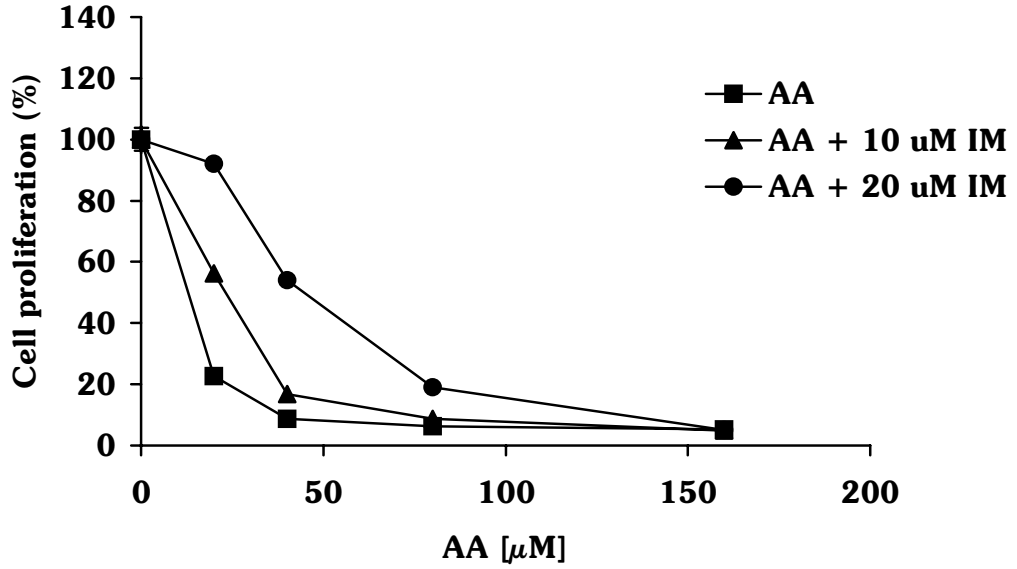


Figure 3.6D



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 3.6 (A&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 hour) and co-incubation (48 hour) of vitamin C (100 μM) could partially reverse the AA and EPA-induced decrease in cell proliferation (data not shown). Because indomethacin 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 indomethacin, a potent inhibitor of cyclooxygenase, for 48 hours. Our studies showed that indomethacin could also partially reverse the EPA and AA-induced decrease in cell proliferation of Caco-2 cells (Figure 3.6 C&D). Indomethacin alone (10 and 20 μM) had no effect on Caco-2 cell proliferation (data not shown).

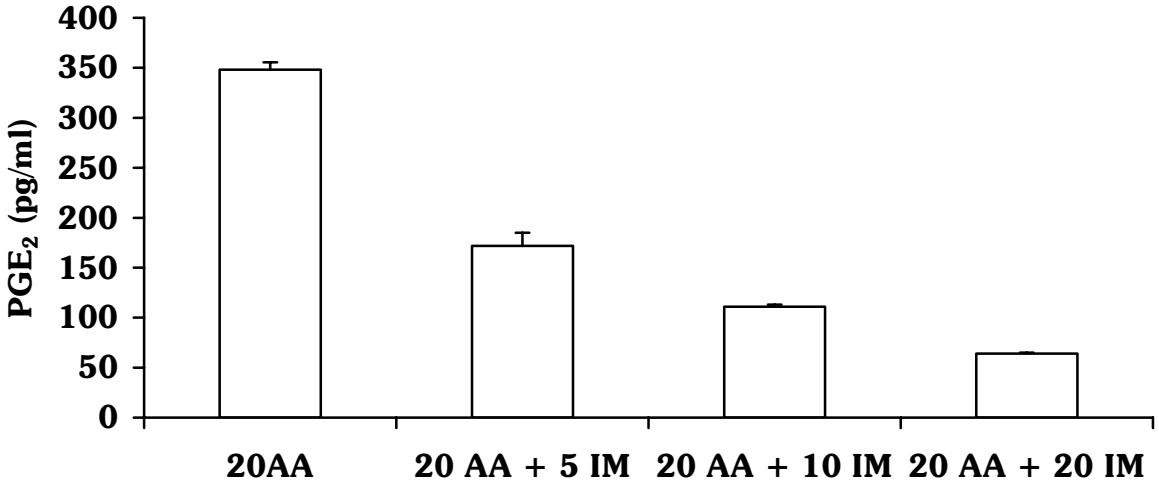


Figure 3.7: Effect of exogenous indomethacin on AA-induced PGE_2 production in human colon carcinoma Caco-2 cells. Cells were treated for 1 hour with 20 μM of AA and indomethacin (IM) at concentrations of 0, 5, 10 and 20 μM. Results represent the means ± SD of duplicate determinations of one of the representative experiments

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

Addition of the non-selective cyclooxygenase inhibitor indomethacin 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 hour after incubation, as shown in Figure 3.7.

Discussion

In several *in vivo* studies, it is hypothesized that corn oil with a high amount of n-6 PUFAs such as linoleic acid (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 eicosapentaenoic acid (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 cancer cell line (39,40).

Therefore, we studied the effect of α-linolenic acid (ALA; 18:3n-3), linoleic acid (LA; 18:2n-6), eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (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 α-linolenic acid (ALA) and linoleic acid (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, regarding 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 comparing to 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.

It is also suggested in *in vivo* studies 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 adenocarcinoma Caco-2 cells, which express high levels of COX-2 (14), produce PGE_2 upon addition of arachidonic acid. HT-29 colon cells on the other hand did not produce high levels of PGE_2 under the same conditions. PGE_2 synthesis in this cell line was rather inducible by IL-1β, a cytokine that is known to 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 linoleic acid could not increase PGE_2 production is in agreement with the finding that the parent essential fatty acid linoleic acid is not converted to the prostaglandin precursor arachidonic acid 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* studies mentioned above that also show that n-3 PUFAs such as EPA inhibit COX activity and arachidonic acid 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 comparing to AA did not lead to differential effects on cell proliferation in human colon adenocarcinoma 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. Malondialdehyde (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 indomethacin, a COX inhibitor that clearly inhibited AA-induced PGE_2 synthesis in Caco-2 cells, could also partially reverse the anti-proliferative effects of both AA and EPA. Also 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 polyunsaturated fatty acid-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 indomethacin could also inhibit AA and EPA-induced MDA formation. Indomethacin 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 indomethacin, on the proliferation of intestinal epithelial cells. These studies indicate that high-dose indomethacin reduces the proliferation rate of colon cancer cells (54,55). Low concentrations of indomethacin (10^{-8} to 10^{-4} M) do not inhibit cell growth, which is in agreement with our result that indomethacin alone (10 and 20 μM) had no effect on Caco-2 cell proliferation. In contrary, we demonstrated that indomethacin in combination with the polyunsaturated fatty acids 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 polyunsaturated fatty acids 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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Chapter 4

Effects of high fat fish oil and high fat corn oil diets on initiation of AOM-induced colonic aberrant crypt foci in male F344 rats

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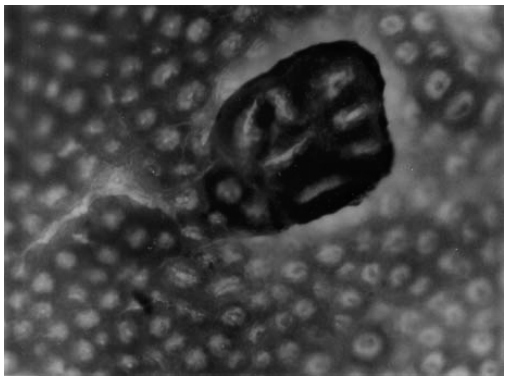


Yvonne E.M. Dommels[1,2]

Suzanne Heemskerk[2]

Hans van den Berg[2]

Gerrit M. Alink[2]

Peter J. van Bladeren[2,3]

Ben van Ommen[4]

[1]WUR/TNO Centre for Food Toxicology
[2]Toxicology Group, Wageningen University, Wageningen, The Netherlands
[3]Present address: Nestlé Research Center, Vers-chez-le-blanc, 1000 Lausanne 26, Switzerland
[4]TNO Nutrition and Food Research, Department of Biomolecular Sciences, Zeist, The Netherlands

Abstract

Modulating effects of high fat fish oil (HFFO) and high fat corn oil (HFCO) diets on azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were studied in male F344 rats following 8 weeks of dietary treatment. The incidence of AOM-induced ACF was significantly lower in the proximal colon of rats fed the HFFO diets compared to rats fed the HFCO diets. No differential effects were found on enzyme activities that are involved in metabolic activation and detoxification of AOM. Activities of hepatic P450 1A1 and P450 2B1 and hepatic and feacal levels of lipid peroxidation were increased by feeding the HFFO diet. Hepatic GST activity and plasma levels of PGE_2 were significantly lower in rats fed the HFFO diets compared to those fed the HFCO diets. In summary, HFFO diets with high levels of n-3 PUFAs are also protective against preneoplastic lesions in the early stages of chemically-induced colon carcinogenesis. It seems unlikely from our results that the inhibitory effect of a HFFO diet can be attributed to an altered metabolic activation and detoxification of AOM. Other mechanisms such as oxidative stress or reduction of PGE_2 levels may play an important role in the anti-carcinogenic effects of n-3 PUFAs.

Introduction

Azoxymethane (AOM), a widely used colon carcinogen in rodents (1) is a synthetic compound, chemically related to the naturally occurring carcinogen cycasin (2). AOM is activated by methyl group hydroxylation to methylazoxy-methanol (MAM) in the liver. It has been proposed that MAM is conjugated with glucuronic acid by means of UDP glucuronyltransferase, immediately upon formation in the liver and transported via the bile to the intestine (3). The glucuronic acid conjugate can be hydrolyzed by bacterial β-glucuronidase to free MAM, producing a relatively high-localized concentration of this compound in the colon (3). The breakdown of MAM, spontaneously or by enzyme-catalyzed reactions, yields the alkylating methyl-diazonium ion, capable of methylating cellular macromolecules such as DNA (3,4). Two major DNA adducts which can be formed are 7-methylguanine and O^6-methylguanine.

The azoxymethane (AOM) model for colon carcinogenesis is often used to study modifying dietary influences on the initiation and promotion stages of colon carcinogenesis in rats under strictly controlled laboratory conditions. Several reviews have appeared on the modulating effects of n-6 and n-3 polyunsaturated fatty acids (PUFAs) on colon tumorigenesis in experimental animal models (5,6). Overall results from these reviews suggest that n-6 fatty acids enhance the risk of colorectal carcinogenesis during the promotion phase, whereas n-3 fatty acids can protect during both the initiation and promotion stages of colorectal carcinogenesis. In comparison to chemically-induced colon tumors, effects of n-3 and n-6 PUFAs on the development of aberrant crypt foci (ACF), precursor lesions of colorectal carcinogenesis are described in only a few studies. Those that have been performed show a decrease in total ACF incidence and multiplicity by perilla oil (7), docosahexaenoic acid (DHA; 22:6n-3) (8) and high fat fish oil (HFFO) diets (9). However, these studies only focused on the protecting effect of n-3 PUFAs during the postinitiation phase of colorectal carcinogenesis. This experimental design superimposes effects on initiation such as carcinogen metabolism.

With respect to the putative chemopreventive mechanism of action of these n-3 PUFAs, Hong *et al.* (10) determined the ability of fish oil to modulate O^6-methylguanine DNA adduct formation (DNA damage), removal (DNA repair) and deletion (apoptosis) during the initiation phase of colonic malignant transformation. They have shown that colonic O^6-methylguanine DNA adduct levels are lower in fish oil fed animals than in corn oil fed animals 6-12 hours after initiation with AOM. The protective mechanism might be related to different metabolic activation and detoxification of AOM. It was unlikely though that the protective effect of fish oil supplementation was related to an effect on O^6-methylguanine-DNA-methyl-transferase (MGMT)-mediated DNA repair because overall,

fish oil feeding had no significant effect on the induction of MGMT in the distal colonic epithelium. Fish oil feeding also resulted in a significantly higher level of apoptosis in the upper one-third of the crypt compared with corn oil treatment. The conclusion of this study was that fish oil reduced O^6-methylguanine DNA adduct levels in rat colon in part by increasing apoptosis during tumor initiation. In addition, Latham *et al.* (11) demonstrated that consumption of n-3 PUFAs in the form of fish oil, fed only for a 48 hour period following administration of the colon carcinogen DMH, significantly reduced the incidence of ACF compared to rats fed a corn oil diet. Consumption of fish oil was also associated with increased apoptotic cell death and suppression of cell proliferation. Fish oil raised the levels of apoptosis and suppressed proliferation also in sham-injected animals, implying that fish oil has the capacity to modulate apoptosis and proliferation even in the absence of DMH. They assumed that effects of dietary fish oil could be due to oxidative stress, since fish oil diets may compromise the antioxidant status of the cell via increased lipid peroxidation, leading to loss of cell viability or selective induction of apoptosis (11).

Rao and Reddy (12) investigated the effect of high fat diets rich in n-3 and n-6 fatty acids during the initiation phase on several cellular events that may play a role in colon tumorigenesis. These results demonstrate that high levels of fish oil in the diet suppress liver, colon and small intestine mucosal ornithine decarboxylase (ODC) activity, tyrosine-specific protein kinase (TPK) activities and plasma, liver and colon prostaglandin E_2 (PGE_2) levels in male F344 rats, 5 days after a second AOM injection, comparing to high dietary levels of corn oil.

In the present study an animal experiment has also been carried out to investigate the modulating effects of high fat fish oil (HFFO) and high fat corn oil (HFCO) diets on rat colon carcinogenesis. Because so far little attention has been paid to the chemopreventive effects of n-3 PUFAs, differential effects of HFFO and HFCO diets fed during both the initiation and postinitiation stages of colon carcinogenesis, were studied on the development of AOM-induced aberrant crypt foci (ACF), precursor lesions of colon carcinogenesis. Differential effects of fish oil and corn oil feeding on metabolic activation and detoxification of AOM, plasma PGE_2 levels, epithelial cell damage and levels of lipid peroxidation were also studied in this animal experiment to gain more insight into the mechanisms by which these fatty acids modulate the early stages of colon carcinogenesis.

Material and methods

Animals and diets

Twenty male F344 rats (~ 3 weeks old) were purchased from Charles River Breeding Laboratory (Someren, The Netherlands) and acclimatized for 1 week. The rats were housed in plastic cages, 2 rats per cage, in an animal holding room using a 12:12-hour light-dark cycle. Temperature and humidity were controlled at 20-24 °C and 50-60%, respectively. Animals were randomly divided into 2 dietary sub-groups of ten animals per group and had free access to water and food. Fresh food in powder form was replenished every day. The experimental diets, high fat fish oil (HFFO) and high fat corn oil (HFCO), were formulated on the basis of a modified AIN-93G diet, as described in Table 4.1. The HFCO diet contained 25% corn oil (Remia, Den Dolder, The Netherlands) at the expense of an isocaloric amount of carbohydrates. The HFFO diet contained 20% Omegapure™ Refined Menhaden Fish oil, which was kindly donated by Omegaprotein (Houston, TX, USA) and 5% corn oil to prevent any essential fatty acid deficiency, also at an isocaloric amount of carbohydrates. 1000 ppm Mixed-tocopherols and 200 ppm tertiary butyl hydroquinone (TBHQ) were added to the Omegapure™ Refined Menhaden Fish oil to prevent lipid peroxidation of the oil. These antioxidants were therefore also added to the corn oil before preparation of the HFCO diet. The amount of L-cystine, cellulose, choline bitartrate, AIN-93 mineral and vitamin mix were adjusted with a factor 1.24 above the recommended amounts (13) to compensate for the reduced food intake of high fat diets (14). Diets were prepared freshly at the beginning of the experiment and after one month. Daily batches were stored in sealed plastic bags in dark containers at -40 °C until use. The peroxide values of the freshly prepared diets and of the diets that were stored for one month were measured to determine the amount of *in vitro* fatty acid peroxidation.

Experimental procedure

Beginning at 4 weeks of age, rats were fed the experimental diets for 8 weeks. All animals were given s.c. injections of AOM (Sigma Chemical Company, St Louis, MO, USA), once weekly for two weeks, at a dose of 15 mg/kg body weight, after two weeks of experimental diet. Animals were placed in an isolator during this period to prevent possible health risks. Body weights were recorded three times a week and daily food intake was measured during three separate periods (day 1-9; day 30-36 and day 48-56). Rats were housed individually during the second and third period. Fresh faeces was collected at several days during the experiment to measure feacal enzyme activities and blood was collected by orbital puncture at day 7 and 29 of the experiment to determine plasma PGE_2 levels. At the end of the experiment (four weeks after the second AOM

injection), blood was collected from the vena cava inferior under anesthesia, which was a combination of isofluran, O_2 and N_2O. Also colon, liver and caecum were removed. Livers and ceacal contents were quickly weighed, snap-frozen in liquid nitrogen and stored at -80 °C until further processing. The animal welfare committee of Wageningen University (The Netherlands) approved the experimental protocol.

Quantification of colonic aberrant crypt foci (ACF)

Colons were rinsed in a 0.9% NaCl solution, slit open lengthwise and fixed flat between filter paper in 4% formalin in phosphate-buffered saline (Dulbecco `A' Tablets, Oxoid, Haarlem, The Netherlands). After storage in 70% ethanol, they were stained for 30 seconds in 0.5% methylene blue according to Bird (15). The number of aberrant crypts per focus (multiplicity) and the total number of ACF were scored in proximal, distal and total colon, under a light microscope at a 40x magnification.

PGE_2 analysis in plasma

PGE_2 levels were analyzed in the plasma of all rats after 1, 4 and 8 weeks of dietary intervention. A PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech UK limited, Buckinghamshire, UK) was used according to protocol 5, recommended by the manufacturer. The results are expressed as picograms of PGE_2 per ml plasma.

Measurement of feacal and ceacal enzyme activities

Fresh feacal and frozen ceacal samples were homogenized in 0.9% NaCl using an Ultra Turrax and centrifuged at 2000 g for 15 minutes at 4 °C. Bacterial β-glucuronidase and β-glucosidase activities were determined spectrophotometrically in the supernatant by measuring paranitrophenol (PNP)-β-D-glucuronide and paranitrophenol-β-D-glucoside (Sigma) hydrolysis, respectively (16). All reactions were performed at 37 °C in 96-wells plates, containing 75 μl supernatant, 25 μl of a 0.1% substrate solution and 25 μl 50 mM phosphate buffer (pH 6.5). After 1 hour of incubation, the reactions were stopped by addition of 0.5% glycine NaOH buffer, 2 mM EDTA (pH 9.5) and the amount of PNP released was measured at 405 nm (Spectramax 340, Molecular Devices, Menlo Park, CA, USA).

Intestinal alkaline phosphatase (ALP) activity was also measured spectrophotometrically in the supernatant by measuring hydrolysis of p-nitrophenolphosphate (17). Release of PNP was also measured at 405 nm. L-phenylalanine (150 mM) was used as a specific inhibitor to determine the intestinal isoenzyme activity (18). All enzyme activities were calculated as μmol PNP released per hour per gram faeces or ceacal content.

Measurement of hepatic enzyme activities

Enzyme activities of cytochrome P4501A1 (EROD), cytochrome P4502B1 (PROD), UDP-glucuronyltransferase (UDPGT) and glutathione transferase (GST) were measured in liver microsomal and cytosolic fractions. Livers were homogenized in three volumes of ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM DTT, using a Potter tube and centrifuged for 30 minutes at 12000 g at 4 °C. The resulting supernatant was centrifuged for 75 minutes at 105000 g at 4 °C. The microsomal pellet was resuspended in ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 3 mM EDTA and 1 mM DTT. Both microsomal and cytosolic fractions were stored in aliquots at -80 °C until further analysis. Protein levels were determined according to Bradford (19). Ethoxyresorufin O-deethylase (EROD) activity was measured according to the method of Burke *et al.* (20) adapted for use of 96 well plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Millipore, Etten-Leur, The Netherlands). Reaction mixtures contained 0.4 μM 7-ethoxyresorufin (ER), 0.1 mM NADPH, 1 mg/ml BSA and 100 μg/ml microsomal protein in 200 μl 0.1 M Tris-HCL, pH 7.8. After preincubation for 5 minutes at 37 °C, reactions were started by the addition of NADPH and after 5 minutes at 37 °C they were stopped by adding 50 μl 1 M NaOH. The formation of the product resorufin (RR) was detected fluorometrically (excitation 530 nm, emission 590 nm) and compared with a calibration curve using 0-150 nM RR in 0.08 M Tris-HCL, 0.8 mg/ml BSA and 0.8 M NaOH. Incubations were carried out in duplicate and results were corrected for blanks without NADPH. Pentoxyresorufin O-deethylase (PROD) was measured following the same procedure as for EROD with a final concentration of 2 μM pentoxyresorufin (PR) in stead of ER. PNP UDPGT activity was measured by incubation of 4 mM PNP for 15 minutes at 37 °C with 0.5 mg microsomal protein/ml, 20 mM UDPGA in 100 mM Tris-HCL (pH 7.4) and 5 mM $MgCl_2$. UDPGT activity was also measured in microsomes that were treated with 0.01% palmitoyl-lysophosphatidylcholine (21). GST activity was measured according to Habig *et al.* (22) adapted for use of 96 well plates and a spectrophotometric plate reader (SpectraMax 340, Molecular Devices, Menlo Park, CA) as described by van Iersel *et al*. (23). Reaction mixtures contained 1 mM GSH, 1 mM CDNB and 1 mg cytosolic protein/ml in 250 μl 0.1 M potassium phosphate buffer (pH 6.5) and 2 mM EDTA. After preincubation for 2 minutes at 25 °C, the reaction was started by addition of CDNB and conjugate formation was followed for 1 minute at 25 °C at 340 nm.

Lipid peroxidation

The thiobarbituric acid assay according to Buege and Aust (24) was used to determine the amount of lipid peroxidation in faeces and caecum supernatant. 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. Lipid peroxidation in liver microsomes was measured at double wavelengths (535 + 520 nm) to avoid interference according to Uchiyama and Mihara (25). Absorbance was converted to μmol MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane. Lipid peroxidation was presented as μmol MDA per gram tissue.

Statistical analysis

Statistical analyses were performed using SPSS version 9.0 for Windows. Data were analyzed using general factorial analysis of variance after testing for normality and homogeneity of variance. In case of homogeneity, the Bonferroni test of significance was used for post-hoc analysis ($p < 0.05$). In case of non-homogeneity, data were tested on significance with the Dunnet T3 test ($p<0.05$). When data were not normally distributed a Kruskall Wallis test was used.

Table 4.1: Composition of the experimental diets

	Diet[1]	
Dietary ingredients	HFCO	HFFO
Casein	25.00	25.00
L-cystine	0.37	0.37
Wheat starch	37.64	37.64
Cellulose	6.18	6.18
Choline bitartrate	0.25	0.25
AIN-93 minerals	4.32	4.32
AIN-93 vitamins	1.24	1.24
Corn oil[2]	25.00	5.00
Fish oil[3]	0.00	20.00
Peroxide values (fresh - stored)[4]		
Batch 1	2.2 - 3.6	1.8 - 3.2
Batch 2	2.8 - 3.8	2.4 - 3.8

[1] Values are expressed as percent by weight

[2] Corn oil contains 34-62% 18:2n-6

[3] Omegapure™ Refined Menhaden fish oil contains ~20-26% n-3 fatty acids (10-17 % EPA and 7-12 % DHA)

[4] Peroxide values are expressed in mEq/kg diet in freshly prepared diets - and diets stored for one month at -40 °C

Results

Omegapure™ Menhaden Fish oil contains high amounts of long-chain n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Corn oil contains high amounts of long-chain n-6 PUFAs, such as linoleic acid (LA; 18:2n-6) (see Table 4.1). Because PUFAs are extremely susceptible to auto-oxidation and peroxidation, antioxidants were added to both of the oils to prevent *in vitro* lipid peroxidation (26). Peroxide values of the HFCO and HFFO diets were < 3 mEq/kg diet (Table 4.1). A small increase up to around 4 mEq/kg diet was seen after storage of the diets for one month at -40 °C, but peroxide values were still below 8-9 mEq/kg, which are found in other normal oils (27). However, the amount of *in vivo* lipid peroxidation, as measured by feacal levels of MDA (Table 4.2), was significantly higher in the rats fed the HFFO diet for 9 days than in the rats fed the HFCO diet for 9 days. This difference was no longer observed in the faeces as well as the caecum, after 57 days of dietary treatment. MDA levels were also higher in the liver microsomes of the HFFO diet group comparing to the HFCO diet group, at the end of the experiment.

Table 4.2: Influence of HFCO and HFFO diets on lipid peroxidation levels in faeces, caecum and liver after 9 or 57 days of dietary treatment[1]

	Diet	
MDA levels[2]	HFCO	HFFO
Faeces, day 9	0.026 ± 0.014[a]	0.054 ± 0.031[a]
Faeces, day 57	0.066 ± 0.051	0.062 ± 0.020
Ceacal contents	0.026 ± 0.007	0.054 ± 0.029
Liver microsomes	0.014 ± 0.002[b]	0.025 ± 0.002[b]

[1] Values are means ± SD per diet group (n=10 per group)

[2] MDA levels are expressed as μmol MDA per gram tissue

[a,b] Means between diet groups sharing the same letter superscripts are significantly different at $p < 0.05$

Food intake and body weights of the animals were not affected by the type of dietary lipid. As seen in Table 4.3, average body weights of the rats in the HFCO and HFFO diet groups were not significantly different at the end of the experiment. Also the amount of ceacal contents was not different between the HFCO and HFFO diet groups. However,

the liver weight was significantly higher for the rats fed the HFFO diet than for the rats fed the HFCO diet (Table 4.3).

Table 4.3: Effect of dietary lipid on body and liver weight, food intake and amount of ceacal content in male F344 rats[1]

	Diet	
	HFCO	HFFO
Initial body weight (g)	45.76 ± 3.05	46.36 ± 3.0
Final body weight (g)	270.36 ± 15.34	278.53 ± 15.73
Food intake (g)[a]	12.1 ± 1.20	12.7 ± 0.68
Liver weight (g)	9.70 ± 0.92[b]	13.04 ± 1.13[b]
Relative liver weight (%)	3.58 ± 0.19[c]	4.68 ± 0.17[c]
Ceacal contents (g)	2.64 ± 0.27	2.56 ± 0.29
Relative ceacal content weight (%)	0.98 ± 0.11	0.92 ± 0.13

[1] Values are mean ± SD per diet group (n=10 per group)

[a] Average food intake per day per rat, as determined in week 8 (day 48-56) of the experiment

[b,c] Means between diet groups sharing the same letter superscripts are significantly different at $p < 0.01$

The total number of AOM-induced ACF in the proximal colon was significantly lower in the HFFO diet group than in the HFCO diet group, after 8 weeks of dietary intervention (Table 4.4). This lower number of ACF in the proximal colon of the HFFO group reflected a lower number of total ACF in the total colon. No significant difference in total number of ACF between the diet groups was observed in the distal colon, although the trend of inhibition of ACF by HFFO diets was also visible. The group of ACF which showed the most reduction had a crypt multiplicity (= number of aberrant crypts per focus) of 1-3. In contrary, the average crypt multiplicity was higher for the HFFO diet group in the distal and total colon, comparing to the HFCO diet group (Table 4.4).
In order to investigate the possible mechanisms of the protective effect of the HFFO diet on the development of AOM-induced ACF, the influence of both HFFO and HFCO diets on the metabolic activation and detoxification of AOM was studied. The activity of liver microsomal UDP glucuronyltransferase, the enzyme that conjugates MAM in the liver to the detoxified MAM-conjugate, is not influenced by the type of dietary lipid (Table 4.5). Activities of other enzymes that are involved in phase I and phase II metabolism were

also measured in livers of HFFO and HFCO treated rats. From these results it became clear that cytosolic GST activity was decreased in livers of the HFFO treated rats comparing to the HFCO treated rats. On the other hand, activities of P450 1A1 and P450 2B1 are elevated in liver microsomes of the HFFO treated rats comparing to the HFCO treated rats (Table 4.5).

Table 4.4: ACF growth characteristics in male F344 rats after 8 weeks of intervention with HFCO and HFFO diets[a-c]

			ACF[d]							
Diet	Total ACF total	CM	1	2	3	4	5	6	7	8
HFCO	108.0 ± 28.2[1]	2.2[2]	26.9	43.6	25.6	8.8	2.7	0.1	0.2	0.1
HFFO	70.3 ± 29.1[1]	2.5[2]	15.4	24.5	16.3	8.1	5.4	0.6	0.0	0.0
			ACF[d]							
Diet	Total ACF proximal	CM	1	2	3	4	5	6	7	8
HFCO	52.3 ± 12.3[3]	2.2	13.8	22.3	10.9	4.3	2.2	0.0	2.0	1.0
HFFO	23.9 ± 11.7[3]	2.3	7.1	8.5	5.2	1.8	1.6	1.0	0.0	0.0
			ACF[d]							
Diet	Total ACF distal	CM	1	2	3	4	5	6	7	8
HFCO	55.7 ± 24.9	2.3[4]	13.1	21.3	14.7	5.4	2.3	1.0	0.0	0.0
HFFO	46.5 ± 20.7	2.7[4]	8.3	16.0	11.1	6.3	4.3	1.3	0.0	0.0

[a] Values are means ± SD per diet group (n=10 per group)

[b] Abbreviations are as follows: ACF; aberrant crypt foci, CM; average crypt multiplicity/group

[c] Animals are terminated after 8 weeks of dietary intervention, 4 weeks after the second AOM injection

[d] ACF are categorized by number of aberrant crypts (1-8) per focus

[1-4] Statistical analyses were only performed for total number of ACF and the average crypt multiplicity/group (CM). Means between diet groups sharing the same number superscripts are significantly different at $p < 0.01$

Table 4.5: Influence of HFCO and HFFO diets on activity of microsomal UDP glucuronyltransferase (UDPGT), cytochrome P450 1A1, cytochrome P450 2B1 and cytosolic glutathione transferase (GST)[1-2]

	Diet	
	HFCO	HFFO
UDPGT (untreated)[3]	25.49 ± 10.28	31.75 ± 3.49
UDPGT (treated)[3]	59.39 ± 4.36	57.64 ± 8.56
GST	685.34 ± 73.11[a]	533. 37 ± 38.56[a]
P450 1A1	7.88 ± 1.06[b]	10.62 ± 1.04[b]
P450 2B1	1.86 ± 0.25[c]	2.78 ± 0.23[c]

[1] Values are means ± SD per diet group (n=10 per group)

[2] UDPGT and GST activities are expressed as nmol/min per mg protein and activities of P450 1A1 and 2B1 are expressed as pmol/min per mg protein

[3] UDPGT activity was measured in untreated microsomes and microsomes treated with 0.01% palmitoyl-lysophosphatidylcholine

[a,b,c] Means between diet groups sharing the same letter superscripts are significantly different at $p < 0.01$

The activity of bacterial β-glucuronidase, the enzyme that deconjugates the MAM-conjugate of AOM into free MAM in the colon, was measured in fresh faeces before (day 0, 6, 9) and after (day 31, 42, 43, 55 and 57) AOM injection. At all days, no differences were noted in the feacal β-glucuronidase activity among the animals fed the different diets (Table 4.6). Also no differences were observed in β-glucosidase activity, another intestinal bacterial enzyme that hydrolyzes cycasin, a pre-carcinogen present in plants, to methylazoxymethanol (MAM)-β-D-glucoside (28). Feacal β-glucuronidase activity was only significantly higher after 55 days of dietary intervention in the HFFO diet group compared to the HFCO diet group. Ceacal β-glucuronidase and β-glucosidase activity, measured at day 57, were not significantly different among the diet groups (data not shown). ALP is an enzyme that is located on the apical membrane of colonic epithelial cells. After disruption of the epithelial cell membrane, ALP will be excreted into the lumen and can be measured in the faeces as a marker of colonic epithelial cell lysis (29). The type of dietary lipid did not influence colonic epithelial cell damage, as measured by ALP activity. No differences were observed in feacal (Table 4.6) as well as ceacal (data not shown) ALP activity between the HFFO and HFCO diet groups at several days during the experiment.

Table 4.6: Influence of HFCO and HFFO diets on fresh feacal activities of β-glucuronidase, β-glucosidase and ALP in Male F344 rats[1-2]

	Days on the diet	Diet	
		HFCO	HFFO
β-glucuronidase	0	11.21 ± 26.10	5.24 ± 7.24
	6	3.23 ± 1.18	7.51 ± 1.85
	9	20.80 ± 42.25	16.76 ± 9.77
	31	7.10 ± 9.49	6.87 ± 2.82
	42	4.78 ± 2.63	6.84 ± 5.75
	43	15.59 ± 15.46	21.28 ± 9.61
	55	9.30 ± 2.79[a]	15.61 ± 6.34[a]
	57	27.54 ± 17.76	27.88 ± 16.15
β-glucosidase	0	9.51 ± 22.35	6.97 ± 11.32
	6	2.06 ± 1.05	3.04 ± 1.33
	9	11.18 ± 21.81	5.16 ± 4.86
	31	3.84 ± 4.03	2.04 ± 0.88
	42	2.89 ± 1.59	1.98 ± 1.00
	43	7.90 ± 8.36	5.77 ± 2.79
	55	4.60 ± 1.44	3.66 ± 0.79
	57	13.69 ± 9.37	5.73 ± 2.79
ALP	0	278.36 ± 217.36	209.55 ± 248.04
	6	45.69 ± 57.37	76.76 ± 65.28
	9	95.59 ± 65.90	241.04 ± 212.95
	31	34.17 ± 17.38	45.55 ± 28.59
	42	25.59 ± 12.03	45.61 ± 21.91
	43	102.13 ± 114.70	100.17 ± 80.34
	55	30.92 ± 9.77	44.82 ± 15.67
	57	120.81 ± 119.08	144.53 ± 112.69

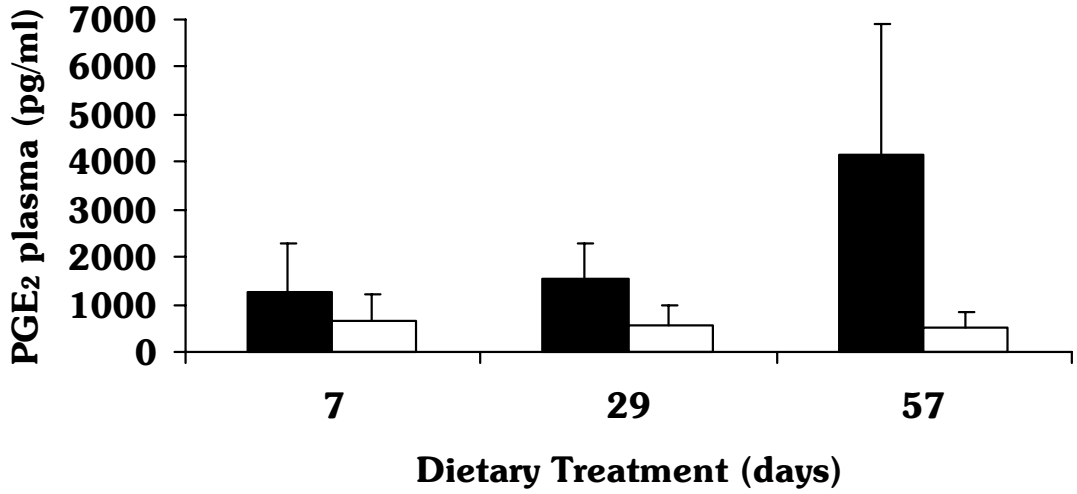
[1] Values are means ± SD per diet group (n=10 per group)

[2] Activities are expressed as μmol PNP released per hour per gram faeces

[a] Means between diet groups sharing the same letter superscripts are significantly different at $p < 0.01$

The effects of HFCO and HFFO diets on PGE_2 levels in plasma are shown in Figure 4.1. High dietary levels of fish oil significantly suppressed PGE_2 levels in plasma compared to high dietary levels of corn oil, after 29 and 57 days of dietary treatment. This trend was already visible after 7 days of dietary treatment, though not statistically significant.

Figure 4.1: PGE_2 levels in plasma of male F344 rats after 1, 4 and 8 weeks of HFCO (black bars) and HFFO (white bars) diets[1]



[1] Values are means $\pm$ SD (n=10 per group). Means between diet groups are significantly different at $p < 0.05$ at day 29 and 57

Discussion

Experimental data indicate that high dietary fish oil can effectively reduce colon tumor incidence in comparison with high dietary corn oil when given during the initiation and postinitiation stages of colorectal carcinogenesis (30). In addition, we have shown in this study that high fat fish oil (HFFO) diets with a high amount of n-3 PUFAs also reduce the incidence of AOM-induced colonic ACF in male F344 rats in comparison with high fat corn oil (HFCO) diets with a high amount of n-6 PUFAs, when fed during both the initiation and postinitiation stages of colorectal carcinogenesis. This protective effect was particularly evident on the total number of ACF in the proximal colon.

The group of ACF which showed the most reduction had a crypt multiplicity (= number of aberrant crypts per focus) of 1-3. These results indicate that high dietary levels of n-3 PUFAs are also protective against preneoplastic lesions in the early stages of chemically-

induced colon carcinogenesis. Our results are consistent with the report of Onogi *et al.* (7) in which perilla oil, rich in the vegetable n-3 PUFA α-linolenic acid, also suppressed the development of ACF, even when it was present in small amounts (3%) in the diet.

In the current study, the rats received daily high amounts of fish oil (20%) + 5% of corn oil for 8 weeks, which provided about 240 mg EPA and 170 mg DHA per day. It must be recognized that these large intakes do not reflect a human diet, although they could be consumed by the use of fish oil supplements. In addition, Reddy and Sugie (31) revealed that feeding high fat diets containing only 5.9% of Menhaden fish oil in combination with 17.6% corn oil significantly inhibited the incidence of colon adenocarcinomas compared to 23.5% corn oil diets. Thus also lower amounts of n-3 PUFAs in high fat diets are effective in inhibition of colon carcinogenesis, though these diets were only fed during the postinitiation phase of colon carcinogenesis.

A possible mechanism to explain the anticancer role of n-3 PUFAs during the initiation phase of colon carcinogenesis could be related to the ability of n-3 PUFAs to positively influence the metabolic activation and detoxification of AOM. Therefore we investigated the effect of a HFFO and a HFCO diet on the activity of rat liver, feacal and ceacal enzymes that are involved in AOM-induced colon carcinogenesis, i.e. microsomal UDP glucuronyltransferase and bacterial β-glucuronidase and β-glucosidase. However, no significant differences in these enzyme activities were observed between the HFFO and HFCO diet groups. Also Bartram *et al.* (32) found that feacal activities of β-glucuronidase and β-glucosidase were not affected by fish oil or corn oil consumption, after four weeks of supplementation in healthy volunteers. On the basis of these and our results, it seems unlikely that the inhibitory effect of a HFFO diet can be attributed to an altered metabolic activation and detoxification of AOM in comparison to what other research groups suggested (10,30,33).

The different fatty acid diets applied in this study did affect other hepatic enzymes that are involved in phase I and phase II metabolism of carcinogens and xenobiotics, such as certain P450 and GST. Activities of P450 1A1 and P450 2B1 are elevated in liver microsomes of the HFFO treated rats comparing to the HFCO treated rats. These results are consistent with those of Chen and co-workers (34,35). Rats fed fish oil also had significantly higher liver weights and relative liver weights compared with those of rats fed corn oil diets. This also suggests that fish oil can exert a stimulatory effect on hepatic microsomal enzymes (36). On the other hand, cytosolic GST activity was decreased in livers of HFFO treated rats in comparison to HFCO treated rats, which is in contrast with results of Lii *et al.* (34), Yang *et al.* (21) and Ko *et al.* (36). They found that hepatic levels of antioxidant enzymes such as GSH reductase and GST were significantly greater in rats

fed fish oil than in rats fed corn oil, which implies an increased antioxidant capacity to defend against oxidative stress.

In the present study, we measured the amount of *in vivo* lipid peroxidation in faeces, caecum and livers of HFFO and HFCO fed rats as a marker of oxidative stress. MDA levels were significantly higher in liver (day 57) and faeces (day 9) of rats fed a HFFO diet than in rats fed a HFCO diet. This increase in lipid peroxidation was not cytotoxic for colonic epithelial cells, since the activity of the apical membrane-marker of colonic epithelial cell lysis ALP was not increased in the faeces and caecum of HFFO fed rats.

The higher lipid peroxidation potential of fish oil diets probably results from the higher number of double bonds in fish oil compared to corn oil (36). Higher *in vivo* MDA levels were not related to *in vitro* peroxidation because the peroxide values of the HFFO diet were comparable to those of the HFCO diet.

As stated in the introduction, Latham *et al.* (11) proposed that fish oil diets compromise the antioxidant status of the cell via increased lipid peroxidation leading to loss of cell viability, selective induction of apoptosis and reduction of carcinogen-induced colonic ACF formation. Also Hong *et al.* (10) concluded that dietary n-3 fatty acids found in fish oil confer protection against experimental induced colon carcinogenesis in part by reducing the level of DNA adducts and by enhancing the deletion of cells through activation of targeted apoptosis. The question remains whether the anticarcinogenic effects of n-3 PUFAs found in this study could be ascribed to the higher oxidative stress caused by the HFFO diets. In this respect it is interesting to note that other *in vivo* studies (37,38) have shown that the addition of vitamin E to the diet diminishes the anticancer effects of n-3 PUFAs in mice bearing mammary tumor or lung carcinoma, suggesting that *in vivo* oxidation of n-3 PUFAs underlies their anticancer action. Also in our own *in vitro* studies, it was found that the colon cancer cell growth inhibitory effects of PUFAs with methylene-interrupted double bonds such as in fish oil are due to peroxidation products that are generated during spontaneous and enzymatic lipid peroxidation (39).

Takahashi *et al.* (8) and also other investigators (7,12,40) proposed that 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 arachidonic acid (AA; 20:4n-6) in the colonic mucosa. We measured plasma levels of PGE_2 in stead of colonic PGE_2, because also paracrine effects of basolaterally released prostaglandins could play a role in the pathogenesis of colorectal neoplasia (41). Our results demonstrate that besides an increase in *in vivo* lipid peroxidation, the rats fed the HFFO diets also had significantly lower plasma levels of PGE_2 than the rats fed the HFCO diets.

It is suggested that PGE_2 can promote rat colon tumors and that cyclooxygenase-inhibitors that prevent prostaglandin production such as indomethacin (IM) can block the

development of colon carcinomas (42). However, in the study of Onogi *et al.* (7) the PGE_2 concentration in the colonic mucosa of olive oil fed rats did not correlate with the development of ACF in that diet group. Also Abou-El-Ela *et al.* (43) found that feeding a 20% corn oil diet containing 0.004% indomethacin, an inhibitor of cyclooxygenase, significantly reduced prostaglandin synthesis but did not inhibit mammary tumorigenesis in female Sprague-Dawley rats. These results are also in line with our own *in vitro* studies in which reductions in PGE_2 synthesis by EPA (20:5n-3) comparing to AA (20:4n-6) did not lead to differential effects on cell proliferation between these fatty acids in human colon adenocarcinoma Caco-2 cells (39). Further studies are therefore needed to elucidate the real causal relation between inhibition of PGE_2 levels and inhibitory effects of n-3 PUFAs in fish oil on the development of ACF.

In summary, our data reveal that HFFO diets with high levels of n-3 PUFAs are also protective against preneoplastic lesions in the early stages of chemically-induced colon carcinogenesis compared to HFCO diets. It seems unlikely from our results that the inhibitory effect of a HFFO diet can be attributed to an altered metabolic activation and detoxification of AOM. The above mentioned results led us to consider that other mechanisms such as oxidative stress or reduction of PGE_2 levels may play an important role in the anticarcinogenic effects of n-3 PUFAs.

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Chapter 5

High fat fish oil diets modulate expression of genes involved in oxidative stress, cell growth, acute phase response and lipid metabolism in normal colonic epithelium of male F344 rats

Submitted

Yvonne E.M. Dommels[1,2]

Petra J. van den Hil[2,3]

Jozef P.H. Linssen[3]

Gerrit M. Alink[2]

Peter J. van Bladeren[2,4]

Ben van Ommen[5]

[1]WUR/TNO Centre for Food Toxicology
[2]Toxicology Group, Wageningen University, Wageningen, The Netherlands
[3]Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands
[4]Present address: Nestlé Research Center, Vers-chez-le-blanc, 1000 Lausanne 26, Switzerland
[5]TNO Nutrition and Food Research, Department of Biomolecular Sciences, Zeist, The Netherlands

Abstract

Experimental data indicate that dietary fish oil with a high amount of n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5n-3) can protect against the development of colorectal carcinogenesis. Although the complete mechanism by which n-3 PUFAs prevent or inhibit colorectal carcinogenesis is currently not known, it seems likely that the protective effects of fish oil may at least be partly due to n-3 PUFA-mediated changes in oxidative stress, PGE_2 metabolism, apoptosis and cell proliferation. To identify genes and molecular pathways responsible for the chemo-preventive effects of n-3 PUFAs, gene expression profiles of normal rat colonic epithelium of high fat fish oil (HFFO) and high fat corn oil (HFCO) fed rats were compared using cDNA microarrays. Data analyses reveal that several key genes involved in oxidative stress response (ferritin; metallothionein; peroxiredoxin 2), cell proliferation (histone 2b), cytoskeleton (beta-actin; alpha adducin), immune response (lectin; RT1 class Ib gene; interferon, alpha-inducible protein 27-like), acute phase response (albumin; hemopexin; transthyretin; transferrin; serine proteinase inhibitor; alpha(1)-inhibitor 3), energy metabolism (cytochrome C oxidase; ATPase inhibitor), lipid metabolism (apolipoprotein C3 and H) and development of the central nervous system (RNB6) were differentially expressed. Supportive physiological data reveal that HFFO diets besides an increase in plasma levels of n-3 PUFAs, a decrease in PGE_2 and an increase in oxidative stress, possibly also could protect against colorectal carcinogenesis through lower total levels of plasma fatty acids.

Introduction

High fat fish oil (HFFO) diets, with a high amount of n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA; 20:5n-3) have been shown to inhibit the initiation of azoxymethane (AOM)-induced colorectal tumors as well as the initiation of AOM-induced colonic aberrant crypt foci in rats (1,2,3,4) compared to high fat corn oil (HFCO) diets, with a high amount of n-6 PUFAs such as linoleic acid (LA; 18:2n-6).

Calviello *et al.* (5) revealed that the growth inhibitory effect of n-3 PUFAs is not specific for tumor cells, but is a general effect in normal tissues with a high degree of cell turnover. They observed that dietary administration of EPA inhibited cell proliferation and enhanced cell differentiation and apoptosis in normal rat colonic mucosa. Bartram *et al.* (6) studied the effect of fish oil and corn oil supplementation on rectal cell proliferation, ornithine decarboxylase (ODC) activity and PGE_2 release in 12 healthy volunteers. They showed that colonic cell proliferation can be reduced by oral fish oil supplementation even in young healthy volunteers who already have a quiescent proliferative activity. Concomitantly, mucosal ODC activity and PGE_2 release from rectal biopsy specimens were significantly lower during the fish oil as compared to the corn oil supplementation period (6). The results of the studies mentioned above support the idea that dietary fish oil may prevent the initiation of colorectal carcinogenesis and suggests a possible use of these fatty acids as dietary chemopreventive agents. Although the exact mechanism by which n-3 PUFAs prevent or inhibit colorectal carcinogenesis is currently not known, it seems likely that the protective effects of fish oil may at least be partly due to n-3 PUFA-mediated changes in oxidative stress (7,8), PGE_2 metabolism (3), apoptosis (7,9,10) and cell proliferation (11,12).

Our previous *in vitro* studies suggest that the number of double bonds in the carbon atom chain of fatty acids, such as EPA in fish oil, is more important for the anti-proliferative and cell death-inducing effects in colon tumor cells than the place of the double bond. The number of double bonds affects the susceptibility for lipid peroxidation whereas the place of the double bond is important for the formation of different types of prostaglandins (for example PGE_2 from n-6 PUFAs and PGE_3 from n-3 PUFAs). Lipid peroxidation products, but not PGE_2 levels seemed to be responsible for the tumor cell growth inhibitory effects of highly polyunsaturated fatty acids such as EPA in our *in vitro* studies (13). Also our previous *in vivo* data (4) suggest that the anticarcinogenic effects of n-3 PUFAs in HFFO diets, could be ascribed to a higher level of lipid peroxidation, which is a marker of oxidative stress.

To study the increased oxidative stress caused by HFFO diets in more detail, another animal experiment was performed in which different (anti)-oxidant parameters in HFFO and HFCO fed rats were compared. Gene expression profiles of normal rat colonic

epithelium of HFFO and HFCO fed rats were compared using cDNA microarrays to identify genes and molecular pathways which are responsible for the chemopreventive effects of HFFO diets. This comprehensive approach can provide more insight into the multiple minor changes in genomic responses by n-3 PUFAs governing the modulation of oxidative stress but also prostaglandin metabolism, apoptosis and cell proliferation. Furthermore these transcriptomics experiments may also provide new mechanistic insights into the molecular effects of n-3 PUFAs on normal rat colonic epithelium. Other biochemical assays were also performed in this study in order to provide additional physiological data that support the identified changes in gene expression between HFFO and HFCO fed rats.

Material and methods

All reagents were obtained from Sigma (St Louis, MO, USA), unless otherwise indicated.

Animals and diets

Twenty male F344 rats (~ 3 weeks old) were purchased from Charles River Breeding Laboratory (Someren, The Netherlands) and acclimatized for 1 week. The rats were housed in plastic cages, 2 rats per cage, in an animal holding room using a 12:12-hour light-dark cycle. Temperature and humidity were controlled at 20-24 °C and 50-60%, respectively. Animals were randomly divided into 2 dietary sub-groups of ten animals per group and had free access to water and food. Fresh food in powder form was replenished every day. The experimental diets, high fat fish oil (HFFO) and high fat corn oil (HFCO), were formulated on the basis of a modified AIN-93G diet, as described in Table 5.1. The HFCO diet contained 25% corn oil (Remia, Den Dolder, The Netherlands) at the expense of an isocaloric amount of carbohydrates. The HFFO diet contained 20% Omegapure™ Refined Menhaden Fish oil, which was kindly donated by Omegaprotein (Houston, TX, USA) and 5% corn oil to prevent any essential fatty acid deficiency, also at the expense of an isocaloric amount of carbohydrates. 1000 ppm Mixed-tocopherols and 200 ppm tertiary butyl hydroquinone (TBHQ) were added to the Omegapure™ Refined Menhaden Fish oil to prevent lipid peroxidation of the oil. These antioxidants were therefore also added to the corn oil before preparation of the HFCO diet. The amount of L-cystine, cellulose, choline bitartrate, AIN-93 mineral and vitamin mix were adjusted with a factor 1.24 above the recommended amounts (14) to compensate for the reduced food intake of high fat diets (15). Diets were prepared freshly at the beginning of the experiment and after one month. Daily batches were stored in sealed plastic bags in dark containers at -40 °C until use. The peroxide values of the

freshly prepared diets and of the diets that were stored for one month were measured to determine the amount of *in vitro* fatty acid peroxidation.

Table 5.1: Composition of the experimental diets

	Diet[1]	
Dietary ingredients	HFCO	HFFO
Casein	25.00	25.00
L-cystine	0.37	0.37
Wheat starch	37.64	37.64
Cellulose	6.18	6.18
Choline bitartrate	0.25	0.25
AIN-93 minerals	4.32	4.32
AIN-93 vitamins	1.24	1.24
Corn oil	25.00	5.00
Fish oil	0.00	20.00
Peroxide values (fresh - stored)[2]		
Batch 1	2.2 - 3.6	1.8 - 3.2
Batch 2	2.8 - 3.8	2.4 - 3.8

[1] Values are expressed as percent by weight

[2] Peroxide values are expressed in mEq/kg diet in freshly prepared diets - and diets stored for one month at -40 °C

Experimental procedure

The experimental protocol was approved by the animal welfare committee of Wageningen University (The Netherlands).

Beginning at 4 weeks of age, rats were fed the experimental diets for 8 weeks. Body weights were recorded three times a week and daily food intake was measured during three separate periods (day 1-9; day 30-36 and day 48-56). Rats were housed individually during the second and third period. Blood was collected by orbital puncture at day 7 and 29 of the experiment. At the end of the experiment, blood was collected from the vena cava inferior under anesthesia, which was a combination of isofluran, O_2 and N_2O. Plasma was separated by centrifugation at 1000 g for 5 minutes. Also liver and colon were removed. Livers were quickly weighed, snap-frozen in liquid nitrogen and

stored at -80 °C until further processing. The colon was laid flat on filter paper and was opened longitudinally. The colonic epithelium was gently scraped using a spatula after removal of the faeces. The scrapings were also snap-frozen in liquid nitrogen and stored at -80 °C until further processing.

RNA isolation

Total RNA was isolated from colonic epithelial scrapings of 5 rats per group using Trizol (Life Technologies S.A., Merelbeke, Belgium) according to the manufacturer's protocol. Total RNA was further purified using the RNeasy Mini Kit (Qiagen, provided by Westburg, Leusden, The Netherlands) and DNA was digested using the Rnase-Free Dnase set (Qiagen). RNA was checked for purity and stability by gel electrophoresis and the concentration was calculated from the extinction at 260 nm as measured spectrophotometrically.

Reference RNA

To allow comparison of all individual expression patterns of the fish oil and corn oil fed rats, a batch of common reference RNA was prepared. Dissected organs of several untreated male rats were flash-frozen in liquid nitrogen and stored at -80 °C. All tissues were pooled together and homogenized in liquid nitrogen. RNA was isolated as described above.

Transcriptomics experimental design

Two animals per group were initially used to assess the difference in colonic epithelial gene expression between fish oil and corn oil fed rats. Each individual rat colonic epithelial RNA sample was co-hybridized with the common reference RNA. Dye-swap replications were used, in which the dye assignments were reversed between the treated (fish / corn oil) and the reference sample in the second hybridization. At a later time-point, a second dataset was generated where 6 other rat colonic epithelial RNA samples (3 fish oil; 3 corn oil) were co-hybridized with the common reference RNA with dye-swaps in order to increase the biological replicates and to confirm previous results. This second dataset thus consisted of 12 arrays (3 x 2 x 2).

cDNA microarray preparation

About 3000 different sequence-verified rat cDNA clones from the I.M.A.G.E. consortium were purchased (Research Genetics, USA) and cDNAs were amplified by PCR using forward (5'-CTGCAAGGCGATTAAGTTGGGTAAC-3') and reverse (5'-GTGAGCGGA-TAACAATTTCACA-CAGGAAACAGC-3') primers containing a 5'-C6-aminolinker

(Isogen Bioscience, Maarsen, The Netherlands) to facilitate crosslinking to the aldehyde coated glass microscope slides. PCR products were purified by ethanol precipitation and checked for purity and fragment size by gel electrophoresis on a 1% agarose gel. Purified PCR products were dissolved in 3x SSC and clones were arrayed in a controlled atmosphere on CSS-100 silylated aldehyde glass slides (Telechem, Sunnyvale, CA, USA). In total, about 3800 cDNAs, including 3000 different rat genes and various control genes were deposited on the slides. After drying, slides were blocked with borohydride and stored in a dark and dust-free cabinet until further use. The second dataset was generated with a new different batch of slides, although the same PCR products as well as the same type of glass slides were used as for the first dataset.

cDNA synthesis, labeling and hybridization

The CyeScribe First-strand cDNA Labeling Kit of Amersham Biosiences (Freiburg, Germany) was used for the generation of Cy3 and Cy5 labeled first-strand cDNA probes utilizing a modified reverse transcriptase CyScript™.

Briefly, 25 μg of total RNA was reverse transcribed using oligo(dT) primers. After annealing of the primers at 70 °C for 5 minutes, Cy3- or Cy5-labeled dUTP was built into the cDNA during reverse transcription. After incubating the reaction for 1.5 hours at 42 °C, RNA was hydrolyzed by adding NaOH. After neutralization, unincorporated nucleotides were removed with spin column chromatography using Autoseq G-50 spin columns (Amersham Biosciences, Freiburg, Germany).

Before hybridization, Cy3- and Cy5-labeled cDNAs were mixed and 4 μl yeast tRNA (25 μg/μl) (Life Technologies S.A., Merelbeke, Belgium), 4 μl poly(dA-dT) (5 μg/μl) (Amersham Biosiences, Freiburg, Germany) and 3 μl Cot-1 DNA (Life Technologies S.A, Merelbeke, Belgium) were added to avoid non-specific binding. The hybridization mix was concentrated, dissolved in 55 μl Easyhyb hybridization buffer (Roche Diagnostics, Mannheim, Germany), denatured for 1.5 minutes at 100 °C and incubated for 30 minutes at 42 °C.

Before adding the hybridization mixture to the slides, slides were prehybridized in prehybridization buffer (1% BSA, 5x SSC and 0.1% SDS, filtered) for 45 minutes at 42 °C, washed in milliQ water, washed with isopropanol and dried.

After pipetting the hybridization mix onto the slides, the slides were covered with a plastic coverslip and hybridized overnight in a slide incubation chamber (Corning, Life Sciences, Schiphol, The Netherlands) submerged in a 42 °C waterbath. After hybridization, slides were washed by submersion and agitation in 1x SSC with 0.2% SDS and in 0.5x SSC wash-solution. Then, slides were washed in a fresh 0.2x SSC wash-solution and shaken

on a rotation plateau for 10 minutes at 300 rpm. This step was repeated once and slides were dried afterwards by centrifugation at 700 rpm for 5 minutes.

Scanning

Slides were scanned with a ScanArray 4000 (Perkin Elmer Life Sciences, USA) and Imagene 4.0 (Biodiscovery Inc., Los Angeles, USA) was used to extract data from the images.

Data analysis

Data were transferred from Imagene into SAS E-guide for analysis. Spots with a signal/background ratio less than 2 or that were flagged in Imagene were not included in the data analysis. For each spot, local background intensity was subtracted from mean signal intensity and the expression ratio was calculated by dividing background corrected signal intensities of the treated samples (fish oil or corn oil) by the background corrected signal intensities of the common reference sample. Expression ratios were then log transformed (base 2) and normalized per slide using an intensity-dependent method (Lowess) (16).

The group average log ratio in gene expression ^{2}log (fish oil / corn oil) is calculated in Microsoft Excel 97. As a consequence of the introduction of the reference sample, comparisons between fish and corn oil fed rats are indirect (the expression levels of corn oil and fish oil samples are measured separately on different slides). Therefore, the log ratio ^{2}log (fish oil / corn oil) is calculated by the difference ^{2}log average (fish oil / reference) - ^{2}log average (corn oil / reference). Group log ratios were calculated for both the first and the second dataset, only if the expression ratios (fish; corn oil / reference) were present in 70% of the total arrays used per group. Also group average of fold changes in gene expression was calculated for both the first and the second dataset.

Principal Component Analysis (PCA), a well-established *unsupervised* statistical method for multivariate interpretation of complex data was used to visualize the highest explained variance in the datasets. This analysis revealed variation, probably technical variation, between the first and second dataset. Therefore, they were treated as two independent datasets.

Principal Component-Discriminant Analysis (PCDA), a *supervised* multivariate statistical analysis was used to discriminate the genes that contributed most (upper 5%) to the differences observed between the fish oil and corn oil treated rats. Statistical significance of the differences in gene expression between the colonic epithelial samples of fish oil and corn oil fed rats were also analyzed with the univariate two-tailed two-sample unequal variance based Student's t-test and with SAM (Statistical Analysis of Microarrays)

(17). Differences in individual gene expression levels were considered significant in the Student's t-test if p-value < 0.05 in both the first and the second dataset. SAM uses repeated permutations of the data to determine if the expressions of any genes are significantly related to the response. A q-value is generated which is the lowest False Discovery Rate at which the gene is called significant. Genes that are significant in the first as well as the second dataset are listed in the results session.

Hepatic lipid peroxidation

The thiobarbituric acid assay according to Uchiyama and Mihara (18) was used to determine the amount of lipid peroxidation in liver microsomes. 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. Lipid peroxidation in liver microsomes was measured at double wavelengths (535 + 520 nm) to avoid interference. The difference in absorbance was converted to μmol MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane. Lipid peroxidation was presented as μmol MDA per gram tissue.

Antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) assay was used to analyze the *in vitro* antioxidant capacity of the plasma samples of both diet groups. The assay used was originally described by Miller *et al.* (19) and was adapted according to Van den Berg *et al*. (20). Briefly, an $ABTS^{\cdot-}$ solution was prepared by mixing 2.5 mM ABAP (2,2'-azobis-(2-amidinopropane)HCL) from Polysciences (Warrington, PA, USA) with 20 mM $ABTS^{2-}$ (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) stock solution in 100 mM phosphate buffer (pH 7.4), containing 150 mM NaCl. The solution was heated for 12 minutes at 60 °C, protected from light and stored at room temperature. For measuring antioxidant capacity, 40 μl plasma (1:10 dilution) was mixed with 1960 μl of the radical solution. The absorbance was monitored at 734 nm for 10 seconds ('fast' TEAC) and for 6 minutes ('slow' TEAC). The decrease in absorption at 6 minutes after addition of the plasma samples was used for calculating the antioxidant capacity, expressed as (+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (TROLOX) (Aldrich, Milwaukee, WI, USA) equivalents.

Hepatic antioxidant enzyme activities

Enzyme activities of catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were measured in liver cytosolic fractions. Livers were homogenized in three volumes of ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and

1 mM DTT, using a Potter tube and centrifuged for 30 minutes at 12000 g at 4 °C. The resulting supernatant was centrifuged for 75 minutes at 105000 g at 4 °C. The microsomal pellet was resuspended in two volumes ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 3 mM EDTA and 1 mM DTT. Both microsomal and cytosolic fractions were stored in aliquots at -80 °C until further analysis. Protein levels were determined according to Bradford (21).

CAT activity was measured according to Aebi (22). The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm in a quartz cuvette. Reaction mixtures contained 1 ml of 0.1 M H_2O_2 (Merck, Hohenbrunn, Germany) and 2 ml of liver cytosol (dilution 1:1000). The blank contained 50 mM phosphate buffer instead of substrate. Catalase activity is expressed as μmol H_2O_2 decomposed / min per mg protein.

GSH-Px activity was assayed with a coupled enzyme system in which GSSG is reduced by glutathione reductase in the presence of an adequate supply of NADPH (23). The reaction mixture contained 500 μl 0.1 M phosphate buffer (pH 7.0), 100 μl enzyme sample (1:10 dilution), 100 μl glutathione reductase (0.24 U) and 100 μl of 10 mM GSH (Acros Organics, New Jersey, USA). This mixture was pre-incubated for 10 minutes at 37 °C. Thereafter, 100 μl NADPH (Roche Diagnostics GmbH, Mannheim, Germany) solution was added and the H_2O_2-independent consumption of NADPH was monitored. The overall reaction was started by adding 100 μl of prewarmed (37 °C) H_2O_2 and the decrease in absorbance was monitored at 340 nm. The non-enzymatic reaction rate was measured by replacing the enzyme sample by buffer. Glutathione peroxidase activity is expressed as μmol NADPH oxidized / min per mg protein.

SOD activity was measured using the RANSOD Superoxide Dismutase Kit of Randox (catalog number SD 125). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The superoxide dismutase activity in the liver cytosol samples (1:500 diluted with RANSOD Sample Diluent, Randox, catalog number SD 124) is then measured by the degree of inhibition of this reaction as compared to SOD standards. Analyses were done in duplicate at the Hitachi 911 analysator at 505 nm. Results are expressed as units / mg protein.

Hepatic levels of GSH and GSSG

GSH and GSSG levels were determined in 5% sulfosalicylic acid supernatant solutions of liver homogenates according to Anderson (24). GSH is oxidized by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to give GSSG and TNB. The rate of TNB formation is measured at 405 nm. The amount of GSH is determined from a standard curve. The same DTNB-GSSG reductase-recycling assay was used to determine hepatic GSSG

levels after derivatisation of GSH with 2-vinylpyridine. Results are expressed as μmol / gram liver.

Determination of vitamin E in plasma

Total lipids in the blood plasma after 8 weeks of dietary intervention were extracted by the method of Bligh and Dyer (25) and dissolved in ethanol. Vitamin E was measured fluorometrically in ethanol (excitation and emission wavelengths, 295 nm and 340 nm respectively) (26). Vitamin E levels were expressed as mmol / l plasma via a standard curve generated with alpha-tocopherol.

Plasma levels of fatty acids

Total lipids in the blood plasma after 8 weeks of dietary intervention were extracted by the method of Bligh and Dyer (25) and phospholipids were isolated according to Kaluzny *et al*. (27). Total extracted phospholipids were methylated according to Morrison and Smith (28). The methyl esters of the fatty acids were analyzed on a Carlo Erbal instruments HRGC 5300 gas chromatograph (Interscience, Breda, The Netherlands) using cold-on-column-injection and flame ionization detection. The temperature program starts at 80 °C, followed by a temperature increase of 20 °C/min until 200 °C and 5 °C/min until 240 °C with a final hold for 10 minutes. Samples were analyzed on an OMEGAWAX™ 320 fused silica capillary column, 30 m x 0.32 mm; 0.25 μm film thickness (Supelco, Bellefonte, USA). Individual fatty acids were identified by comparison with two standard fatty acid mixtures, PUFA nr. 2 Animal source (Sigma 47015U) and PUFA nr. 3 from Menhaden oil (Sigma, 47085U). Identification was verified and confirmed by GC-MS (Finnigan MAT 95). For fatty acid quantification of plasma phospholipids, a known amount of the phospholipid 1,2-Dinonadecanoyl-*sn*-Glycero-3-Phosphocholine (Avanti Polar Lipids, Alabaster, USA) was added to each plasma sample as an internal standard. Fatty acid levels in plasma phospholipids are expressed as mg / ml plasma. The fatty acid composition of the Omegapure™ Refined Menhaden Fish Oil and the corn oil was checked by the same method as described above. Fatty acid levels in both of the oils are expressed as % of total fatty acids detected.

PGE_2 levels in plasma

PGE_2 levels were analyzed in the plasma of all rats after 1, 4 and 8 weeks of dietary intervention. A PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech UK limited, Buckinghamshire, UK) was used according to protocol 5, recommended by the manufacturer. The results are expressed as picograms of PGE_2 per ml plasma.

Statistical analysis

Statistical analyses for the physiological data were performed using SPSS version 10.1 for Windows. Data were analyzed using one-way ANOVA after testing for normality and homogeneity of variance. When data were not normally distributed the non-parametric Mann-Whitney U test was used.

Results

Body + liver weight and food intake

The type of dietary lipid did not affect body weights of the rats. As seen in Table 5.2, average body weights of the HFCO and HFFO diet groups were not significantly different at the end of the experiment. Also the food intake was not affected by the type of dietary lipid. Liver weight as well as the liver-to-body weight ratio was significantly higher for the rats fed the HFFO diet than for the rats fed the HFCO diet, at the end of the experiment.

Table 5.2: Effect of dietary lipid on body and liver weight and food intake in male F344 rats[1]

	Diet	
	HFCO	HFFO
Initial body weight (g)	45.39 ± 2.74	46.01 ± 3.15
Final body weight (g)	280.17 ± 12.83	286.61 ± 17.71
Liver weight (g)	10.10 ± 0.70[a]	13.17 ± 1.00[a]
Relative liver weight (%)	3.61 ± 0.17[b]	4.60 ± 0.22[b]
Food intake (g)[2]	10.19 ± 3.13	10.60 ± 3.77

[1] Values are means ± SD per diet group (n=10 per group)

[2] Food intake is defined as the average food intake per day per rat, measured during three separate periods of the experiment (day 1-9, day 30-36 and day 48-56)

[a,b] Means between diet groups sharing the same letter superscripts are significantly different at $p < 0.01$

Transcription profiling results

Several statistical methods were applied to identify differentially expressed genes between colonic epithelial samples of fish oil and corn oil fed rats, in order to identify genes and molecular pathways responsible for the anti-colon carcinogenic effects of HFFO diets.

Genes that were differentially expressed in the first dataset and that were confirmed as differentially expressed in the second dataset are reported in Table 5.3.
Table 5.3 summarizes the results of the PCDA analysis, with the different diet groups used as discriminant. These results indicate that 21 genes belong to the upper 5% of the genes that contributed most to the differences in group means in both sets of slides. Biochemical functions of the differential expressed genes are diverse and involve oxidative stress response (ferritin; metallothionein), cell adhesion/cytoskeleton (actin, beta; alpha adducin), immune response (lectin; RT1 class Ib gene), acute phase response (albumin; hemopexin; transthyretin; transferrin; serine proteinase inhibitor; alpha(1)-inhibitor 3), energy metabolism (cytochrome C oxidase; ATPase inhibitor) and lipid metabolism (apolipoprotein C3 and H).
Significantly differentially expressed genes, as determined by the Student's t-test (see Table 5.4) also include genes that are involved in oxidative stress response (ferritin light chain 1; peroxiredoxin 2) and immune response (interferon, alpha-inducible protein 27-like). Furthermore, histone 2b, a gene that is involved in cell proliferation is significantly decreased and RNB6, a gene that is involved in the development of the central nervous system is significantly increased in the colonic epithelium of HFFO fed rats compared to HFCO fed rats. SAM also indicated interferon, alpha-inducible protein 27-like as well as ferritin light chain 1 as significantly differentially expressed genes.

Table 5.3: Differentially expressed genes in rat colonic epithelium between HFFO and HFCO fed rats, as determined by PCDA analyses[1]

AC #	Gene Name	IF FO/CO first dataset	IF FO/CO second dataset
AA859846	Actin, beta	1.27	1.33
AA818469	ESTs, highly similar to S54147 alpha-adducin	1.42	1.20
AA859797	Lectin, galactose binding, soluble 3	1.49	1.22
AA924062	RT1 class Ib gene	1.54	1.33
AA818960	Albumin	0.33	0.76
AA819042	Hemopexin	0.61	0.76
AA818637	Transthyretin (pre-albumin, amyloidosis type I)	0.41	0.81
AA858975	Transferrrin	0.42	0.77
AA819467	Serine (or cysteine) proteinase inhibitor, clade A, member 1	0.72	0.86
AA817963	Alpha(1)-inhibitor 3, variant I	0.60	0.80
AA818441	Ferritin, heavy polypeptide 1	1.57	1.25
AA924281	ESTs, highly similar to MT2_RAT metallothionein-II	1.66	1.19
AA859399	Metallothionein	1.84	1.20
AA900218	Metallothionein	1.43	1.19
AA998201	Cytochrome C oxidase, subunit Va	1.63	1.20
AA819164	ATPase inhibitor	0.72	0.67
AA874885	ESTs, highly similar to RS4_HUMAN 40S ribosomal protein S4, X isoform	1.53	1.11
AA819465	Apolipoprotein C3	0.79	0.98
AA819890	ESTs, highly similar to APOH_RAT Beta-2-glycoprotein I precursor (Apolipoprotein H)	0.77	0.98
AA818706	Group-specific component (vitamin D-binding protein)	0.41	0.92
AA901407	Betaine-homocysteine methyltransferase	0.66	0.83

[1] The table contains the GenBank accession numbers (AC#) of the cDNA fragments present on the microarray, the name of the gene, the average induction factor (IF) in gene expression of fish oil (FO) versus corn oil (CO) fed rats of both the first and the second dataset

Table 5.4: Differentially expressed genes in rat colonic epithelium between HFFO and HFCO fed rats, as determined by the Student's t-test[1]

AC#	Gene Name	Average FO/CO *first dataset* IF	p-value	Average FO/CO *second dataset* IF	p-value
AA955192	Interferon, alpha-inducible protein 27-like	1.33	0.026	1.32	0.001
AA819034	ESTs, weakly similar to interferon, alpha-inducible protein 27-like	2.09	0.003	1.42	0.001
AA817693	Ferritin light chain 1	1.52	0.027	1.39	0.008
AA819472	Ferritin light chain 1	1.58	0.037	1.24	0.015
AA858972	Peroxiredoxin 2	1.34	0.034	1.30	0.042
AA964055	Histone 2b	0.82	0.003	0.84	0.046
AA997968	RNB6	1.31	0.032	1.21	0.044

[1] The table contains the GenBank accession numbers (AC#) of the cDNA fragments present on the microarray, the name of the gene, the average induction factor (IF) in gene expression of fish oil (FO) versus corn oil (CO) fed rats of both the first and the second dataset, as well as the p-value of the Student's t-test

Plasma fatty acid + PGE_2 levels

Omegapure™ Menhaden Fish oil contains high amounts of long-chain n-3 PUFAs, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Corn oil contains high amounts of long-chain n-6 PUFAs, such as linoleic acid (LA; 18:2n-6) (Table 5.5). These differences in fatty acid composition of the oils reflected the differences in fatty acid composition of the plasma phospholipids after 8 weeks of dietary treatment. Table 5.6 shows that fish oil feeding increased the levels of EPA and DHA and decreased the levels of linoleic acid and arachidonic acid (AA; 20:4n-6) compared to corn oil feeding, resulting in a lower total n-6 / n-3 ratio. Overall, the total levels of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) as well as the total level of fatty acids in plasma phospholipids were significantly lower at 8 weeks in the HFFO diet group compared to the HFCO diet group. Addition of fish oil to the diet also altered the plasma levels of prostaglandins. As shown in Figure 5.1, high dietary levels of fish oil suppressed PGE_2 levels in plasma compared to high dietary levels of corn oil after 1, 4 and 8 weeks of dietary treatment.

Table 5.5: Fatty acid composition of Corn Oil and Omegapure™ Refined Menhaden Fish oil[1]

Fatty acid	Corn Oil	Fish Oil
14:0	nd	9.75 ± 0.31
16:0	11.61 ± 0.23	18.88 ± 0.17
16:1n-7	nd	13.82 ± 0.38
16:2n-4	nd	2.55 ± 0.09
16:3n-4	nd	3.14 ± 0.12
18:0	1.45 ± 0.01	3.14 ± 0.38
18:1n-9	26.46 ± 0.12	9.61 ± 0.89
18:1n-7	nd	3.36 ± 0.04
18:2n-6	59.61 ± 0.11	1.63 ± 0.55
18:3n-6	nd	0.43 ± 0.09
18:3n-4	nd	0.73 ± 0.05
18:3n-3	0.87 ± 0.00	1.41 ± 0.06
18:4n-3	nd	3.16 ± 0.07
20:1n-9	nd	1.04 ± 0.02
20:4n-6	nd	1.19 ± 0.43
20:4n-3	nd	1.47 ± 0.08
20:5n-3	nd	13.59 ± 0.82
22:4n-6	nd	0.30 ± 0.10
22:5n-3	nd	2.07 ± 0.15
22:6n-3	nd	8.72 ± 0.63

[1] Values are means ± SD of 2 determinations

Data are expressed as % of total fatty acids detected

nd = not detected

Table 5.6: Influence of HFCO and HFFO diets on plasma levels of fatty acids after 8 weeks of dietary intervention[1]

	Diet	
Fatty acids[2]	HFCO	HFFO
14:0	0.036 ± 0.018[a]	nd[a]
16:0	0.390 ± 0.065[a]	0.180 ± 0.004[a]
18:0	0.389 ± 0.057[a]	0.140 ± 0.007[a]
18:1n-9	0.196 ± 0.066[a]	0.033 ± 0.002[a]
18:1n-7	0.034 ± 0.019[a]	0.012 ± 0.001[b]
18:2n-6	0.152 ± 0.028[a]	0.088 ± 0.005[a]
20:4n-6	0.234 ± 0.031[a]	0.058 ± 0.003[a]
20:5n-3	nd[a]	0.023 ± 0.002[a]
22:6n-3	0.025 ± 0.004[a]	0.042 ± 0.002[a]
SFA	0.815 ± 0.134[a]	0.320 ± 0.010[a]
MUFA	0.230 ± 0.076[a]	0.045 ± 0.002[a]
PUFA	0.412 ± 0.056[a]	0.210 ± 0.008[a]
Total n-6	0.387 ± 0.053[a]	0.146 ± 0.006[a]
Total n-3	0.025 ± 0.004[a]	0.065 ± 0.003[a]
Ratio n-6 / n-3	15.82 ± 1.15[a]	2.26 ± 0.05[a]
Total	1.458 ± 0.242[a]	0.576 ± 0.019[a]

[1] Values are means ± SEM per diet group (n=10 per group)

[2] Fatty acid levels in plasma phospholipids are expressed as mg / ml plasma

[a] Means between diet groups sharing the same letter superscripts within a row are significantly different at $p < 0.05$

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detected

Oxidative stress response

Because PUFAs are very susceptible to auto-oxidation, antioxidants were added to both oils to prevent *in vitro* lipid peroxidation (29). Peroxide values of the freshly prepared HFCO and HFFO diets were < 3 mEq/kg diet (Table 5.1). A small increase up to around 4 mEq/kg diet was observed after storage of the diets for one month at -40 °C, but peroxide values were still below 8-9 mEq/kg, which are found in other normal oils (30). The amount of *in vivo* lipid peroxidation, as measured by microsomal levels of MDA in the liver (Table 5.7), was significantly higher in the rats fed the HFFO diet than in the rats

fed the HFCO diet. This was not accompanied with a decreased antioxidant capacity, as measured by TEAC in the plasma of the HFFO fed rats, although plasma vitamin E levels were lower, though not statistically significant. Liver GSH and GSSG contents were not significantly different in rats fed either HFFO or HFCO diets. Dietary lipid source also had no effect on hepatic glutathione peroxidase and superoxide dismutase activity. However, animals fed the HFFO diet expressed higher catalase activity in the liver compared to HFCO fed rats (Table 5.7).

Table 5.7: Influence of HFCO and HFFO diets on hepatic lipid peroxidation (TBARs), plasma antioxidant capacity (TEAC), hepatic levels of GSH and GSSG, hepatic antioxidant enzyme activities and plasma levels of vitamin E[1]

	Diet	
	HFCO	HFFO
TBARs[2]	0.026 ± 0.01[a]	0.038 ± 0.007[a]
TEAC[3]	2.79 ± 0.59	2.63 ± 0.46
GSH[4]	4.34 ± 0.56	4.47 ± 0.60
GSSG[4]	0.065 ± 0.01	0.062 ± 0.01
Glutathione peroxidase[5]	0.364 ± 0.07	0.346 ± 0.07
Catalase[6]	0.435 ± 0.09[b]	0.531 ± 0.09[b]
Superoxide dismutase[7]	22.11 ± 3.54	19.48 ± 4.94
Vitamin E[8]	109.4 ± 30.26	85.1 ± 25.37

[1] Values are means ± SD per diet group (n=10 per group)

[2] TBARs are measured in liver microsomes and are expressed as μmol MDA per gram liver

[3] The TEAC of the plasma is expressed as mmol TE equivalents / liter plasma

[4] Hepatic GSH and GSSG levels are expressed as μmol / gram liver

[5] Hepatic glutathione peroxidase activity is expressed as μmol NADPH oxidized / min per mg protein

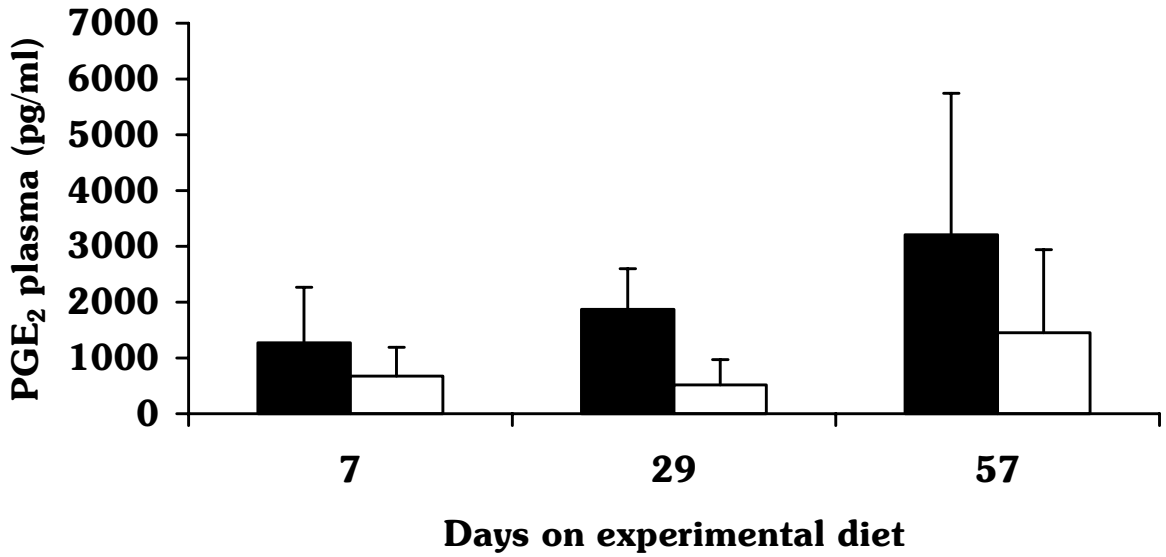
[6] Hepatic catalase activity is expressed as μmol H_2O_2 decomposed / min per mg protein

[7] Hepatic superoxide dismutase activity is expressed as units / mg protein

[8] Vitamin E levels in plasma are expressed as mmol / l plasma

[a,b] Means between diet groups sharing the same letter superscripts are significantly different at $p < 0.01$

Figure 5.1: PGE_2 levels in plasma of male F344 rats after 1, 4 and 8 weeks of HFCO (black bars) and HFFO (white bars) diets[1]



[1]Values are means ± SD (n=10 per group). Means between diet groups are significantly different ($p < 0.05$) at day 29

Discussion

Fish oil feeding resulted in significantly increased levels of EPA (20:5n-3) and DHA (22:5n-6) in plasma phospholipids and decreased levels of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) compared to corn oil feeding. This increase in EPA and DHA was accompanied with an increase in oxidative stress, as measured by microsomal levels of MDA, a decrease in plasma levels of vitamin E and a decrease in plasma levels of PGE_2. These results are in line with our previous *in vivo* data (4) and agree with a.o. Ko *et al.* (31) who also found that fish oil feeding induced changes in fatty acid composition, hepatic vitamin E and PGE_2 content and hepatic lipid peroxidation.

Fish oil is thus an oxidative stress-causing factor, which might enhance antioxidant capacities to defend against oxidative stress (31,32,33,34,35). This is confirmed by the fact that the normal liver seems to be able to adapt to the HFFO diet by increasing the activity of catalase, thereby increasing the decomposition of H_2O_2 to H_2O.

Our gene expression analysis revealed that fish oil feeding also increased the expression of antioxidant genes in the normal colonic epithelium of male F344 rats compared to corn oil feeding. The expression of for example ferritin light chain and heavy chain was induced after fish oil feeding. Ferritin is a 480-kDa intracellular protein that can store up

to 4500 atoms of iron (36). Because iron functions as a catalyst in the formation of oxygen free radicals and lipid peroxidation (36), upregulation of ferritin by fish oil may have a cytoprotective function. Also, metallothionein and peroxiredoxin 2 were upregulated by fish oil feeding in the rat colonic epithelium. Peroxiredoxins also called thiol-specific antioxidants or thioredoxin peroxidases play a key role in the antioxidant defense against hydroperoxides (37). Metallothioneins, a group of proteins characterized by a high thiol content and that bind Zn^{2+} and Cu^{2+}, are also involved in the protection against oxidative stress and are considered to have reactive oxygen scavenging activity (38,39). Mechanisms responsible for the induction of antioxidant genes in response to oxidative stress are mediated via an antioxidant-responsive element (ARE); also known as electrophile-responsive element (EpRE) (36).

Functional EpRE/ARE sequences have been identified in glutathione S-transferase Ya (GSTYa), glutathione S-transferase P1 (GSTP1), NAD(P)H:quinone oxidoreductase (NQO1), γ-glutamylcysteine synthetase heavy and light chain subunit, heme oxygenase (HO), thioredoxin, NF-E2-related factor 2 (Nrf2), ferritin heavy and light chain and metallothionein (36,38). From these genes, besides ferritin, metallothionein and peroxiredoxin 2 also NQO1 and GSTP1 were significantly upregulated by fish oil feeding. This was however only observed in the first dataset and not confirmed in the second dataset.

The above mentioned results implicate thus indeed that fish oil is an oxidative stress-causing agent that can enhance antioxidant capacities in normal healthy tissue in order to defend against oxidative stress. The increased antioxidant capacity seems however specific for normal cells, because tumor cells have been found to have reduced antioxidant defense mechanisms (40) and seemed to have lost the ability to upregulate antioxidant defensive enzymes (32). Deficiencies in antioxidant defense systems in tumor cells may be responsible for the fact that n-3 PUFAs have been shown to selectively kill tumor cells. Higher levels of oxidative stress in for example colon tumor cells could cause these cells to die by apoptosis or necrosis (41), while causing no real harm to normal cells (42,43,44). Several lines of *in vitro* and *in vivo* evidence (44,45) including ours (13) indeed suggest that lipid peroxidation is involved in cytotoxic and tumor cell-growth inhibiting effects of PUFAs, such as EPA in fish oil.

Aw (41) proposed an interesting working hypothesis of lipid peroxide-induced cell proliferation and apoptosis in the intestine. Low levels of oxidative stress are required for normal tissue homeostasis (e.g. is involved in cell proliferation). Intermediate levels of oxidative stress might cause selective gene activation and protein expression, which can be considered as an adaptive response to this increased oxidative stress (e.g. antioxidants, stress proteins). If the oxidant dose is high enough and antioxidants are not

able to sufficiently protect, the cells may be pushed beyond the maximal point of cell division into the apoptotic phase. At high cytotoxic levels of oxidative stress cells can die by necrosis.

Our transcriptomics experiments also reveal that genes involved in growth control pathways such as cell proliferation are significantly altered in colonic epithelium of HFFO fed rats compared to HFCO fed rats. For example histone 2b, a gene whose expression has been associated with cellular proliferation is significantly decreased in the normal rat colon after fish oil feeding. Thompson *et al.* (46) found that histone 2b mRNA levels increase during S phase in continuously growing cells and decrease when cell replication ceases in density-arrested cultures. Lower mRNA levels of histone 2b might thus imply that the cellular proliferation is decreased in the colonic epithelium of HFFO fed rats compared to HFCO fed rats. Although the hypothesis that HFFO diets could prevent or inhibit colorectal carcinogenesis via oxidative stress-related modulation of cell proliferation and apoptosis is still speculative and only a few genes in these pathways were differentially expressed in our study, our results do reveal that genes involved in oxidative stress and cell growth pathways are indeed modulated by a HFFO diet.

Okamura *et al*. (47) found different genes whose transcription is modulated in the process of growth arrest induced by wild-type p53. These genes are related to cell cycle regulation (transferrin), cell respiration (cytochrome C oxidase) and cytoskeleton structure. We also found differentially expressed genes related to the cytoskeleton (↑) and cell respiration (energy metabolism) (↑), but also genes related to the immune response (↑), acute phase response (↑/↓) and lipid metabolism (↓) were markedly altered in the colonic epithelium after fish oil feeding. For example, interferon alpha-inducible protein 27-like is significantly upregulated by fish oil feeding. Upregulation of interferon-alpha can cause profound physiological changes in the cell, which include induction of an antiviral state but also inhibition of cellular proliferation and modulation of differentiation via induction of interferon alpha-stimulated genes (48). Tumor suppressor gene p53, which is induced by reactive oxygen-induced damage (49), was also increased (IF FO/CO 1.270, p-value = 0.0589) after fish oil feeding in this study. This was however also only observed in the first dataset and not confirmed in second dataset.

Fish oil feeding also clearly elicited a response well known as the acute-phase protein response (APPR). The APPR is a defensive response, seen following various types of stress including trauma, infection, inflammation and cancer (50). APPR-genes that are altered in the colonic epithelium after fish oil feeding include the negative acute phase proteins such as albumin, prealbumin (transthyretin) and transferrin and positive acute phase proteins such as hemopexin, α-1-inhibitor 3, α-1-antitrypsin (serine proteinase inhibitor, clade A, member 1), ferritin and metallothioneins. Nuclear transcription factor

κB (NF-κB) is one of the main factors, which is involved in the induction of gene transcription during the acute-phase response (51). Activation of an APPR by fish oil might thus be mediated via activation of the oxidant- and redox-sensitive NF-κB.

Another group of genes, which are differentially expressed in the normal rat colonic epithelium after fish oil feeding are related to lipid metabolism. Both apolipoprotein C3 and H are downregulated after fish oil feeding compared to corn oil feeding. Apolipoprotein C3 (apoC3) is a 79 amino acid long glycoprotein that is synthesized predominantly in the liver and to a lesser extent in the intestine (52). It is present on very low lipoproteins (VLDLs) and chylomicron remnants and to some extent on high-density lipoproteins (HDLs). Although the precise, function of apoC3 is not clearly understood, several lines of evidence suggest that increased expression is associated with hypertriglyceridemia, whereas the absence of the gene in knock out mice lead to reduced plasma triglyceride (TG) levels (52). β2-Glycoprotein (β2GP), also known as apolipoprotein H, is a plasma glycoprotein of approximately 50 kDa. It is also associated with VLDLs, HDLs and chylomicrons. β2GP has been implicated in a variety of physiological pathways, including blood coagulation and haemostasis (53).

Fish oil thus alters apolipoprotein gene expression, which may lead to lower plasma triglyceride concentrations (54,55). In this study we also found that fish oil feeding had a hypolipidemic effect in the plasma of male F344 rats compared to corn oil feeding. Overall, the total levels of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) as well as the total level of fatty acids in plasma phospholipids were significantly lower at 8 weeks in the HFFO diet group compared to the HFCO diet group. Besides the n-3 PUFA-induced hypolipidemia in the circulation also changes in liver metabolism may occur that favor lipid deposition. In accordance with this, hepatomegaly has been observed in the rats fed the HFFO diets. This may be caused, at least in part, by excess liver lipid accumulation due to a decreased capacity of the rat liver to transport triacylglycerol out of the hepatocyte after a diet rich in fish oil (56).

n-3 PUFA-induced hypolipidemia in the circulation already appeared to have an important role in the control of coronary heart disease and other atherosclerotic disorders (57,58). Also experimental animal studies have shown that high dietary fat is a risk factor in colon cancer and that low fat diets reduce the risk of colon carcinogenesis (59). A possible mechanism that has been proposed to explain this protective effect of a low fat diet on colon carcinogenesis includes the lowering of secondary bile acids, which can act as tumor promoters by inducing cell proliferation (59,60). HFFO diet might thus, besides an increase in plasma levels of n-3 PUFAs, a decrease of PGE_2 and an increase in oxidative stress possibly also protect against colon carcinogenesis through lower

basolateral levels of total fatty acids. The exact role of this hypolipidemic effect in the chemopreventive effects of HFFO diets on colon cancer however remains still to be elucidated.

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Chapter 6

n-6 and n-3 polyunsaturated fatty acid-induced transcriptome changes in Caco-2 cells

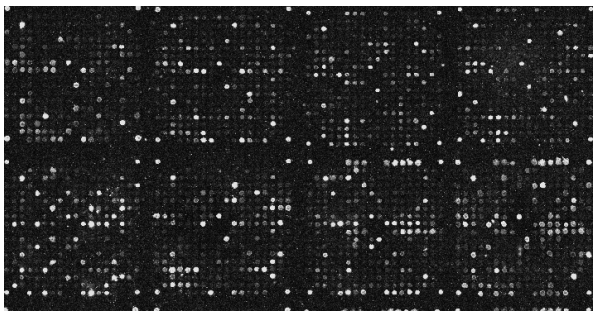
In preparation

Yvonne E.M. Dommels[1,2]

Gerrit M. Alink[2]

Peter J. van Bladeren[2,3]

Ben van Ommen[4]



[1]WUR/TNO Centre for Food Toxicology
[2]Toxicology Group, Wageningen University, Wageningen, The Netherlands
[3]Present address: Nestlé Research Center, Vers-chez-le-blanc, 1000 Lausanne 26, Switzerland
[4]TNO Nutrition and Food Research, Department of Biomolecular Sciences, Zeist, The Netherlands

Abstract

Transcriptome changes induced in human adenocarcinoma Caco-2 cells, after 48 hour incubation of two concentrations of linoleic acid (LA; 18:2n-6), arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3), were studied to identify molecular mechanisms explaining the physiological differences in cell growth inhibiting and cytotoxic effects between n-6 and n-3 PUFAs. Incubation of Caco-2 cells with 20 μM LA, AA or EPA resulted in similar changes in expression of genes involved in oxidative stress, cell growth, differentiation and apoptosis, cell adhesion, lipid metabolism, immune system and energy metabolism. No differences in expression of genes involved in COX-mediated PGE_2 signaling such as EP receptor subtypes, prostaglandin E synthase, phospholipase A2, a prostaglandin transporter and COX-2 were observed between EPA and AA. Down- and upregulated genes between EPA and LA could be classified in functional groups related to colon carcinogenesis such as cell proliferation, differentiation and apoptosis, and extracellular matrix and cell migration. However, although transcriptomic expression of 17.000 genes was measured, no dramatic and consistent changes in biological pathways between EPA and LA were observed after 48 hours of incubation, which makes it difficult to predict the physiological effect. In conclusion, measuring subtle changes in mRNA expression by microarrays at a single time-point does not fully explain or predict the mechanism of action of the colon cancer inhibiting effects of n-3 PUFAs in Caco-2 cells.

Introduction

Many experimental animal studies have demonstrated that diets containing fish oil with a high amount of n-3 polyunsaturated fatty acids (PUFAs) have a protective effect on chemically-induced colon carcinogenesis (1,2,3). N-3 PUFAs also suppress colon tumor cell growth *in vitro* (4,5,6).

It has been hypothesized that the suppression of colon tumor cell growth by dietary n-3 PUFAs, as compared with n-6 PUFAs, is mediated through inhibition of cyclooxygenase (COX)-mediated PGE_2 synthesis from arachidonic acid (AA; 20:4n-6). However, Boudreau *et al.* (5) demonstrated that proliferation of the colon tumor cell line HCT-116, which does not express COX, was inhibited by eicosapentaenoic acid (EPA; 20:5n-3), whereas it was not inhibited by linoleic acid (LA; 18:2n-6). These results of Boudreau *et al.* (5) suggest that the inhibitory effects of n-3 PUFAs on tumor cell growth are not mediated through inhibition of PGE_2 synthesis. Also our previous *in vitro* study demonstrated that the n-3 fatty acid EPA inhibited proliferation of human colorectal adenocarcinoma Caco-2 cells compared with the n-6 fatty acid LA (4). Also not PGE_2, but peroxidation products that are generated during lipid peroxidation and COX activity seemed to be responsible for the colon tumor cell growth inhibitory and cytotoxic effects of n-3 PUFAs (7).

Compared to functional physiological assays, nutrigenomics experiments can provide mechanistic insights into the molecular action of nutrients in isolated systems (e.g. cell culture or in animal models) (8). DNA microarray analyses of normal rat colonic epithelium of high fat fish oil (HFFO) and high fat corn oil (HFCO) fed rats revealed that genes involved in oxidative stress and cell growth pathways are differentially modulated by a HFFO diet with a high amount of EPA compared with a high fat corn oil (HFCO) diet with a high amount of LA (9). Earlier studies of Narayanan *et al.* (10) demonstrated that the n-3 fatty acid docosahexaenoic acid (DHA; 22:6n-3) is capable of modulating a panel of cell cycle and apoptosis-regulating genes in Caco-2 colon cancer cells.

In the present study, 17.000 element DNA microarrays (oligonucleotide arrays) were used to get more insight into the molecular mechanisms underlying the *in vitro* colon tumor cell growth inhibitory effects of n-3 PUFAs compared with n-6 PUFAs. Transcriptome changes induced by LA and EPA were compared to identify subsets of induced or repressed genes that are likely to underlie the physiological differences in cell growth inhibiting and lipid peroxidation-induced cytotoxic effects between these n-6 and n-3 PUFAs in Caco-2 cells. The question whether the beneficial effects of n-3 PUFAs such as EPA are due to modulation of COX-mediated PGE_2 signaling or are independent of such downstream metabolic pathways in Caco-2 cells was also addressed. Furthermore, the results of this study will reveal whether nutrigenomics experiments can

really predict the mechanism of action of the colon cancer inhibiting effects of n-3 PUFAs and can thus be used as a screening tool for the selection of chemopreventive nutrients.

Material and methods

Reagents

Bovine serum albumin (BSA; essential fatty acid-free), linoleic acid (LA; 18:2n-6), arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) were obtained from Sigma Chemical Company (St Louis, MO, USA). Fetal calf serum (FCS), non-essential amino acids, penicillin-streptomycin, DMEM and Trizol were obtained from Life Technologies (Breda, The Netherlands).

Cell culture and fatty acid treatment

Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in an atmosphere of 5% CO_2/95% air at 37 °C. Caco-2 cells were grown in DMEM, supplemented with 10% heat-inactivated FCS, 1% non-essential amino acids and 2% penicillin-streptomycin. The cells were sub-cultured at a ratio of 2:10, after they had reached about 70% confluence in 75 cm^2 culture flasks (Costar, Cambridge, MA, USA).

For the transcriptomics experiments, T-75 cell culture flasks were plated with Caco-2 cells (passage 38) at a concentration of 3.5 x 10^4 / cm^2 (≅ 2.5 x 10^6 cells in 10 ml of medium). After 24 hours, the medium was removed and fresh serum-free medium supplemented with 1 mg/ml fatty acid-free BSA was added. This medium was supplemented with 10 or 20 µM linoleic acid (LA; 18:2n-6), arachidonic acid (AA; 20:4n-6) or eicosapentaenoic acid (EPA; 20:5n-3). The concentrations chosen were based on our previous *in vitro* study in which 10 μM AA and EPA inhibited the growth of Caco-2 cells compared with 10 μM LA. 20 μM AA and EPA showed both growth inhibitory and cytotoxic effects compared with 20 μM LA (4).

The polyunsaturated fatty acids (PUFAs) were dissolved in ethanol (EtOH) up to a final concentration in the medium of 0.5%. Before addition to the cultures, the fatty acids were pre-incubated in the BSA-containing medium for 30 minutes at 37 °C. Control samples received serum-free medium supplemented with 1 mg/ml fatty acid-free BSA plus 0.5% ethanol. Unsupplemented Caco-2 cells (passage 45) were used to make a batch of reference RNA.

RNA isolation, purification, probe preparation and hybridization

After 48 hours of incubation, total RNA was isolated from both fatty acid and ethanol treated Caco-2 cells as well as from the untreated cells using Trizol, according to the manufacturer's protocol. Total RNA was further purified using the RNeasy Mini Kit (Qiagen, provided by Westburg, Leusden, The Netherlands) and DNA was digested using the Rnase-Free Dnase set (Qiagen). RNA was checked for purity and stability by gel electrophoresis and the concentration was calculated from the extinction at 260 nm as measured spectrophotometrically.
After purification, total RNA of two fatty acid or ethanol treated T-75 cell culture flasks was pooled in order to get enough RNA for labeling and hybridization. The total RNA of 15 untreated T-75 Caco-2 cell culture flasks was pooled to create the batch of reference RNA. Labelings were performed by ServiceXS in Leiden (The Netherlands) according to the fluorescent direct label kit of Agilent Technologies. RNA samples from PUFA (10 or 20 μM LA, AA or EPA) or ethanol treated Caco-2 cells were converted to fluorescent labeled Cy5-cDNA. RNA from the untreated Caco-2 cells was converted to fluorescent labeled Cy3-cDNA and used as the control probe. Cy5 and Cy3-labeled cDNAs were hybridized in triplo according to the Agilent Oligonucleotide Microarray hybridization protocol.

Human oligonucleotide array, microarray scanning and data extraction

The effect of PUFAs on Caco-2 gene expression was evaluated using the Human 1A Oligo Microarray Kit from Agilent Technologies. The Human 1A Oligo Microarray, based on Agilent's SurePrint technology, is comprised of 22,575 (60-mer) oligonucleotide probes representing 17,803 well characterized human genes from the Incyte Genomics Foundation Database.
Microarray slides were scanned using Agilent's dual-laser (532 and 633 nm laser lines) Microarray scanner. Agilent Feature Extraction software with default parameters for custom oligonucleotide microarrays was used to extract the data from the microarray images.

Data analysis

The LOWESS normalized data, obtained by the Agilent Feature Extraction software were transferred into Microsoft Excel for further data analysis. Spots that were flagged in the Agilent Feature Extraction software were not included in the dataset. As a consequence

of the introduction of the reference control, comparisons between the different PUFAs and between PUFAs and ethanol treatment are indirect (the expression levels of either 10 or 20 μM LA, AA, EPA and ethanol treated Caco-2 cells compared to the reference control (untreated Caco-2 cells) are measured on separate slides). The average fold induction values of PUFAs/ethanol, PUFA/PUFA were determined from the indirect average log ratios. Genes with an average induction factor (IF) > 2 or < 0.5 were considered differentially expressed. Genes with a p-value < 0.01 between two different treatments, as determined by the Student's t-test, were considered significantly differentially expressed. Also SAM (Statistical Analysis of Microarrays) was used to determine if the expression of any genes between two different treatments and multiclass experiments were significantly related to the response. Genes with a p-value < 0.05 in both AA and EPA and LA versus ethanol treated Caco-2 cells, as determined by the Student's t-test, were also considered as significantly differentially expressed. The program Genmapp was used to view and analyze the microarray data into biological pathways (11).

Results

Effects of LA, AA and EPA on Caco-2 gene expression

Gene expression of 20 μM LA, AA and EPA treated Caco-2 cells were compared with ethanol treated Caco-2 cells to determine n-6 and n-3 fatty acid-induced changes in gene expression. Various genes were found significantly differentially ($p<0.01$) expressed after 48 hours of treatment with the different fatty acids compared to ethanol (EtOH) treatment: 20 µM LA - EtOH (225 genes); 20 µM AA - EtOH (83 genes) and 20 µM EPA - EtOH (113 genes) (see Table 6.1). Genes with a p-value < 0.05 in both 20 μM LA and AA and EPA versus ethanol treated Caco-2 cells, as determined by the Student's t-test are shown in Table 6.2 as well as the genes that were significantly differentially expressed as determined by SAM. Also genes with an induction factor (IF) > 2 or < 0.5 in both 20 μM LA and AA and EPA versus ethanol treated Caco-2 cells were selected as differentially expressed and are listed in Table 6.2. In total, 91 genes fulfilled these criteria, of which 46 genes could be predefined in functional groups based on reported gene classifications that are published in PubMed Medline (12).

Table 6.1: Number of significant gene expression changes ($p<0.01$) induced by 10 and 20 μM LA, AA and EPA in Caco-2 cells after 48 hours of incubation

Number of significant gene expression changes	LA	AA	EPA	ethanol
LA	X	72	43	61
AA	34	X	28	84
EPA	49	24	X	62
ethanol	225	83	113	X

Values for 10 μM incubations have an underscore and are displayed in gray, the other values are of the 20 μM incubations

Biochemical functions of the genes in the expression profiles are diverse and include genes related to oxidative stress response, oxidative protein damage/protein degradation, cell growth, differentiation and apoptosis, extracellular matrix and cell migration, lipid metabolism, immune system and energy metabolism. The similarities in transcriptional responses suggest that LA, AA and EPA are related in terms of mechanism of action. Effects of LA, AA and EPA on gene expression tend to be dose-related (see Table 6.1), since no differentially expressed genes related to oxidative stress response and oxidative protein damage were observed after 48 hours of incubation with the low concentration (10 μM) of LA, AA or EPA versus ethanol. Likewise, fewer genes involved in cell growth, differentiation and apoptosis, cell adhesion, lipid metabolism, immune system and energy metabolism were differentially expressed after 48 hours of treatment with 10 μM LA, AA or EPA versus ethanol treatment.

Table 6.2: Selected functional groups of (significantly) differentially expressed genes from microarray gene expression analysis in Caco-2 cells treated with 20 μM LA, AA or EPA versus ethanol (EtOH) for 48 hours[1]

Probe Name	Gene Name	20 μM LA - EtOH		20 μM AA - EtOH		20 μM EPA - EtOH	
		IF	p-value	IF	p-value	IF	p-value
Oxidative stress response							
A_23_P252413	Member of metallothionein family	3.01		3.15		2.88	
A_23_P60933	Metallothionein 1H	2.13		2.28		2.05	

A_23_P163782	*Metallothionein 1H*	2.39	0.00	3.11	0.03	2.56	0.00
A_23_P206701	*Metallothionein 1G*	1.83	0.03	2.31	0.03	1.87	0.03
A_23_P983	Protein with strong similarity to anti-oxidant proteins	1.35	0.03	1.38	0.01	1.35	0.00
A_23_P51009	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3	1.28	0.03	1.38	0.02	1.26	0.04
Oxidative protein damage / protein degradation							
A_23_P111132	*Heat shock 70 kDa protein 1A*	1.93	0.00	1.73	0.03	1.66	0.01
A_23_P15705	Proteasome subunit beta 6	1.18	0.03	1.36	0.04	1.18	0.04
A_23_P769	Proteasome subunit beta 4	1.40	0.01	1.46	0.01	1.35	0.01
A_23_P164141	Proteasome activator subunit 3	1.49	0.01	1.30	0.02	1.33	0.02
A_23_P56188	Ubiquitin A-52 residue ribosomal protein fusion product 1	1.36	0.00	1.45	0.02	1.37	0.01
A_23_P83438	Member of the ubiquitin-conjugating enzyme (E2) family	1.44	0.01	1.40	0.01	1.39	0.01
A_23_P208856	Ribosomal protein S16	1.23	0.02	1.37	0.01	1.33	0.01
A_23_P256933	Ribosomal protein L24	1.20	0.04	1.23	0.05	1.20	0.04
Cell growth, differentiation and apoptosis							
A_23_P86779	Protein phosphatase 1 catalytic subunit alpha	3.20		3.95		3.42	
A_23_P158176	Protein with high similarity to caspase 5	2.58		2.21		2.29	
A_23_P70458	*H4 histone family member E*	0.46		0.66		0.61	
A_23_P128215	STAT induced STAT inhibitor-2	1.60	0.03	1.56	0.02	1.40	0.04
A_23_P209459	N-acetylgalactosaminyl-transferase T3	0.68	0.04	0.55	0.02	0.65	0.03
A_23_P215944	Cathepsin B	1.48	0.00	1.51	0.01	1.38	0.01
A_23_P97206	Small protein effector 1 of cell division cycle 42	1.27	0.03	1.30	0.03	1.27	0.04
A_23_P107401	Tissue inhibitor of metalloproteinase 2	1.21	0.04	1.20	0.01	1.20	0.00

A_23_P65031	Dynein cytoplasmic light polypeptide	1.58	0.00	1.58	0.00	1.43	0.00
A_23_P93282	H3 histone family member I	1.28	0.05	1.26	0.00	1.23	0.03
A_23_P63067	Death-associated protein 3	1.33	0.02	1.26	0.02	1.21	0.04
A_23_P92196	Resistin-like molecule beta	0.70	0.02	0.73	0.04	0.75	0.05
A_23_P75568	Protein with high similarity to human ICEBERG	0.65	0.01	0.57	0.00	0.55	0.00
A_23_P62659	Palmitoyl-protein thioesterase	1.35	0.00	1.33	0.01	1.22	0.05
A_23_P56865	DEAD box protein 18	1.21	0.02	1.15	0.01	1.14	0.01
A_23_25194	Harakiri	0.84	0.02	0.83	0.04	0.86	0.03
A_23_P140219	BCL2 like 2	1.62	0.03	1.77	0.01	1.82	0.02
Extracellular matrix and cell migration							
A_23_P28388	Protein containing a C-type lectin domain	2.41		2.26		2.50	
A_23_P36985	*Protocadherin 8*	0.61	0.03	0.54	0.01	0.55	0.03
A_23_P254522	Alpha 4 subunit of type IV collagen	2.08	0.03	2.01	0.04	2.06	0.04
Lipid metabolism							
A_23_P210900	*Acetyl-coenzyme A synthetase 2*	0.78		0.62		0.67	
A_23_P24444	*7-dehydrocholesterol reductase*	0.94		0.74		0.73	
Immune system							
A_23_P59035	*Lymphocyte antigen 95*	0.61		0.74		0.82	
A_23_P19510	*Protein with high similarity to beta chain of A class II molecule*	0.70		0.64		0.68	
A_23_P117971	B-cell growth factor 1	0.65	0.04	0.50	0.01	0.62	0.02
A_23_P3532	LPS-induced TNF factor	1.26	0.03	1.16	0.02	1.17	0.02
A_23_P256487	B7-H1 protein	0.85	0.00	0.85	0.04	0.83	0.03
Energy metabolism							
A_23_P32384	Cytochrome c1	1.47	0.01	1.31	0.02	1.36	0.04
A_23_P64879	Inwardly rectifying potassium channel	1.40	0.01	1.42	0.01	1.37	0.03

A_23_P56678	ATP synthase H+ transporting mitochondrial F0 complex subunit c	1.74	0.04	1.51	0.05	1.52	0.05
A_23_P75622	ATP synthase H+ transporting mitochondrial F0 complex subunit g	1.50	0.02	1.38	0.03	1.44	0.02
A_23_P213718	Ubiquinone binding protein	1.40	0.04	1.45	0.01	1.40	0.03

[1]The table contains the Human 1A oligonucleotide probe IDs, the gene name, the average induction factor (IF) and the p-value obtained by the Student's t-test.
Genes without a p-value are not significantly differentially expressed ($p > 0.05$). Gene names which are formatted in *italic* are significantly differentially expressed, as determined by SAM.

Effects of LA, AA and EPA on COX-mediated prostaglandin metabolism

Analysis of the effects of LA, AA and EPA on changes in Caco-2 gene expression was also performed to obtain information on n-6 and n-3 PUFA-induced molecular effects on COX-mediated PGE_2 signaling. Transcriptome analyses revealed the expression of the four different EP receptors (EP1, EP2, EP3 and EP4), all of which are specific for PGE_2-mediated cell signaling in Caco-2 cells. However, no differential effects in transcriptional responses of the different EP receptors were observed between 20 μM LA, AA and EPA (Table 6.3). Also no differences in gene expression of COX-2, prostaglandin E synthase, prostaglandin D synthase, phospholipase A2; the enzyme that releases AA from membrane phospholipids to produce free AA for prostaglandin production and solute carrier family 21 member 2; a gene that transports many different prostaglandins, were observed between the different n-6 and n-3 PUFAs (Table 6.3).

Table 6.3: Genes involved in COX-mediated prostaglandin metabolism, selected from microarray gene expression analysis in Caco-2 cells treated with 20 μM LA, AA or EPA versus ethanol (EtOH) for 48 hours

Probe Name	Gene Name	20 μM LA - ETOH		20 μM AA - ETOH		20 μM EPA - ETOH	
		IF	p-value	IF	p-value	IF	p-value
A_23_P200345	COX-2	0.85		0.98		0.88	
A_23_P169446	Prostaglandin E synthase	1.43		1.40		1.27	
A_23_P10506	Prostaglandin D2 synthase	0.96		0.91		0.86	
A_23_P88766	Phospholipase A2	1.01		1.01		0.96	
A_23_P88766	Phospholipase A2 group X	1.01		1.01		0.96	
A_23_P4808	Prostaglandin E2 receptor 1	0.87		0.85		0.86	
A_23_P151710	Prostaglandin E2 receptor 2	1.49		1.24		1.17	
A_23_P103328	Prostaglandin E2 receptor 3	1.56		1.18		1.36	0.02
A_23_P158857	Prostaglandin E2 receptor 3	1.09		1.04		1.03	
A_23_P148047	Prostaglandin E2 receptor 4	0.80		0.86		0.74	
A_23_P135990	Solute carrier family 21 (prostaglandin transporter) member 2	0.98		1.05		1.03	

[1]The table contains the Human 1A oligonucleotide probe IDs, the gene name, the average induction factor (IF) and the p-value obtained by the Student's t-test.
Genes without a p-value are not significantly differentially expressed ($p > 0.05$)

Differential effects between LA, EPA and AA on Caco-2 gene expression

Transcriptome changes induced by EPA and LA were compared to identify subsets of induced or repressed genes that are likely to underlie the physiological differences in cell growth inhibiting and cytotoxic effects between these n-3 and n-6 PUFAs in Caco-2 cells after 48 hours of incubation. The average log ratios of 20 μM LA treated Caco-2 cells

versus Caco-2 reference cells are plotted against the average log ratios of 20 μM EPA treated Caco-2 cells versus Caco-2 reference cells (Figure 6.1). This scatter plot reveals that only a few genes are differentially expressed between 20 μM EPA and LA after 48 hours of incubation in Caco-2 cells. Also the number of significantly differentially expressed genes, selected with the Student's t-test ($p < 0.01$), between 20 μM EPA and 20 μM LA was considerably lower (49 genes) compared to changes in gene expression by 20 μM LA vs. ethanol (225 genes) or 20 μM EPA vs. ethanol (113 genes) (Table 6.1).

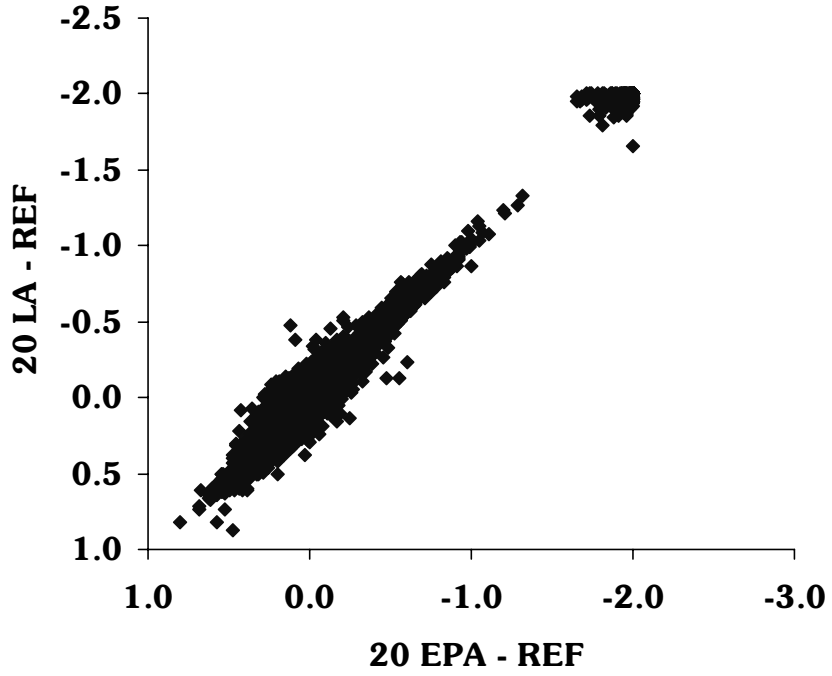


Figure 6.1: Scatter plot view of gene expression: Average log ratios of 20 μM LA treated Caco-2 cells versus Caco-2 reference cells against average log ratios of 20 μM EPA treated Caco-2 cells versus Caco-2 reference cells

This was also the case for changes between 10 μM EPA and 10 μM LA (43 genes) as compared to changes in gene expression by 10 μM EPA versus ethanol (62 genes) or 10 μM LA versus ethanol (61 genes) (see Table 6.1).
Genes with a p-value < 0.01 or an IF > 2 or < 0.5 in 20 μM EPA versus 20 μM LA treated Caco-2 cells, which could be classified in functional groups related to colon carcinogenesis are listed in Table 6.4. Specific downregulated genes involved in cell proliferation, differentiation and apoptosis are for example tumor suppressing subtransferable candidate 3 (TSSC3), which is implicated in Fas-mediated apoptosis (13), the P19(arf) tumor suppressor, a nucleolar protein that binds to Mdm2 to induce

p53-dependent cell cycle arrest (14) and a member of the cyclin family, which regulates the nuclear cell division cycle. Several genes involved in the extracellular matrix and cell migration, such as two protocadherins, which are suggested to be tumor suppressors, are upregulated after incubation with EPA compared to LA (15).

Table 6.4: Selected functional groups of (significantly) differentially expressed genes from microarray gene expression analysis in Caco-2 cells treated with 20 μM EPA versus 20 μM LA or 48 hours[1]

Probe Name	Gene Name	20 μM EPA - 20 μM LA	
		IF	p-value
Cell proliferation, differentiation and apoptosis			
A_23_P154235	N myc STAT interactor	0.37	
A_23_P114185	Transmembrane 4 superfamily member 2	0.48	
A_23_P90634	Member of the cyclin family	0.77	0.0038
A_23_P47614	Tumor suppressing subtransferable candidate 3	0.79	0.0051
A_23_P43490	Tumor suppressor P19(Arf)	0.87	0.0081
A_23_P250701	Protein kinase lysine deficient 1, a serine/threonine kinase	0.92	0.0074
A_23_P48807	Galactokinase 2	0.85	0.0098
A_23_P118946	Beta 1-4 galactosyltrans-ferase polypeptide 6	2.11	
Extracellular matrix and cell migration			
A_23_P156010	Protocadherin beta 12	2.00	
A_23_P98640	Protocadherin 16	1.07	0.0074
A_23_P208182	Sialic acid-binding Ig-like lectin-10	2.13	
A_23_P168616	Reelin	2.24	
Xenobiotic and drug detoxification			
A_23_P132644	Protein with moderate similarity to arylacetamide deacetylase	0.75	0.0056
A_23_P48713	Member of the zinc-containing alcohol dehydrogenase family	0.83	0.0009

Gene translation and degradation			
A_23_P152297	DNA directed RNA polymerase III polypeptide K	0.83	0.0013
A_23_P202844	Putative subunit of the SCF ubiquitin ligase	0.93	0.0023
A_23_P86182	Mitochondrial ribosomal protein S21	1.06	0.0081
A_23_P_100088	Proteasome subunit alpha type 4	0.85	0.0089
A_23_P118642	Eukaryotic translation initiation factor 4E	0.85	0.0031
A_23_P45945	Translation initiation factor 1	0.85	0.0047
A_23_P129786	Sterol regulatory element binding transcription factor 1	0.70	0.0059
A_23_P99700	Menage a trois 1	0.92	0.0092
A_23_P42718	Nuclear factor erythroid-derived 2 like 3	0.83	0.0018
Fatty acid and cholesterol metabolism			
A_23_P3402	Electron transfer flavoprotein alpha subunit	0.85	0.0072
A_23_P24444	7-dehydrocholesterol reductase	0.77	0.0013

[1]The table contains the Human 1A oligonucleotide probe IDs, the gene name, the average induction factor (IF) and the p-value obtained by the Student's t-test.
Genes without a p-value are not significantly differentially expressed ($p > 0.01$)

Furthermore genes involved in gene translation, transcription and degradation are down-regulated in 20 μM EPA treated Caco-2 cells compared to 20 μM LA treated Caco-2 cells. Among the downregulated genes is for example the sterol regulatory element binding transcription factor 1 (SREBF1), which is a transcription factor that plays a key role in the regulation of fatty acid and cholesterol metabolism. Also other genes involved in fatty acid and cholesterol metabolism, such as electron transfer flavoprotein alpha subunit and 7-dehydrocholesterol reductase are down-regulated in Caco-2 cells after EPA treatment. Up- and downregulated genes, involved in apoptosis, between 20 μM EPA and LA are shown in Figure 6.2. This figure reveals that although many genes in the apoptosis pathway are expressed in Caco-2 cells, no consistent differential effect on

apoptosis related genes is seen between incubations with EPA and LA in this biological pathway. This was also the case for other biological pathways visualized by means of the program Genmapp, such as the cell cycle, inflammatory response and proteasome degradation.

Gene expression changes between EPA and AA in Caco-2 cells were even less compared to changes between EPA and LA. Only 28 genes were significantly differentially expressed ($p < 0.01$) between 10 μM EPA and 10 μM AA (Table 6.1). 24 Genes were significantly altered ($p < 0.01$) between 20 μM EPA and 20 μM AA. Among these genes were a high amount of proteins of unknown function, which made it very difficult to classify these genes in functional biological pathways (data not shown). Furthermore, genes involved in COX-mediated PGE_2 signaling were not differentially expressed between AA and EPA, as shown above (see Table 6.3).

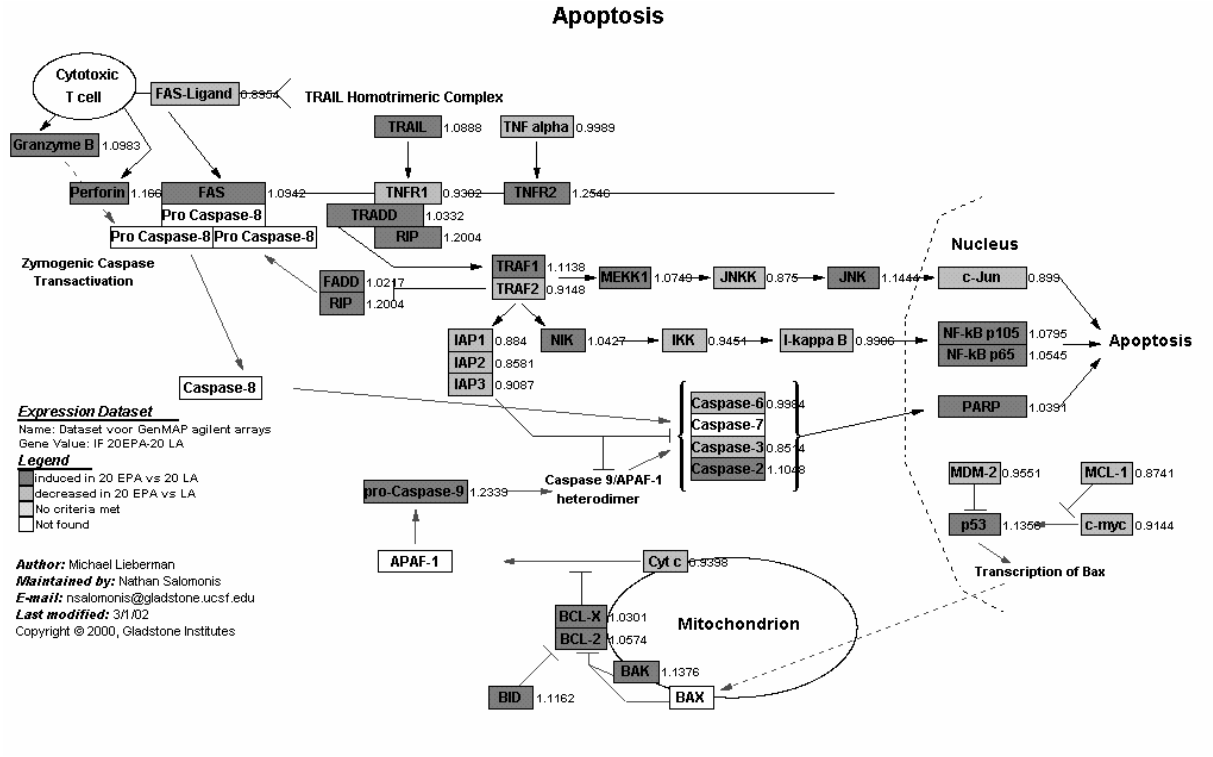


Figure 6.2: Biological pathway created by Genmapp (www.Genmapp.org): overview of up- and downregulated genes involved in apoptosis in 24 hours treated Caco-2 cells with 20 μM EPA versus 20 μM LA (gene expression ratios are given besides each gene name)

Discussion

In the present study, transcriptome changes induced by LA (18:2n-6), AA (20:4n-6) and EPA (20:5n-3) were studied in an attempt to link the physiological differences in cell growth inhibiting and cytotoxic effects between n-3 and n-6 PUFAs observed in human colon adenocarcinoma Caco-2 cells to genomic and molecular processes. Our results indicate that the different n-6 and n-3 PUFAs LA, AA and EPA show similarities in transcriptional responses in Caco-2 cells and thus seem to be related in terms of mechanism of action. Activation of genes involved in antioxidant defense mechanisms such as metallothioneins, anti-oxidant proteins and NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3, which is a subunit of NADH-ubiquinone oxidoreductase (NQO1), suggest for example a role for LA, AA and EPA in stimulation of the oxidative stress response. Upregulation of repair mechanisms such as heat shock proteins that renature damaged proteins or resolubilize aggregates of damaged proteins and the proteasome, that recognizes and degrades oxidatively damaged proteins also supports the role of the different n-6 and n-3 PUFAs in oxidative stress and cell damage (16). Furthermore genes involved in cell growth, differentiation and apoptosis, cell adhesion, lipid metabolism, immune system and energy metabolism were similarly altered after 48 hours of incubation with 20 μM LA, AA or EPA in Caco-2 cells. These results are in line with our previous *in vivo* data that also revealed that genes involved in oxidative stress, cell growth, cell adhesion, immune response, lipid metabolism and energy metabolism are modulated by high fat fish oil (HFFO) diets with a high amount of EPA and high fat corn oil (HFCO) diets with a high amount of LA (9). Also Jump and Clarke (17) demonstrated that PUFAs have pronounced effects on gene expression involved in carbohydrate and lipid metabolism, cell differentiation and growth, inflammation and cell adhesion.

Fatty acids can either directly bind to transcription factors such as PPARα, PPARβ, PPARγ, HNF4α, NFκB and SREBP, modulating gene expression or can modulate cell signaling via cyclooxygenase (COX) products such as prostaglandins (17). For example, PGE_2, the COX-mediated metabolite of AA, is thought to stimulate proliferation and motility of colorectal carcinoma cells via activation of the PI3/Akt pathway, an effect likely due to activation of the PGE_2 receptor subtype EP4 (18). It remains however to be determined whether n-3 PUFA-mediated inhibiting effects on colon tumor cell growth reflects a decrease in AA content and PGE_2 synthesis, or is independent of PGE_2 synthesis. Results from our previous *in vitro* study clearly demonstrated that EPA could competitively inhibit AA-induced PGE_2 synthesis in Caco-2 cells between 1 and 24 hours of incubation. Reductions in PGE_2 synthesis by EPA compared to AA did not lead to differential effects on cell proliferation in Caco-2 cells. Both AA and EPA inhibited the growth of Caco-2 cells after 48 hours of incubation. These results suggest that regulation

of cell proliferation by n-3 and n-6 PUFAs in colon cancer cell lines is not mediated via PGE_2 levels (4).

The transcriptome analysis performed in this study revealed that differences in Caco-2 gene expression between AA and EPA were very minimal, which is in line with the similarities in physiological effect between AA and EPA. Caco-2 cells express all four EP receptor subtypes, but no differences in transcriptional responses of the different EP receptors were observed between AA and EPA. Also no differences in gene expression of other genes involved in COX-mediated prostaglandin signaling such as prostaglandin E synthase, prostaglandin D synthase, phospholipase A2, solute carrier family 21 member 2 and COX-2 were observed between the different n-6 and n-3 PUFAs, which further supports the idea that the colon cancer inhibiting effects of n-3 PUFAs is independent of COX-mediated PGE_2 signaling in Caco-2 cells.

It would be expected that differences between EPA and LA on Caco-2 gene expression are more pronounced, based on the physiological differences in cell growth inhibiting and cytotoxic effects between EPA and LA in Caco-2 cells (4). Gene expression profiling displayed that down- and upregulated genes between EPA and LA could be classified in functional groups related to cell proliferation, differentiation and apoptosis, extracellular matrix and cell migration, xenobiotic and drug detoxification, gene translation and degradation and fatty acid and cholesterol metabolism. However dramatic differences as well as consistent changes in biological pathways between EPA and LA are not observed after 48 hours of incubation, which makes it difficult to predict the physiological effect. A possible explanation could be that the physiological *in vitro* effect not primarily originates from differences in cell signaling resulting in transcriptome changes, but is more influenced by direct cytotoxic cell damage or post-translational modifications. Furthermore, individual responsive genes could vary considerably in the kinetics of their regulation and gene expression changes proceed as a function of time. Therefore, profiling gene expression changes at multiple time points could greatly help to reveal patterns of consistent changes in biological pathways, or clusters of genes that show similarities in their time-dependent response (19,20).

In summary, besides the fact that LA, AA and EPA show similarities in transcriptional responses and that several genes involved in different biological pathways are altered after 48 hours of incubation with EPA or LA, it is still very difficult to predict the mechanism of action of the colon cancer inhibiting effects of n-3 PUFAs from measuring subtle changes in mRNA expression by means of microarrays at a single time-point. So far, the use of nutrigenomics as a screening tool for the selection of chemopreventive nutrients as a superior alternative to traditional *in vitro* and *in vivo* physiological assays is still in its infancy. Therefore, it is very important that experience, especially in

experimental design and bio-informatics, will be extended and shared to advance the utility of gene expression profiling in predictive and mechanistic nutrigenomics.

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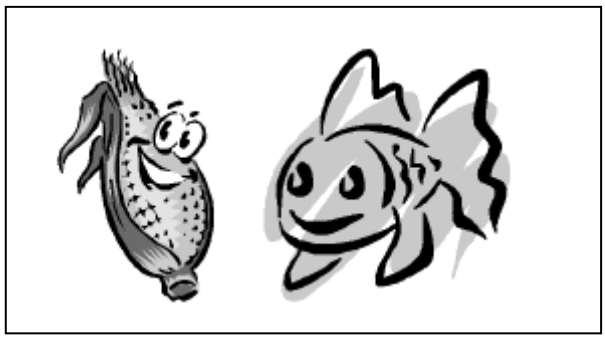
Chapter 7

Summary and general discussion



7.1 Summary of results

Previous studies (as summarized in the introduction) led us to suggest that both modulation of COX-mediated PGE_2 signaling as well as lipid peroxidation-induced oxidative stress may play a role in the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs. The exact mechanisms by which n-3 PUFAs prevent or inhibit colorectal carcinogenesis compared with n-6 PUFAs is however currently poorly understood. The aim of this thesis therefore was to investigate the cellular, biochemical and molecular mechanisms of the adverse and beneficial effects of n-6 and n-3 PUFAs on colorectal carcinogenesis, with special emphasis on PGE_2 and the oxidative stress response.

Effects of n-6 and n-3 PUFAs on PGE_2 synthesis and cell proliferation

The n-6 fatty acid arachidonic acid (AA; 20:4n-6) is the precursor fatty acid of the 2-series of prostaglandins, including PGE_2. In chapter 3, experiments are described demonstrating that human colon adenocarcinoma Caco-2 cells, which express high levels of cyclooxygenase 2 (COX-2), produce PGE_2 upon addition of AA. HT-29 colon tumor cells on the other hand did not produce high levels of PGE_2 under the same conditions. However, PGE_2 synthesis in this cell line was inducible by IL-1β, a cytokine that is known to increase COX-2 expression. The observation that linoleic acid (LA; 18:2n-6) did not increase PGE_2 production in Caco-2 as well as in HT-29 cells is in agreement with the finding that the parent essential fatty acid LA is not converted to the prostaglandin precursor AA in these colon cancer cell lines (chapter 2).

The n-3 fatty acid eicosapentaenoic acid (EPA; 20:5n-3) is the precursor fatty acid of the 3-series of prostaglandins, including PGE_3. Because EPA and AA are both substrates for prostaglandin synthesis, they can compete for COX enzyme activity. In chapter 3 is shown that EPA competitively inhibited AA-induced PGE_2 synthesis in Caco-2 cells. The *in vivo* studies described in chapter 4 and 5 also show that high fat fish oil (HFFO) diets with a high amount of EPA result in lower levels of PGE_2 in the plasma of F344 rats compared with high fat corn oil (HFCO) diets with a high amount of LA.

In order to investigate the hypothesis that the inhibitory effects of n-3 PUFAs such as EPA on colon tumor cell growth may partly be related to inhibition of PGE_2 synthesis from AA, differential effects of AA and EPA on colon tumor cell proliferation were compared (chapter 3). Reductions in PGE_2 synthesis by EPA compared to AA did however not lead to differential effects on cell proliferation in human colon adenocarcinoma Caco-2 cells. EPA inhibited the cell division of Caco-2 cells, but the n-6 fatty acid AA showed the same growth inhibitory and cytotoxic effects. Also differences in transcriptome changes induced by AA and EPA were minimal in Caco-2 cells. No differential expression of genes involved in COX-mediated PGE_2 signaling such as

prostaglandin receptors (EP1, EP2, EP3 and EP4), prostaglandin E synthase, phospholipase A2, a prostaglandin transporter and COX-2 were observed between EPA and AA (chapter 6).
In addition, AA and EPA did not influence the proliferation rate of HT-29 cells. Induction of COX-2 expression and PGE_2 synthesis in HT-29 cells by IL-1β did not result in an increased cell proliferation, but on the contrary a decrease in cell proliferation by both AA and EPA was observed (chapter 3).
Moreover the causal relationship between PGE_2 and colonic epithelial cell proliferation is also questioned because direct addition of exogenous PGE_2 failed to stimulate proliferation of Caco-2, HT-29, LIM1215 and HCA-7 colon cancer cells (chapter 3). These negative results could for example be due to the absence of EP receptors in these cell lines, but based on the results described in chapter 6 (detectable gene expression of all four EP receptors subtypes in Caco-2 cells), this seems not to be very likely.
Thus, based on the results of our studies, regulation of cell proliferation in colon cancer cell lines seems not to be mediated via PGE_2 levels. These results are not in line with the above-mentioned hypothesis and thus question the role of PGE_2 in the (anti)-colon carcinogenic effects of n-3 and n-6 PUFAs.

Effects of n-6 and n-3 PUFAs on oxidative stress response

Human colon adenocarcinoma Caco-2 cells were used as an *in vitro* model to study the effects of n-3 and n-6 PUFAs on gap junctional intercellular communication (GJIC) during spontaneous proliferation and differentiation (chapter 2). The GJIC capacity increased during spontaneous Caco-2 cell growth and differentiation. However, no differential effects between n-6 and n-3 PUFAs on GJIC were observed. Short-term incubation (24 hours) with linoleic acid (LA; 18:2n-6), α-linolenic acid (ALA; 18:3n-3), arachidonic acid (AA; 20:4n-6) or eicosapentaenoic acid (EPA; 20:5n-3) did not influence GJIC capacity, while long-term incubation during differentiation (> 10 days of incubation) with LA or ALA inhibited GJIC of these colon cells. Long-chain metabolites such as AA and EPA were not formed after incubation with LA and ALA, thus excluding the involvement of prostaglandins in the observed effects (chapter 2).
Although the exact mechanism of GJIC inhibition is unclear, cytotoxicity probably mediated by lipid peroxidation products seems to affect GJIC because incubation with more polyunsaturated fatty acids (AA and EPA) completely abolished GJIC after only a few days (chapter 2). The more polyunsaturated fatty acids AA (20:4n-6) and EPA (20:5n-3) also inhibited proliferation of Caco-2 cells and increased cytotoxicity compared to the longer chain fatty acids LA (18:2n-6) and ALA (18:3n-3) (chapter 3). This suggests

that the number of double bonds in the carbon atom chain of fatty acids is more important for the anti-proliferative and cytotoxic effects of n-6 and n-3 PUFAs on colon tumor cells than the place of the double bond.

PUFAs with methylene-interrupted double bonds, such as AA and EPA, are highly susceptible to enzymatic and non-enzymatic peroxidation. Peroxidation products of PUFAs may have played an important role in the AA and EPA-induced anti-proliferative and cytotoxic effects, because levels of malondialdehyde (MDA), which is an end product of lipid peroxidation, were increased after incubation of Caco-2 cells with AA and EPA. Addition of antioxidants, such as vitamin E reduced MDA formation in Caco-2 cells and also partially reversed the cell proliferation inhibiting effects of both AA and EPA (chapter 3). In addition, indomethacin, a COX enzyme inhibitor that clearly inhibited AA-induced PGE_2 synthesis in Caco-2 cells, also partially reversed the anti-proliferative effects of both AA and EPA. Furthermore, induction of COX-2 expression and PGE_2 synthesis in HT-29 cells by IL-1β did not result in an increased cell proliferation but rather caused a decrease in cell proliferation and an induction of cytotoxicity by both AA and EPA. Both results suggest that besides spontaneous oxidation, the inhibitory effects of AA and EPA on cell proliferation found in the *in vitro* studies (chapter 3) could also be due to peroxidation products, generated during the peroxidase activity of the COX enzyme.

The *in vivo* study, described in chapter 4, reveals that high fat fish oil (HFFO) diets with a high amount of EPA inhibit the initiation of AOM-induced colonic aberrant crypt foci (ACF; precursor lesions of colon cancer) compared to high fat corn oil (HFCO) diets with a high amount of LA, when fed during both the initiation and postinitiation stages of colorectal carcinogenesis. This chemopreventive effect could possibly also be related to higher levels of oxidative stress caused by HFFO diets. Levels of MDA, which is a marker of oxidative stress, were increased in livers (chapter 4+5) and faeces (chapter 4) of HFFO fed F344 rats compared to HFCO fed rats, which proves that HFFO diets can indeed cause an increase in tissue levels of lipid peroxidation. Also lower levels of vitamin E were found in the plasma of HFFO fed rats compared to HFCO fed rats (chapter 5), possibly because of higher vitamin E consumption to protect the HFFO fed rats against the increased levels of oxidative stress caused by these diets. Fish oil is an oxidative stress-causing factor that also enhanced the activity of the antioxidant enzyme catalase to defend against the increased levels of oxidative stress that were e.g. found in the liver (chapter 5).

HFFO diet induced-oxidative stress is probably also involved in signal transduction and the regulation of gene expression via redox-sensitive mechanisms. Mechanisms responsible for the induction of antioxidant genes in response to oxidative stress are mediated via an antioxidant-responsive element (ARE), also known as electrophile-

responsive element (EpRE). Functional EpRE/ARE sequences have been identified in glutathione S-transferase Ya (GSTYa), glutathione S-transferase P1 (GSTP1), NAD(P)H:quinone oxidoreductase (NQO1), γ-glutamylcysteine synthetase heavy and light chain subunit, heme oxygenase (HO), thioredoxin, NF-E2-related factor 2 (Nrf2), ferritin heavy and light chain and metallothionein (1,2). From these genes, ferritin and metallothionein, but also peroxiredoxin 2, NQO1 and GSTP1 were significantly upregulated by fish oil feeding compared with corn oil feeding (chapter 5). Also the *in vitro* study, described in chapter 6, demonstrates that antioxidant genes, such as metallothioneins and NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3, which is a subunit of NQO1, are significantly upregulated by higher levels of PUFAs (20 μM). Also repair mechanisms such as heat shock proteins that renature damaged proteins or resolubilize aggregates of damaged proteins and the proteasome, that recognizes and degrades oxidatively damaged proteins (3) are significantly upregulated in Caco-2 cells after incubation with n-3 and n-6 PUFAs (chapter 6). Induction of antioxidant and stress proteins in both the *in vivo* and *in vitro* studies could be considered as an adaptive response to the increased oxidative stress, i.e. a cytoprotective function.

If the oxidant dose is however large enough and antioxidants and repair proteins are not able anymore to sufficiently protect against the oxidative stress-induced cytotoxicity, proliferation of for example colon cells may become inhibited or apoptosis induced to prevent clonal expansion and stimulate disposal of damaged colonic epithelial cells (4). The transcriptomics experiments revealed that genes involved in growth control pathways such as cell proliferation and apoptosis are indeed modulated by HFFO diets (chapter 5) as well as by single PUFAs in the *in vitro* experiments (chapter 6). For example histone 2b, a gene whose expression has been associated with cellular proliferation (5) is significantly decreased in the normal rat colon after fish oil feeding. Lower mRNA levels of histone 2b might imply that the cellular proliferation is indeed decreased in the colonic epithelium of HFFO fed rats compared to HFCO fed rats. A gene involved in the induction of apoptosis is caspase 5. This pro-apoptotic gene is upregulated by 10 and 20 μM LA, AA and EPA after 48 hours of incubation in human colon adenocarcinoma Caco-2 cells.

Although the oxidative stress related hypothesis is still speculative and only a few genes in these pathways were differentially expressed, the transcriptomics results do reveal that genes involved in oxidative stress and cell growth pathways are indeed modulated in the *in vivo* as well as the *in vitro* microarray studies. Overall, the results might imply that n-3 PUFAs could protect against colon carcinogenesis via increased levels of oxidative stress that can ultimately lead to higher levels of apoptosis and decreased levels of cell proliferation in for example the colonic epithelium.

7.2 General discussion

Fatty acids from the diet are absorbed in the small intestine in the form of mixed micelles and transported via the lymph into the plasma as chylomicrons (6). Consumption of HFFO diets can in this way lead to increased levels of EPA and DHA in the plasma, whereas consumption of HFCO diets can lead to increased plasma levels of LA and AA. Plasma phospholipids and triglycerides can be hydrolyzed by the enzyme lipoprotein lipase which enables free fatty acids to enter target cells through membrane-associated fatty acid transporters. In this way basolateral free fatty acids can be taken up by colon cells, but also other organs such as liver, breast, heart, brain, prostate, lung and adipose tissue (7,8), where they can influence important body processes. Besides the protective effects of n-3 PUFAs on colorectal carcinogenesis (the subject of this thesis), it is published that consumption of n-3 PUFAs may also contribute to a lower risk of breast cancer, prostate cancer and liver cancer (9,10,11). Also anti-inflammatory, anti-thrombotic, anti-arrhythmic, hypolipidemic and vasodilatory properties of n-3 PUFAs have been described (12).

It was generally believed that n-3 PUFAs as well as nonsteroidal anti-inflammatory drugs (NSAIDs) protect against colorectal carcinogenesis through inhibition of COX-mediated PGE_2 synthesis. However, our results and those of others (13,14) question the dominant role of PGE_2 in the colon cancer inhibiting effects of n-3 PUFAs. Also recent studies on selective COX-2 inhibitors demonstrated that much higher doses of these compounds were required to achieve inhibition of colon tumor cell growth than to inhibit PGE_2 production (15). New insights reveal that NSAIDs as well as n-3 fatty acids can act via prostaglandin-independent pathways for example by direct activation of the peroxisome proliferator-activated receptor-γ (PPARγ) (15). PPARγ is expressed in rodent colon tumors (16) and in selected colon cancer cell lines including HT-29 (15), HCA-7 (17) and Caco-2 (18) and selective PPARγ ligands have been shown to cause growth inhibiting and apoptosis-inducing effects both *in vitro* and *in vivo* (10,19). Another potential mechanism of the chemopreventive effects of NSAIDs not related to a reduction in prostaglandins is related to an elevation of free AA in the medium. Chan *et al.* (20) have shown that NSAID treatment of colon tumor cells results in a dramatic increase in free AA that in turn stimulates the conversion of sphingomyelin to ceramide, a known mediator of apoptosis. This could also be a possible reason for the AA-induced inhibition of Caco-2 cell growth, found in our studies.

However, the findings described above are based on mechanistic studies in colon cancer cell lines. Lack of interaction *in vitro* with other cell systems and systemic effects involved in the process of carcinogenesis could lead to oversimplification of the observed effects. Cutler *et al.* (17) for example have demonstrated that PGI_2, another COX-mediated

prostaglandin, produced by fibroblast cells in the colonic stroma, promotes survival of surrounding colonic epithelial cells through PPARδ. Chapple *et al*. (21) have shown that COX-2 is predominantly expressed in macrophages within colorectal adenomas. Also besides PGE_2 and PGE_3, AA and EPA can be metabolized by COX into various prostaglandins, thromboxanes and leukotrienes, collectively called eicosanoids. These eicosanoids possess a broad spectrum of biological activities. For example, AA can be metabolized to leukotriene B_4, which has considerable inflammatory potential, while EPA is metabolized to leukotriene B_5, which is one to two orders of magnitude less inflammatory (6). Also PGI_2 and TXA_2 which are primarily synthesized by vascular endothelial cells have important roles in cancer biology because they are involved in tumor cell-vascular endothelial cell and tumor cell-platelet interactions (8). Overall, these observations reveal that oxidative metabolism of AA and EPA by the cyclooxygenase pathway can lead to a variety of intercellular and intracellular signals and suggest that COX-2 mediated paracrine signaling between other cell types in the colon such as macrophages, endothelial cells or fibroblasts, and colonic epithelial cells could also play an important role in the colon cancer modulating effects of n-6 and n-3 PUFAs.

Furthermore our results suggest that lipid peroxidation-induced oxidative stress which could lead to higher levels of apoptosis and decreased levels of colonic cell proliferation could be an important pathway for the anti-colon carcinogenic effects of PUFAs. HFFO diets with a high amount of EPA increased lipid peroxidation compared to HFCO diets with a high amount of LA, not only locally in the colon, as measured by feacal levels of MDA, but also in other organs such as the liver. In this respect it is interesting to note that other *in vivo* studies (22,23,24) have shown that the addition of vitamin E to the diet diminishes the anticancer effects of n-3 PUFAs in mice bearing mammary tumor or lung carcinoma, suggesting that *in vivo* oxidation of n-3 PUFAs also underlies the chemopreventive effects in other types of cancer.

In summary, the oxidative stress related pathway seems to be more important for the anti-colon carcinogenic effects of n-3 PUFAs than modulation of PGE_2 synthesis. However, complete understanding of the molecular, cellular and biochemical mechanisms of the adverse and beneficial effects of n-6 and n-3 PUFAs on the multistage process of colorectal carcinogenesis seems to be a lot more complicated.

7.3 Implications of n-3 PUFA consumption for humans

Epidemiological studies have also suggested an inverse relation between fish and n-3 PUFA intake and the risk of colorectal carcinogenesis. Migration studies have shown that the low mortality rates from colon cancer in Japan increase when Japanese migrate to

the US and adapt to a Western diet, which contains for example higher levels of n-6 PUFAs, such as linoleic acid. Like Japanese, also Eskimos have a relatively low incidence of colon cancer (25). Both populations consume large quantities of n-3 PUFAs (1-3 gram per day) due to a high intake of marine foods, including fish, whereas the average consumption of n-3 PUFAs in Western countries is only 100 mg per day (26). The rats in our *in vivo* experiments also received daily high amounts of fish oil (20%) for 8 weeks, which provided about 0.24 gram EPA and 0.17 gram DHA per 100-200 gram rat per day. It must be recognized that these large intakes, adjusted to the weight of humans, do not reflect a human Western diet, although they could be consumed by the use of fish oil supplements. Reddy and Sugie (27) revealed though that also lower amounts of n-3 PUFAs in high-fat diets (only 5.9% of Menhaden fish oil in combination with 17.6% corn oil) are effective in inhibition of incidence as well as multiplicity of adenocarcinomas in the rat colon.

Data on the role of n-3 PUFA intake in the etiology of human colorectal cancer are however inconclusive. In a study of Hursting *et al.* (28), fish n-3 PUFAs showed a negative association, although not-significant, with the incidence of cancers of the breast, prostate, lung, cervix and colon. Terry *et al.* (29) on the other hand found no clear association between specific fatty acids, such as α-linolenic acid, EPA, DHA or the sum of n-6 fatty acids or n-3 fatty acids and the development of colorectal cancer in Sweden. Daily consumption of n-3 PUFAs (EPA + DHA) seems however to be very low in this study (EPA: 0.03-0.09 gram / DHA: 0.08-0.18 gram) (29) when compared to the daily intake of n-3 PUFAs in Japanese female dietitians (EPA: 0.19-0.49 gram / DHA: 0.36 - 0.87 gram) (30). The low level of n-3 PUFA intake in the Swedish study was probably insufficient to exert anti-colon carcinogenic effects and may have caused the negative results in their study. The efficacy of dietary n-3 PUFAs may depend on the levels of n-6 PUFAs that are also found in mixed diets and may account for some of the variability of results observed among studies using human subjects (31). Bartram *et al.* (32,33) clearly demonstrated that the dietary n-6/n-3 ratio is indeed an important determinant of the beneficial effects of n-3 PUFAs on colorectal carcinogenesis. Two human studies were carried out in which fish oil supplementation (4.4 gram / day) was given in order to suppress rectal epithelial cell proliferation and PGE_2 synthesis. This was only achieved when the dietary n-6/n-3 ratio was 2.5/1 but not with the same absolute level of fish oil intake and an n-6/n-3 ratio of 4/1.

The Dutch Health Council (2001) has advised an average daily intake of 0.2 gram n-3 PUFAs, which can be provided by consumption of one portion of fatty fish, such as herring or mackerel per week. These levels should be adequate to decrease the risk of

cardiovascular diseases. It can however not be excluded that a higher intake of n-3 PUFAs is necessary to profit from the beneficial effects on colorectal carcinogenesis.
If oxidative stress is indeed involved in the anti-colon carcinogenic effects of n-3 PUFAs in HFFO diets, as already seems reasonable from the results of our studies and others (22,23,24), this would implicate that attention should be paid to the amount of antioxidants that are also consumed in addition to HFFO diets. So, in contrary to the reported beneficial protective effects of vitamin E on colon cancer (34), controversial results are observed upon antioxidant supplementation and the anticancer activity of n-3 PUFAs (22,23).
In summary, human diets enriched with high levels of n-3 PUFAs may have beneficial colon cancer inhibiting effects, in particular when containing only basal essential amounts of n-6 PUFAs and antioxidants, such as vitamin E or C.

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Nederlandse samenvatting

Samenvatting van de resultaten

Eerder uitgevoerde wetenschappelijke onderzoeken suggereren dat zowel modulatie van cyclooxygenase (COX)-gemedieerde synthese van prostaglandine E_2 (PGE_2) als lipide peroxidatie-geïnduceerde oxidatieve stres een rol kunnen spelen in de remming van colonkanker door n-3 meervoudig onverzadigde vetzuren (MOV). Het exacte werkingsmechanisme waarop n-3 vetzuren colonkanker kunnen voorkomen of remmen ten opzichte van n-6 vetzuren is echter tot op heden nog niet goed bekend. Het doel van dit promotieonderzoek was daarom de cellulaire, biochemische en moleculaire mechanismen van de gunstige effecten van n-3 MOV en de nadelige effecten van n-6 MOV op de ontwikkeling van colorectaal kanker te onderzoeken, met speciale aandacht voor PGE_2 en de oxidatieve stres respons.

Effecten van n-6 en n-3 meervoudig onverzadigde vetzuren op PGE_2 synthese en celproliferatie

Het n-6 vetzuur arachidonzuur (AA; 20:4n-6) is het precursor vetzuur van de prostaglandines van de 2-serie, zoals PGE_2. In hoofdstuk 3 van dit proefschrift staan experimenten beschreven waaruit blijkt dat humane coloncarcinoma Caco-2 cellen, die veel COX-2 tot expressie brengen, PGE_2 kunnen produceren na blootstelling aan AA. HT-29 coloncarcinoma cellen produceren daarentegen bijna geen PGE_2 onder dezelfde condities. De PGE_2 synthese kan echter wel geïnduceerd worden in deze cellijn door toevoeging van IL-1β, een cytokine waarvan bekend is dat het de COX-2 expressie kan verhogen. Het gegeven dat linolzuur (LA; 18:2n-6) de PGE_2 productie in zowel Caco-2 als HT-29 cellen niet kan verhogen is in overeenstemming met het feit dat linolzuur, de voorloper van de n-6 vetzuurfamilie niet omgezet wordt in de prostaglandine precursor arachidonzuur in deze humane coloncarcinoma cellijnen (zie hoofdstuk 2).

Het n-3 vetzuur eicosapentaeenzuur (EPA; 20:5n-3) is het precursor vetzuur van de prostaglandines van de 3-serie, zoals PGE_3. EPA en AA kunnen competitie aangaan voor COX enzymactiviteit omdat ze beide substraten zijn voor de prostaglandine synthese. In hoofdstuk 3 staan experimenten beschreven waaruit blijkt dat EPA de AA-geïnduceerde PGE_2 synthese competatief remt in Caco-2 cellen. De dierproeven die beschreven staan in zowel hoofdstuk 4 als hoofdstuk 5 laten ook zien dat hoog vet visolie diëten met een hoog gehalte aan EPA resulteren in lagere hoeveelheden PGE_2 in het bloedplasma van F344 ratten, vergeleken met hoog vet maisolie diëten met een hoog gehalte aan linolzuur.

Om de hypothese te kunnen onderzoeken dat n-3 MOV zoals EPA de groei van colon-tumorcellen kunnen remmen via remming van AA-geïnduceerde PGE_2 synthese zijn de

effecten van AA en EPA op de proliferatie van de colontumorcellen Caco-2 en HT-29 vergeleken. Remming van PGE_2 synthese door EPA ten opzichte van AA leidt echter niet tot een verschillend effect op celproliferatie van Caco-2 cellen (zie hoofdstuk 3). EPA remt de celdeling van Caco-2 cellen, maar het n-6 vetzuur AA heeft dezelfde celgroei-inhiberende en cytotoxische effecten. Verschillen in AA en EPA-geïnduceerde genexpressie in Caco-2 cellen waren ook minimaal. Er werd bijvoorbeeld geen verschillend effect tussen AA en EPA gevonden op de expressie van genen die betrokken zijn bij de COX-gemedieerde PGE_2 signaaltransductie, zoals prostaglandine-receptoren, prostaglandine E synthase, fosfolipase A2, een prostaglandine transporter en het COX-2 gen (zie hoofdstuk 6).
Daarnaast hebben AA en EPA geen invloed op de celdeling van HT-29 cellen. Inductie van COX-2 expressie en PGE_2 synthese in HT-29 cellen door IL-1β resulteert niet in een toename van de celproliferatie, maar in een remming van de celproliferatie door zowel AA als EPA (zie hoofdstuk 3).
Het causale verband tussen PGE_2 en proliferatie van colonepitheelcellen wordt nog meer in twijfel gebracht door het feit dat blootstelling aan exogeen PGE_2 geen stimulerend effect blijkt te hebben op de proliferatie van Caco-2, HT-29, LIM1215 en HCA-7 colon-tumorcellen (zie hoofdstuk 3). Deze negatieve effecten zouden mogelijk veroorzaakt kunnen worden door de afwezigheid van prostaglandine E (EP) receptoren in deze cellijnen, maar door de resultaten van hoofdstuk 6, waaruit blijkt dat alle 4 de EP receptor subtypen aanwezig zijn in Caco-2 cellen, lijkt dit niet erg waarschijnlijk.
Regulatie van celproliferatie in colonkanker cellijnen blijkt dus niet gemedieerd te worden via PGE_2. Deze resultaten komen niet overeen met de eerder genoemde hypothese en trekken de rol van PGE_2 in de anti-colon carcinogene effecten van n-3 vetzuren en carcinogene effecten van n-6 vetzuren in twijfel.

Effecten van n-6 en n-3 meervoudig onverzadigde vetzuren op de oxidatieve stres respons

Humane coloncarcinoma Caco-2 cellen zijn gebruikt als een *in vitro* model om de effecten van n-6 en n-3 MOV op gap junction intercellulaire communicatie (GJIC) tijdens spontane proliferatie en differentiatie van Caco-2 cellen te onderzoeken (zie hoofdstuk 2). De GJIC capaciteit van Caco-2 cellen neemt toe tijdens spontane differentiatie. Daarentegen worden er geen verschillende effecten op GJIC capaciteit gevonden tussen n-3 en n-6 MOV. Kortdurige blootstelling (24 uur) aan linolzuur (LA; 18:2n-6), α-linoleenzuur (ALA; 18:3n-3), arachidonzuur (AA; 20:4n-6) en eicosapentaeenzuur (EPA; 20:5n-3) beïnvloedt de GJIC niet, terwijl langdurige incubatie met LA of ALA tijdens differentiatie (> 10 dagen) de GJIC van deze coloncellen remt. Lange-keten

metabolieten van LA en ALA zoals AA en EPA worden niet gevormd na blootstelling aan LA en ALA. Het kan daarom worden uitgesloten dat prostaglandines een rol hebben gespeeld in de waargenomen effecten van zowel LA als ALA op de GJIC (zie hoofdstuk 2).

Alhoewel het precieze mechanisme van GJIC remming bij langdurige blootstelling van Caco-2 cellen aan LA en ALA onduidelijk is, lijkt het waarschijnlijk dat cytotoxiciteit, vermoedelijk gemedieerd door vetzuuroxidatie producten, een rol speelt omdat blootstelling aan vetzuren met meerdere dubbele bindingen in de koolstofketen zoals AA en EPA de GJIC capaciteit van Caco-2 cellen al na een paar dagen volledig remt (zie hoofdstuk 2). De meervoudig onverzadigde vetzuren AA en EPA remmen ook de proliferatie van Caco-2 cellen en verhogen de cytotoxiciteit ten opzichte van de langere keten vetzuren LA (18:2n-6) en ALA (18:3n-3) (zie hoofdstuk 3). Dit suggereert dat het aantal dubbele bindingen in de koolstofketen van vetzuren belangrijker is voor de anti-proliferatieve en cytotoxische effecten op colontumorcellen dan de plaats van de dubbele binding in de koolstofketen.

MOV zoals AA en EPA zijn erg gevoelig voor enzymatisch- en niet-enzymatisch-geïnduceerde vetzuuroxidatie. Peroxidatieprodukten van MOV hebben waarschijnlijk een belangrijke rol gespeeld in AA en EPA-geïnduceerde anti-proliferatieve en cytotoxische effecten, omdat malondialdehyde (MDA), één van de eindprodukten van vetzuuroxidatie, verhoogd is in Caco-2 cellen na blootstelling aan AA en EPA. Simultane blootstelling aan MOV en antioxidanten, zoals vitamine E, vermindert de MDA vorming in Caco-2 cellen. Ook de celgroei remmende effecten van zowel AA als EPA worden gedeeltelijk opgeheven door co-incubatie van vetzuren met antioxidanten (zie hoofdstuk 3). Daarnaast heeft blootstelling aan indomethacine (IM), een COX enzym-remmer die de AA-geïnduceerde PGE_2 synthese in Caco-2 cellen duidelijk remt, ook tot gevolg dat de anti-proliferatieve effecten van zowel AA en EPA gedeeltelijk worden opgeheven. Verder resulteert verhoging van de COX-2 expressie en PGE_2 synthese in HT-29 cellen door toevoeging van IL-1β niet in een toename van de proliferatie, maar wordt de celproliferatie juist geremd en neemt de cytotoxiciteit van zowel AA als EPA toe. Beide resultaten suggereren dat naast spontane vetzuuroxidatie, de remmende effecten van AA en EPA op de groei van colontumorcellen ook veroorzaakt kunnen worden door peroxidatieprodukten die gegenereert worden tijdens COX activatie.

De dierproef die beschreven staat in hoofdstuk 4 laat zien dat hoog vet visolie diëten (HFFO) met een hoog gehalte aan EPA de initiatie van AOM-geïnduceerde aberrante crypt foci (ACF; beginstadium van colonkanker) in het colon kunnen remmen ten opzichte van hoog vet maisolie diëten met een hoog gehalte aan LA, wanneer deze diëten gevoerd worden tijdens de initiatie- en postinitiatie fase in de ontwikkeling van

colonkanker. Dit beschermende effect van HFFO diëten op colonkanker wordt mogelijk ook veroorzaakt door een hoger gehalte aan oxidatieve stres. De hoeveelheid MDA, een indicator van de hoeveelheid oxidatieve stres in het lichaam, is namelijk verhoogd in de lever (zie hoofdstuk 4+5) en de ontlasting (zie hoofdstuk 4) van HFFO gevoerde F344 ratten ten opzichte van HFCO gevoerde ratten. Dit geeft aan dat HFFO diëten de hoeveelheid vetzuuroxidatie in het lichaam inderdaad kunnen verhogen. Ook lagere gehalten aan vitamine E in het plasma van HFFO gevoerde ratten kunnen er op duiden dat HFFO gevoerde ratten meer vitamine E verbruikt hebben om zich te beschermen tegen de hogere gehalten aan oxidatieve stres die door deze diëten veroorzaakt worden. Ook de verhoogde activiteit van het antioxidant enzym catalase in de lever van HFFO gevoerde ratten kan mogelijk toegeschreven worden aan de beschermende reactie tegen deze hogere gehalten aan oxidatieve stres in onder andere de lever (zie hoofdstuk 5).
HFFO-geïnduceerde oxidatieve stres speelt waarschijnlijk ook een rol in signaal-transductie en regulatie van genexpressie via redox-gevoelige mechanismen. Mechanismen die verantwoordelijk zijn voor inductie van antioxidant genen als reactie op oxidatieve stres worden gemedieerd via een antioxidant-responsief element (ARE), ook wel een electrofiel-responsief element (EpRE) genoemd. Functionele EpRE/ARE sequenties zijn aangetoond in glutathion S-transferase Ya (GSTYa), glutathion S-transferase P1 (GSTP1), NAD(P)H:chinon oxidoreductase (NQO1), γ-glutamylcysteine synthetase (de zware en de lichte subeenheid), heme oxygenase (HO), thioredoxine, NF-E2-gerelateerde factor 2 (Nrf2), ferritine (zware en lichte keten) en metallothioneïnes. Van deze genen zijn naast ferritine en metallothioneïne, ook peroxiredoxine 2, NQO1 en GSTP1 significant verhoogd in rat colon epitheel na een hoog vet visolie dieet in vergelijking tot een hoog vet maisolie dieet. Ook het Caco-2 genexpressie experiment beschreven in hoofdstuk 6 laat zien dat de expressie van antioxidant genen zoals metallothioneïnes en NADH dehydrogenase (ubichinon 1) beta subcomplex 3, wat een onderdeel is van NQO1, significant verhoogd kan worden door hoge gehalten aan MOV (20 μM). Ook herstelmechanismen zoals heat shock eiwitten die beschadigde eiwitten herstellen of stukjes van beschadigde eiwitten opruimen en het proteasoom dat oxidatieve schade aan eiwitten herkent en opruimt zijn significant verhoogd in Caco-2 cellen na blootstelling aan n-3 en n-6 MOV (zie hoofdstuk 6). Inductie van antioxidant- en stres-eiwitten in zowel de *in vitro* als *in vivo* experimenten kan worden gezien als een adaptatie aan de toegenomen hoeveelheid oxidatieve stres, met andere woorden een soort celbeschermend vermogen.
Wanneer de hoeveelheid oxidatieve stres echter te groot wordt en antioxidanten en hersteleiwitten niet langer voldoende bescherming bieden tegen de oxidatieve stres-geïnduceerde cytotoxiciteit, kan de proliferatie van bijvoorbeeld coloncellen geremd

worden of de apoptose (geprogrammeerde celdood) verhoogd worden om op deze manier verdere uitgroei van beschadigde cellen te voorkomen en dood en verwijdering van deze cellen te stimuleren. De microarray experimenten laten zien dat genen die betrokken zijn bij de regulering van celgroei en celdood inderdaad gemoduleerd worden door HFFO diëten (zie hoofdstuk 5) en MOV in de celkweek-experimenten (zie hoofdstuk 6). Bijvoorbeeld histon 2b, een gen dat betrokken is bij celproliferatie, is significant verlaagd in de gezonde dikke darm van de rat na consumptie van visolie. Een lager mRNA niveau van histon 2b kan impliceren dat de celdeling in het colonepitheel van hoog vet visolie gevoerde ratten inderdaad lager is dan in hoog vet maisolie gevoerde ratten. Een gen dat betrokken is bij de inductie van apoptose is bijvoorbeeld caspase 5. Dit pro-apoptotisch gen wordt verhoogd door zowel 10 als 20 μM LA, AA en EPA na 48 uur blootstelling in humane coloncarcinoma Caco-2 cellen.
Alhoewel de oxidatieve stres gerelateerde hypothese nog speculatief is en slechts van een paar genen in dit proces de expressie verandert, geven de transcriptoom analyses wel aan dat genen die een rol spelen bij oxidatieve stres en cel-groei en dood inderdaad gemoduleerd worden in ratten en Caco-2 cellen.
Over het algemeen suggereren de resultaten dat n-3 vetzuren kunnen beschermen tegen het ontstaan en de ontwikkeling van dikke darmkanker via verhoging van oxidatieve stres, wat een verhoging van apoptose en een vermindering van de celproliferatie in het colon tot gevolg kan hebben.

Curriculum Vitae and List of publications

Curriculum Vitae

Yvonne Elizabeth Maria Dommels werd geboren op 18 juni 1975 te Geldrop. Na het behalen van het VWO diploma op het Hertog Jan College te Valkenswaard in 1993, is ze begonnen aan de studie Gezondheidswetenschappen (afstudeerrichting Biologische Gezondheidkunde) aan de Universiteit van Maastricht. Tijdens deze studie heeft ze 2 stage onderzoeken verricht, waarvan de eerste is uitgevoerd in Maastricht op de afdeling Humane Biologie in samenwerking met de afdeling Interne Geneeskunde van het academisch ziekenhuis in Maastricht. Voor de tweede stage is ze 8 maanden naar Boston (USA) geweest om onderzoek te doen op de afdeling Toxicologie van het Massachusetts Institute of Technology. In augustus 1998 behaalde zij haar doctoraal diploma.
Vanaf november 1998 werd ze voor 4 maanden aangesteld bij TNO Voeding te Zeist om het onderzoeksvoorstel voor haar promotieonderzoek te schrijven. Dit promotie-onderzoek, beschreven in dit proefschrift, werd vanaf februari 1999 uitgevoerd bij de leerstoelgroep Toxicologie van de Wageningen Universiteit in het kader van het TNO-WUR Centrum voor Voedingstoxicologie. In dezelfde periode heeft Yvonne de certificaten 'Stralingshygiene, deskundigheidsniveau 5b' en 'Proefdierkunde op grond van art.9 van de Wet op Proefdieren' behaald. Daarnaast heeft ze enkele modules van de Postdoctorale Opleiding Toxicologie gevolgd om voor erkenning als toxicoloog in aanmerking te komen.
Sinds half augustus 2003 is Yvonne werkzaam als wetenschappelijk onderzoeker op het Rikilt Instituut voor Voedselveiligheid in Wageningen, in de onderzoeksunit 'Effects of Food Bioactives'.

List of publications

Yvonne E.M. Dommels, Gerrit M. Alink, Peter J. van Bladeren and Ben van Ommen. (2002) Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results of cultured colon cells, animal models and human studies. *Environmental Toxicology and Pharmacology*, **12(4)**, 233-244

Yvonne E.M. Dommels, Gerrit M. Alink, Jozef P.H. Linssen and Ben van Ommen. (2002) Effects of n-6 and n-3 polyunsaturated fatty acids on gap junctional intercellular communication during spontaneous differentiation of the human colon adenocarcinoma cell line Caco-2. *Nutrition and Cancer*, **42(1)**, 125-130

Yvonne E.M. Dommels, Merel M.G. Haring, Nynke G.M. Keestra, Gerrit M. Alink, Peter J. van Bladeren and Ben van Ommen. (2003) 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. *Carcinogenesis*, **24(3)**, 385-392

Yvonne E.M. Dommels, Suzanne Heemskerk, Hans van den Berg, Gerrit M. Alink, Peter J. van Bladeren and Ben van Ommen. (2003) Effects of high fat fish oil and high fat corn oil diets on initiation of AOM-induced colonic aberrant crypt foci in male F344 rats. *Accepted for publication in Food and Chemical Toxicology*

Yvonne E.M. Dommels, Petra van den Hil, Jozef P.H. Linssen, Gerrit M. Alink, Peter J van Bladeren and Ben van Ommen. (2003) High fat fish oil diets modulate expression of genes involved in oxidative stress, cell growth, acute phase response and lipid metabolism in normal rat colonic epithelium of male F344 rats. *Submitted*

Yvonne E.M. Dommels, Gerrit M. Alink, Peter J. van Bladeren and Ben van Ommen. (2003) n-6 and n-3 polyunsaturated fatty acid-induced transcriptome changes in Caco-2 cells. *In preparation*

Rob Stierum, Marco Gaspari, Yvonne Dommels, Taoufik Quatas, Helma Pluk, Sonja Jespersen, Jack Vogels, Kitty Verhoeckx, John Groten and Ben van Ommen. (2003) Proteome analysis reveals novel proteins associated with proliferation and differentiation of the colorectal cancer cell line Caco-2. *Accepted for publication in Biochimica et Biophysica Acta*

Harald Moonen, Yvonne Dommels, Marloes van Zwam, Marcel van Herwijnen, Gerrit Alink, Jos Kleinjans and Theo de Kok. (2003) Effects of polyunsaturated fatty acids on DNA adduct formation by heterocyclic aromatic amines in human adenocarcinoma colon cells. *Submitted*

Abstracts

Yvonne Dommels, Gerrit Alink, Peter van Bladeren and Ben van Ommen. The Caco-2 cell line as an *in vitro* model to study the influence of n-6 and n-3 polyunsaturated fatty acids on colorectal carcinogenesis. Proceedings of the scientific meeting of The Netherlands Society of Toxicology, p38, Kerkrade, The Netherlands, 2000

Yvonne EM Dommels, Rob H Stierum, Marlou LPS van Iersel, Gerrit M Alink, Peter J van Bladeren and Ben van Ommen. The Caco-2 cell line as an *in vitro* model to study the influence of n-6 and n-3 polyunsaturated fatty acids on colorectal carcinogenesis. 91st Annual Meeting of the American Association for Cancer Research, p343, AACR, San Francisco, USA, 2000

Yvonne EM Dommels, Gerrit M Alink, Peter J van Bladeren and Ben van Ommen. Colon cell lines as *in vitro* models to study the influence of n-6 and n-3 polyunsaturated fatty acids on colorectal carcinogenesis. 7th International Conference on Eicosanoids & other Bioactive Lipids in Cancer, Inflammation and Related Diseases, p108, Nashville, Tennessee, USA, 2001

Yvonne EM Dommels, Gerrit M Alink, Peter J van Bladeren and Ben van Ommen. Colon cell lines as *in vitro* models to study the influence of n-6 and n-3 polyunsaturated fatty acids on colorectal carcinogenesis. Proceedings of the scientific meeting of The Netherlands Society of Toxicology, p12, Nijmegen, The Netherlands, 2001

HJJ Moonen, YEM Dommels, M van Zwam, MHM van Herwijnen, GM Alink, IMCM Rietjens, JCS Kleinjans and TMCM de Kok. Effects of polyunsaturated fatty acids on DNA adduct formation by heterocyclic amines in HCA-7 cells. Proceedings of the scientific meeting of The Netherlands Society of Toxicology, p19, De Bilt, The Netherlands, 2003

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Dankwoord

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AA	arachidonic acid (20:4n-6)
ACF	aberrant crypt foci
ALA	alpha-linolenic acid (18:3n-3)
ALP	alkaline phosphatase
AOM	azoxymethane
ApoC3	apolipoprotein C3
APPR	acute phase protein response
ARE	antioxidant responsive element
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CAT	catalase
COX	cyclooxygenase
DBA	*Dolichos biflorus* agglutinin
DHA	docosahexaenoic acid (22:6n-3)
DMH	1,2-dimethylhydrazine
EPA	eicosapentaenoic acid (20:5n-3)
EpRE	electrophile responsive element
EROD	ethoxyresorufin O-deethylase
EtOH	ethanol
FAP	familial adenomatous polyposis
FCS	fetal calf serum
FPTase	farnesyl protein transferase
GJIC	gap junctional intercellular communication
GLA	gamma-linolenic acid
β2GP	β2 glycoprotein
GSH-Px	glutathione peroxidase
GST	glutathione transferase
GSTP1	glutathione S-transferase P1
GSTYa	glutathione S-transferase Ya
HDL	high density lipoprotein
HFCO	high fat corn oil
HFFO	high fat fish oil
HNPCC	hereditary non-polyposis colorectal cancer
HO	heme oxygenase
IF	induction factor
IL-1β	interleukin 1β
IM	indomethacin
LA	linoleic acid (18:2n-6)
LDH	lactate dehydrogenase
LFCO	low fat corn oil
MAM	methylazoxy-methanol
MDA	malondialdehyde
MGMT	O^6-methylguanine-DNA-methyltransferase
MUFA	monounsaturated fatty acids
NQO1	NAD(P)H:quinone oxidoreductase
Nrf2	NF-E2-related factor 2
NSAID	nonsteroidal anti-inflammatory drug
ODC	ornithine decarboxylase
PCA	principal component analysis
PCDA	principal component discriminant analysis
PCNA	proliferating cell nuclear antigen
PGE	prostaglandine E
PNP	paranitrophenol
PPAR	peroxisome proliferator activated receptor
PROD	pentoxyresorufin O-deethylase
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SAM	statistical analysis of microarrays
SFA	saturated fatty acids
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TBHQ	tertiary butyl hydroquinone
TEAC	trolox equivalent antioxidant capacity
TG	triglyceride
TPK	tyrosine protein kinase
TSSC3	tumor suppressing subtransferable candidate 3
UDPGT	UDP-glucuronyltransferase
Vit E	vitamin E
VLDL	very low density lipoprotein

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