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Heinrich-Heine-Universität Düsseldorf
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"The influence of dietary compounds like polyunsaturated fatty acids in colon cancer development: role of Cox-2 and PPARs"

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Content

1. Introduction

1.1. Colorectal Cancer

Colorectal cancer is the second common cancer in developed countries and the second most common death from cancers in United States. Colonic tumours are generally thought to arise sequentially from precancerous polyps or adenomas. Understanding the aetiology of colorectal cancer has advanced considerably in recent years, but important gaps still remain. Fearon and Vogelstein reconstructed a sequence of alterations leading to malignancy and the development of colorectal cancer. The different stages of colon cancer development are shown in figure 1.1. A small proportion of cases of colon cancer are due to inheritance of mutations in certain genes, such as the adenomatous polyposis coli (*apc*) gene, but the majority of cases are sporadic (i.e. due to mutations arising in somatic cells). Studies of cells from sporadic colorectal cancers have shown that mutations of several genes, including the apc gene, are common and are probably the cause of neoplastic behaviour of the cells, but the causes of the mutations are not yet known (Franks and Teich, Cellular and Molecular Biology of Cancer, 1997).

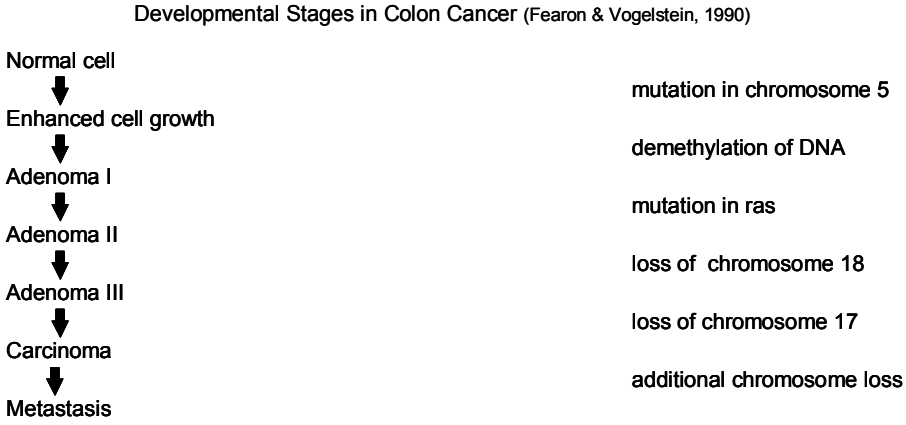


Figure 1.1: Stages in colon carcinogenesis (adapted by Fearon and Vogelstein, 1990)

The interest in the role of nutritional factors in the aetiology of colorectal cancer stems largely from the wide variations in disease rates between populations with different diets, plus the plausibility of the hypothesis that the contents of the colon and rectum could affect the risk of cancer. Burkitt originally suggested that dietary fibre may protect against colorectal cancer, while subsequent ecological analyses of diet and cancer rates suggested

that meat or animal fat might increase risk and that fibre may be protective (Burkitt D P, 1971, 1978).

Although fat intake was early linked to the incidence of colorectal cancer, subsequent studies also highlighted total calorie intake (Mai V et al., 2003). Furthermore, a distinction has been made between saturated fat from animal sources, which seems to increase the incidence of cancer, and the highly unsaturated ω-3 fish oils, which seem to have protective effect (Bruckner H W, Pitrelli J and Merrick M, 2000, www.cancer.org).

A number of studies have shown that experiment rat colon carcinogenesis is inhibited by feeding a diet containing a high level of fish oil or supplemented with ω-3 fatty acids (reviewed in Rose D P and Connolly J M, 1999). Various germ line mutations of the murine *apc* gene result in multiple intestinal polyposis and provide a model for human familial adenomatous polyposis. The inhibitory effects of DHA and DHA/EPA enriched fish oil concentrate on the development and progression of intestinal polyps have been demonstrated in two of these models (Rose D P and Connolly J M, 1999). On the other hand, experimental data have shown that n-6 fatty acids stimulate carcinogenesis, tumour growth and metastasis, whereas n-3 fatty acids exert suppressive effects (Boudreau M D et al., 2001, Rao C V et al., 2001).

This suggests that the composition of ingested dietary fat is probably more critical concerning cancer risk than the total amount of fat.

1.2. Poly-unsaturated fatty acids: Nomenclature, dietary sources and metabolic conversion

The unsaturated fatty acids (USFAs) comprise monounsaturates and polyunsaturates. The conventional chemical nomenclature is to begin the systematic numbering of carbon atoms from the carboxyl terminal group. The carbon atoms number 2 and 3 from the carboxyl group are referred to as α and β carbons, respectively, the last carbon is the ω- or *n*-carbon, and the position of the double bond is indicated by the symbol Δ, followed by a number (for example: Δ^9 refers to a double bond between carbon atoms 9 and 10 from the carboxyl group). However, an accepted practice in describing the chemical structure of fatty acid molecules is to start numbering the carbons at the methyl group (ω- or *n*-) (Rose D P and Connolly J M, 1999). In figure 1.2 the formula and structure of some important dietary fatty acids is shown.

The ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) cannot be synthesized by mammals, and because they must be obtained from the diet, they are referred to as "essential fatty acids".

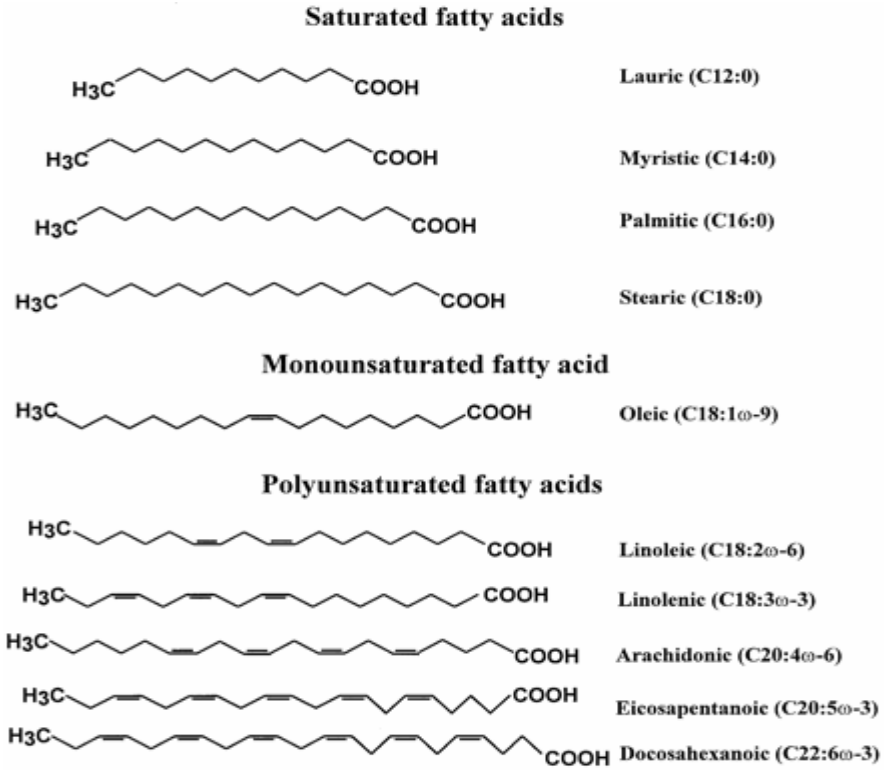


Fig. 1.2: Formulae and structures of some important saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids. The number before the colon indicates the number of carbon atoms in the fatty acid chain and the number after the colon indicates the number of double bonds. Double bond geometry is indicated by the prefix *cis* or *trans*. The family to which an unsaturated fatty acid belongs is indicated by ω-3, ω-6 or ω-9. (Adapted by Bartsch H et al., 1999)

Linoleic acid (LA) is the principal polyunsaturated fatty acid in human diet. Vegetable oils such as those from soybeans, corn and sunflowers are rich in LA. Dietary LA is considered to be the major source of tissue Arachidonic acid (AA), although meat fat and lean meat are direct sources in human diet. Linolenic acid (LNA) is found in dark green leafy plants and in linseed, walnut and blackcurrant oils. Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are abundant in deep cold-water fish. Both classes of fatty acids accumulate in cell membranes (Bartsch H et al., 1999).

LNA and LA are metabolised to longer chain fatty acids, largely in the liver. LNA is converted to DHA and EPA, while LA is the metabolic precursor of AA. The metabolic pathways involve increases in chain length and degree of unsaturation that are achieved by

adding extra double bonds between the existing double bond and the carboxyl group. Production of AA from LA and EPA from LNA respectively proceeds preferentially by Δ^6-desaturation, followed by two carbon atom chain elongation and Δ^5-desaturation. Metabolism of ω-3 and ω-6 fatty acids proceeds through a series of competitive desaturation and elongation steps, which is limited by the activity of Δ^{6-}desaturase. ω-3 fatty acids have greater affinities for the enzyme, which means that increasing the dietary intake of ω-3 PUFAs reduces the desaturation of LA and consequently the production of AA (Rose D P and Connolly J M, 1999). These metabolic steps are summarised in figure 1.3.

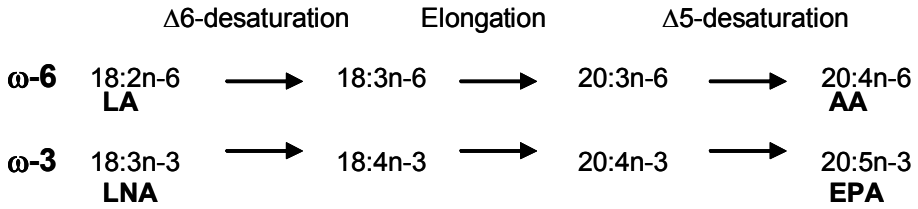


Figure 1.3: Metabolism of ω-6 and ω-3 fatty acids.

AA and EPA are further metabolised to eicosanoids by the action of two classes of enzymes: the prostaglandin synthases or cyclooxygenases (Cox), which produce prostaglandins, prostacyclin and thromboxanes, and the lipoxygenases (Lox), which catalyse the biosynthesis of hydroxyeicosatetraenoic acids (HETEs) and leukotrienes. These products, called eicosanoids because of their common origin from a 20-carbon PUFA, act as local hormones and are chemical transmitters for a variety of intercellular and intracellular signals (Krause W and DuBois R N, 2000). The biosynthesis of eicosanoids is initiated by the release of AA or EPA from the cell membrane. This cleavage from membrane phospholipids is mediated by the activity of Phospholipase A_2. The relative proportions of PUFAs on cell membranes, as well as the cell type, are the primary factors in regulating which eicosanoid will be generated. In general, AA-derived eicosanoids have proinflammatory properties, whereas EPA-derived eicosanoids have anti-inflammatory effects. Eicosanoids generated from AA, such as PGE2, leukotriene B4, thromboxane A2 and 12-hydroxyeicosatetraenoic acid (12-HETE) have been positively linked to carcinogenesis (Fischer S M, 1997).

Because of the increased amounts of ω-6 fatty acids in the western diet, the eicosanoid products from AA are formed in larger quantities than those formed from ω-3 fatty acids.

The eicosanoids from AA are biologically active in small quantities and if they are formed in large amounts they contribute to cell proliferation, as well as inflammatory disorders (Simopoulos A P, 1999).

1.3. Eicosanoid metabolism: The Cyclooxygenase and Lipoxygenase pathways

After AA is cleaved from the cell membrane, it can be metabolised through one of three major pathways: the cyclooxygenase pathway, the lipoxygenase pathway and the cytochrome P-450 monooxygenase pathway. AA can also be converted non-enzymatically to isoprostanes via free radical oxidation.

The Cox pathway of AA metabolism has been extensively studied (Krause W and DuBois R N, 2000). There are two Cox enzymes: Cox-1, which is constitutively expressed, and Cox-2. Cox-2 expression is induced by phorbol esters, hormones and growth factors. Cox-2 overexpression has been detected in many types of cancers, including colorectal cancer. A schematic overview of the Cox-2 pathway of AA metabolism is shown in figure 1.4.

The conversion of AA to prostaglandin G_2 occurs via a two step process. In the first step two molecules of oxygen are added to the arachidonate, forming the bicyclic peroxidate intermediate PGG_2. In the second step, peroxidation of PGG_2 results in PGH_2. PGH_2 is in turn converted to prostaglandins, prostacyclins and thromboxanes, depending on the cell type and the enzymes present. An expanding body of evidence indicates that the prostaglandin products of the Cox-2 pathway enhance cell proliferation and growth in both normal and tumour cells (Fisher S M, 1997). The main product of Cox-2 metabolism of AA is PGE_2. High levels of Cox-2 expression and PGE_2 have been found in many cancers. Human colon cancer tissues produce more PGE_2 than the surrounding normal tissue. It has been shown that the level of PGE_2 in polyps was elevated by about 40% and in colon cancer by almost 3-fold. Moreover, studies in the early 1980s indicated that non-steroidal anti-inflamatory drugs (NSAIDs) were chemopreventive in animal models of colon cancer (Fisher S M, 1997). Epidemiological studies also revealed that the use of aspirin reduced the relative risk for colon cancer of individuals by 40%. The same effect was shown for indomethacin, whereas acetaminophen, which does not affect Cox activity, was shown to provide no protective effect. The results from these studies, in combination with the studies showing increased Cox-2 expression and PGE_2 production in colon cancers suggested that

Cox-2 may be involved in colorectal carcinogenesis. Since then, results from many researchers have confirmed these findings. However, the role of Cox-2 in neoplasia has yet to be understood completely (reviewed in Krause W and DuBois R N, 2000).

ω-6 and ω-3 fatty acids show substrate competition not only for Δ^6-desaturase, but also for Cox enzymatic activity. EPA is the precursor for the prostaglandins of the 3-series. This ω-3 fatty acid is converted by the Cox enzymes to PGH_3, through PGG_3, and finally to PGE_3. These metabolites are biologically less active than the corresponding AA metabolites (Rose D P and Connolly J M, 1999). Yang et al. (Yang P et al., 2004) showed that EPA is a better substrate for Cox-2 than for Cox-1. When they simultaneously exposed A549 cells to AA and EPA, PGE_2 production was reduced, when PGE_3 production was similar in cells treated either with AA alone or with both fatty acids. The same investigators showed that PGE_3 inhibits growth of A549 cells (lung cancer epithelial cells) but not of normal human bronchial epithelial cells. Many other studies have reported the growth inhibitory effects of PGE_3 in cancer cells of lung and prostate (Yang P et al., 2004). In normal murine epithelial cells and 3T3 fibroblasts PGE_3 has been shown to stimulate growth but with much less potency than PGE_2 (Bagga D et al., 2003). These results suggest that the mechanism for the anticancer activity of ω-3 fatty acids could be the inhibition of AA-derived eicosanoids.

The second important pathway of fatty acid metabolism is the lipoxygenase pathway. This pathway has also been well characterised by Krause and DuBois (Krause W and DuBois R N, 2000). There are three major lipoxygenases: 5, 12 and 15, named according to the specific carbon of AA to which molecular oxygen is added. Lipoxygenases metabolise PUFAs to a wide spectrum of biologically active products. The 15-Lox-1 pathway has been implicated in several forms of epithelial cell cancers, but the results on the role of 15-Lox-1 in colorectal carcinogenesis are controversial. Some studies have shown that the enzyme is overexpressed in colon and prostate tumours (Ikawa H et al., 1999, Kelavkar U P et al., 2001). On the other hand, it has also been shown that sodium butyrate, which induces differentiation in Caco-2 cells, and NSAIDs upregulate the expression of 15-Lox-1 (Kamitani H et al., 1998, Shureiqi I et al., 2000). Moreover, Shureiqi I et al. have shown that the 15-Lox-1 product 13-S-hydroxyoctadecadienoic acid downregulates PPARδ to induce apoptosis in colorectal cancer cells (Shureiqi I et al., 2003).

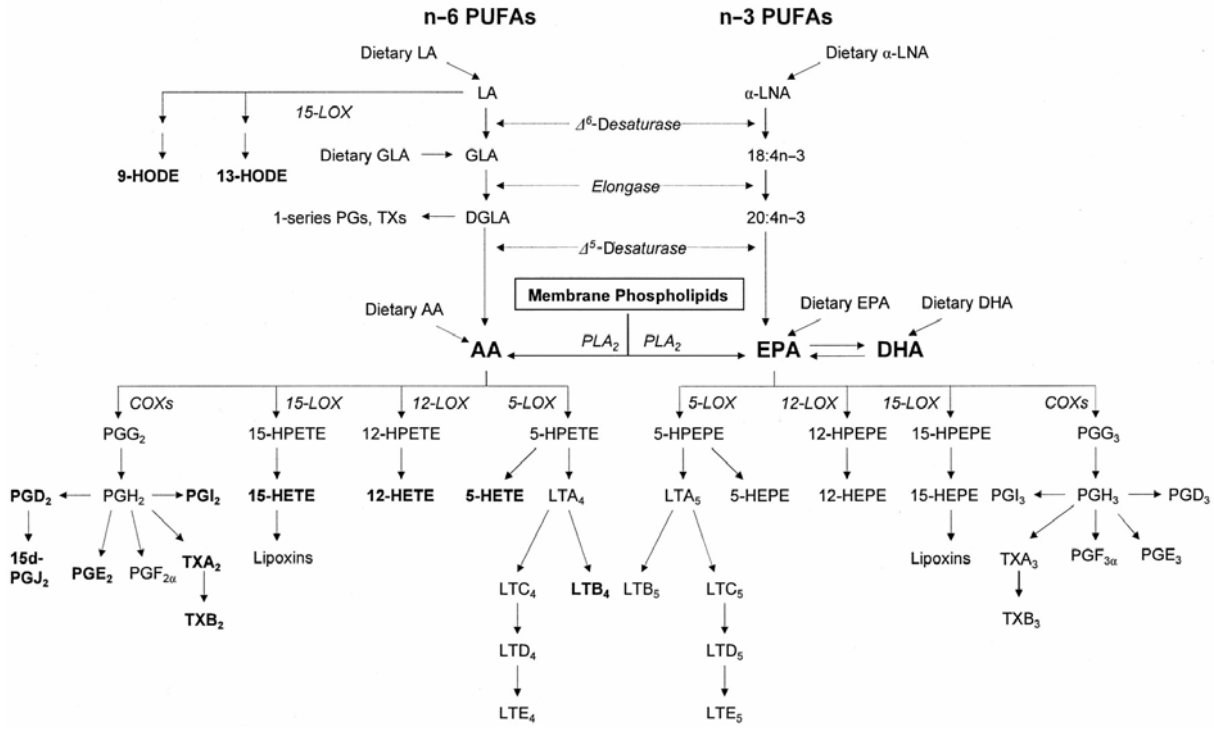


Figure 1.4: Overview of the metabolism of n–6 and n–3 polyunsaturated fatty acids (PUFAs) into eicosanoids involved in inflammation and carcinogenesis. The names of these eicosanoids are shown in bold. LA, linoleic acid (18:2n–6); α-LNA, α-linolenic acid (18:3n–3); GLA, γ-linolenic acid (18:3n–6); DGLA, dihomo-γ-linolenic acid (20:3n–6); AA, arachidonic acid (20:4n–6); EPA, eicosapentaenoic acid (20:5n–3); DHA, docosahexaenoic acid (22:6n–3); PLA_2, phospholipase A_2; LOX, lipoxygenase; COXs, cyclooxygenases (COX-1 and COX-2); 15-HETE, 15(*S*)-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPEPE, hydroperoxyeicosapentaenoic acid; LT, leukotriene; HODE, hydroxyoctadecadienoic acid; PG, prostaglandin; TX, thromboxane (adapted by Larsson et al., 2004).

1.4. Genes involved in colon carcinogenesis: PPARγ, mPGES and Nag-1

Several genes have been found to be involved in colon carcinogenesis, either as tumour supressors or as oncogenes. Among them PPARγ and mPGES have been shown to play important roles in the process of colorectal carcinogenesis.

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear hormone receptor superfamily member. It functions as a transcription factor after it heterodimerizes with the Retinoid-X receptor (RXR) and binds to specific response elements, called peroxisome proliferating response elements (PPREs). In order to act as a transcription factor, PPARγ has to be activated by specific ligands. Different ligands of PPARγ appear to be able to recruit different co-activators, which might provide specificity of biological activity of the

receptor. A natural occurring PPARγ ligand with high affinity for the receptor is a metabolite of arachidonic acid, 15-doexy-$\Delta^{12,14}$-prostaglandin J_2 (Koeffler P H, 2003). PUFAs are natural ligands for PPARγ, such as LA, α-LNA, AA and EPA. Synthetic ligands include substances used for the treatment of type II diabetes melitis, such as troglitazone, rosiglitazone and pioglitazone. EPA has been show to be not only a PPARγ agonist, but also to induce the expression of the receptor in adipocytes (Larsson S C et al., 2004). Adipocyte tissue has the highest levels of PPARγ but the receptor is also expressed in many other tissues, including monocytes and macrophages, liver, skeletal muscle, breast, prostate, and colon. Most of the known target genes of PPARγ belong to the pathways of lipid metabolism and transport, but the target genes that mediate the anticancer activity of the receptor are unclear (Koeffler P H, 2003).

PPARγ regulates differentiation and/or cell growth in a large and increasing number of cell types. It is expressed in human colon carcinomas and ligand activation of the receptor causes many colon cancer cell lines to undergo differentiation and reverse their malignant phenotype (Sarraf P et al., 1998). ω-3 polyunsaturated fatty acids, EPA, as well as NSAIDs have been shown to activate PPARγ and their consumption is associated with prevention of colon cancer (Koeffler P H, 2003, Lehmann J M et al., 1997). Moreover, colon cancer in humans is associated with loss-of-function mutations in PPARγ (Sarraf P et al., 1999).

Microsomal prostaglandin E synthase 2 has also been shown to be involved in colon carcinogenesis. Human prostaglandin E synthase is a member of a recently recognised protein superfamily, consisting of membrane associated proteins involved in eicosanoid and glutathione metabolism. mPGES is an inducible terminal enzyme in the biosynthetic pathway for prostaglandin E_2 from arachidonic acid (Brock T G et al., 1999). The activity of the enzyme is glutathione-dependent (Jakobsson P-J et al., 1999) and has been shown to be functionally linked with Cox-2 (Murakami M et al., 2000). Increased amounts of PGE_2 have been detected in colorectal adenomas and cancer, resulting in stimulation of angiogenesis and enhancement of motility of colon cancer cells (Sheng H et al., 2001). It has also been shown that the enzyme is overexpressed in colorectal adenomas and cancer (Yoshimatsu K et al., 2001).

Another gene found to be involved in colon carcinogenesis is the Nonsteroidal Anti-inflammatory drugs-activated gene-1 (Nag-1). NSAIDs have been proven affective in reducing human and rodent colorectal cancer and this effect has been linked with their ability of inhibiting Cox-2 activation, but also Cox-2-independent mechanisms of action have been reported (Smith M-L et al., 2000, Yoon J-B et al., 2003). In their attempt to

elucidate the mechanism of action of NSAIDs, Baek et al. recently reported the identification of a TGFβ superfamily member induced by NSAIDs in the human colon cancer cell line HCT116 (Baek S J et al., 2001a). They showed that Nag-1 expression is induced after treatment of the cells with sulindac and that this induction is independent of Cox-2, PGE2 or AA (Baek S J et al., 2002). The expression of the protein is increased in human colon cancer cell lines treated with NSAIDs, inducing apoptosis (Baek S J et al., 2001a), and also in *Min* mice after sulindac treatment (Kim K-S et al., 2002). Many transcription factors are involved in the regulation of Nag-1 expression, like Sp1 and Sp3 (Baek S J et al., 2001b).

The same group has shown that dietary phenolic compounts like resveratrol and genistein, and dialyl disulfide, a component of garlic, induce Nag-1 expression in HCT116 cells (Baek S J et al., 2002, Bottone F G et al., 2002, Wilson L C et al., 2003). However, there is no reference so far about the effects of dietary fatty acids on Nag-1 expression.

1.5. Polyunsaturated fatty acids and Lipid Peroxidation

Fatty acids in biological membranes are mostly unsaturated. They are therefore highly sensitive to oxidation by singlet oxygen and hydroxyl radicals. The oxidation consists of a chain of reactions which is known as *lipid peroxidation*. The process through which lipid peroxidation proceeds, involves the following steps:

- abstraction of a hydrogen from a PUFA chain by singlet oxygen and hydroxyl radicals (radical formation, *initiation*)
- reaction of the resulting fatty acid radical with molecular oxygen (*peroxidation*)
- a detoxification process which may follow, in which the reaction chain is stopped (*termination*).

Figure 1.5 summarises these steps of lipid peroxidation. During this process many products are formed, like hydroperoxides, alkanes, semi-aldehydes, or endoperoxides. Endoperoxides disintegrate to yield *malondialdehyde* and other products. Malondialdehyde (MDA) is biologically very active. It has been found to be mutagenic in bacterial and mammalian cells and carcinogenic in rats. It can react with DNA to form DNA-adducts, mainly pyrimidopurinone-deoxyguanosine adducts (Sharma R A et al., 2001).

Lipid peroxidation causes serious membrane damage and may therefore lead to cell death. Membrane components with antioxidant effect may block the injurious chain reaction.

A well known example is *α-tocopherol*. This compound reacts with a lipid radical to yield the α-tocopherol radical and a harmless fatty acid. The α-tocopherol radical, which is less reactive, can then be reduced to α-tocopherol by glutathione (GSH), and thus be transformed to the original vitamin (Toxicology, Principles and Applications, CRC).

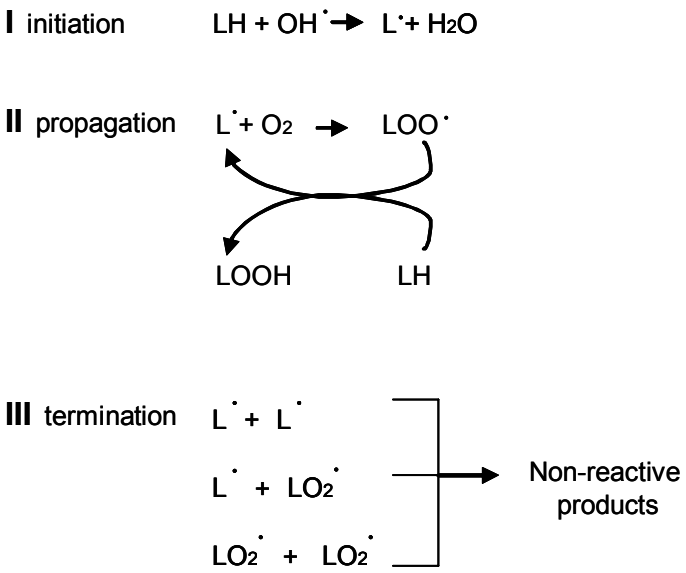


Figure 1.5: Stages in lipid peroxidation. LH: polyunsaturated fatty acid, L•: lipid radical (Adapted from: Toxicology, Principles and Applications, Niesink R, de Vries J, Hollinger M, 1996, CRC).

Various methods are applied for the determination of lipid peroxidation. A generally accepted method is the *thiobarbituric acid test* (Buege J. A. and Aust S. D, 1978). With this test, lipid peroxidation products, such as MDA, are condensed with 2-thiobarbituric acid. The resulting condensation products have an absorption maximum at 535nm. The method is not specific for MDA; however it is widely used for the determination of lipid peroxidation in biological samples.

Ito et al. measured serum concentrations of fatty acids and lipid peroxides between Japanese people in Japan and Japanese and Caucasians in USA. They found that thiobarbituric acid reactive species (TBARS) were highest among Japanese people in Japan, followed by Japanese people in USA and Caucasians in USA (Ito Y et al., 1999). These findings suggest that serum levels of TBARS are higher among people who consume higher amounts of fish.

1.6. Proliferation and cell death of colon cells

The concept of rapid renewal of cells in the gastrointestinal tract has now become well established. In the colon of man the area of proliferating activity occupies about ¾ of the crypt column. As cells reach the upper portion of the crypt fewer remain in the proliferative state and they start to differentiate. During their migration towards the crypt, the cells differentiate into absorptive enterocytes, mucous secreting goblet cells or enteroendocrine cells. These populations remain in steady state, whereas increase in number of cells migrating from the crypt to the luminal surface balances cell loss. Cells die by shedding at the surface or by undergoing apoptosis *in situ*, followed by shedding or phagocytosis (Lipkin M, 1973).

The organisation of the colonic epithelium is directed towards maintaining a continuous layer of cells with functional maturity at the surface, with a constant supply of epithelial cells of sufficient maturity from the crypts (Gibson P R, 2004). Control of cell proliferation is important, since cell proliferation plays essential roles in carcinogenesis, including the process of initiation and promotion. Tissue homeostasis of the total cell number is maintained by a balanced rate of proliferation and elimination. While the latter can be achieved directly through an apoptotic process, in most tissues cells go through terminal differentiation as a transition stage between proliferation and cell death. Fully differentiated cells in a constantly renewing tissue usually loose their proliferative capability and have a defined, relatively short life span. Colonic cells are exposed to a variety of dietary and environmental factors that are potentially carcinogenic. Disturbance of the balance between proliferation, differentiation and cell death by dietary factors for example, can lead to colorectal carcinogenesis (Cai J et al., 2004).

Many methods are available for the determination of cell proliferation, such as for example 5-bromo-2´-deoxyuridine incorporation, ^{3}H-thymidine labelling, detection of expression of proliferating cell nuclear antigen (PCNA) or Ki67 (Roche Biochemicals).

Cell death on the other hand can occur through two different mechanisms: apoptosis or necrosis. Apoptosis or programmed cell death is the physiological process by which damaged or useless cells are eliminated during colonic crypt cell homeostasis. Necrosis (or “accidental” cell death) in contrast is a pathological process that occurs after exposure of cells to serious physical or chemical insult. Certain chemical or dietary compounds are considered to be cytotoxic to the cells. In contrast to apoptosis and necrosis, cytotoxicity does not indicate a specific cell death mechanism.

In order to determine cytotoxicity, most assays are based on the alterations of membrane permeability of damaged cells and subsequent leakage of cytoplasmic components into the supernatant. An example of such assay, very widely used in order to determine cytotoxicity, is the LDH assay (Roche Biochemicals). Lactate dehydrogenase is a cytoplasmic enzyme present in all cells, which is rapidly released out of the cell when there is membrane damage. The assay is based on the measurement of LDH in the culture medium after exposure of the cells to a possible cytotoxic compound.
Apoptosis can also be determined by several methods (Roche Biochemicals). A widely used method is the determination of caspase-3 activity. Caspases are crucial mediators of programmed cell death. Among them, caspase-3 is a frequently activated death protease, catalysing the specific cleavage of many key cellular proteins. Activation of caspase-3 is either dependent or independent on cytochrome c release and caspase-9 function. Recent work has revealed that caspase-3 is important for cell death in a remarkable tissue, cell type, or death stimulus-specific manner and is essential for some of the characteristic changes in cell morphology and certain biochemical events associated with apoptosis (reviewed in Porter A G and Jänicke R U, 1999).

1.7. Possible mechanisms for the (anti)-carcinogenic effects of ω-6 and ω-3 fatty acids: role of Cyclooxygenase-2 and lipid peroxidation

Mounting evidence shows that Cox-2 plays an important role in the process of carcinogenesis and that the prostaglandin products of the Cox-2 pathway (mainly PGE_2) enhance cell proliferation and growth in both normal and tumour cells. Another important action of Cox-2 on cellular processes is the inhibition of apoptosis, which in many cases constitutes a mechanism to promote tumour cell growth (Cao Y and Prescott S M, 2002).
Various mechanisms of Cox-2 related tumour promotion have been identified. Some of them depend on PGE_2 production, while others do not. Effects of Cox-2 expression that may contribute to colorectal tumour development independently of PGE_2 production are the activation of carcinogens (polycyclic hydrocarbons for example), production of MDA, which is a direct mutagen, or reduction of free AA levels. The level of free AA, which is the main Cox-2 substrate and has pro-apoptotic action, is reduced by Cox-2 activation (Wendum D et al., 2004).

On the other hand, PGE_2 levels are elevated in colorectal cancer. PGE_2 can promote tumour development by inducing cell proliferation and inhibiting apoptosis. In addition, PGE_2 increases the motility and metastatic potential of cancer cells (Wendum D et al., 2004). Four receptors for PGE_2 have been identified in the gastrointestinal tract, named EP1, EP2, EP3 and EP4. These receptors are associated with different signal transduction pathways. While the classical PG receptors are cell surface, PPARs may also function as receptors, at least for some of the eicosanoids (Fischer S M, 1997).

One of the most important functions of ω-6 and ω-3 fatty acids is related to their enzymatic conversion to eicosanoids, with AA-derived eicosanoids considered as positively linked to carcinogenesis and EPA-derived eicosanoids considered as anticarcinogenic. Several molecular mechanisms have been proposed, whereby ω-3 fatty acids potentially affect carcinogenesis. One of these mechanisms is the inhibition of AA-derived eicosanoids from ω-3 fatty acids. This effect is achieved at several levels. First, high intake of ω-3 fatty acids results in their incorporation into membrane phospholipids, where they potentially replace AA. By decreasing the availability of AA, the synthesis of AA-derived eicosanoids is also suppressed, in favour to EPA-derived eicosanoids. Second, ω-3 fatty acids compete with ω-6 fatty acids for desaturases and elongases, with ω-3 fatty acids having greater affinities for the enzymes. That means that higher intake of ω-3 fatty acids reduces the conversion of LA to AA and thus the production of AA-derived eicosanoids. Third, ω-3 fatty acids suppress Cox-2 and compete with ω-6 for Cox-2. Compared with AA, EPA is also the preferential substrate for lipoxygenase, and finally ω-3 fatty acids enhance eicosanoid catabolism, probably through induction of peroxisomal enzymes (Larsson S C et al., 2004).

Another mechanism that has been proposed for the anticarcinogenic effects of ω-3 fatty acids is through their effect on signal transduction pathways. The first transcription factor that was identified as being regulated by fatty acids is PPARα, a member of the Peroxisome Proliferator-Activated Receptor superfamily, which includes also PPARβ and PPARγ. The preferred ligands for PPARγ are PUFAs, including LA, α-LNA, AA, and EPA. In addition to be a PPARγ ligand, EPA has been shown to increase PPARγ expression in adipocytes. Agonists of PPARγ have been found to have antiproliferative effects both *in vivo* and *in vitro* (Larsson S C et al., 2004).

However, the exact role of Cox-2 and PUFAs in colon carcinogenesis has not been fully clarified. Moreover, studies comparing the effect of different fatty acids on normal colon

cells and different colon cancer cell lines, with different levels of Cox-2 expression, have not been reported.

Besides to their metabolism to eicosanoids, PUFAs are also subjected to lipid peroxidation reactions (Grammatikos S I et al., 1994, Tang D G et al., 2002). Susceptibility to oxidative damage increases with the degree of unsaturation, which means that not only the position but also the number of the double bonds might be important for the effects of the different fatty acids. Increased lipid peroxidation may lead to oxidative stress. Oxidative stress is the situation in which there is continuous interaction with oxidizing agents inside the cell, and consistently more reducing equivalents are used than in the case of normal functioning of the cell (Toxicology, Principles and Applications, CRC).

Many studies have recently shown that ω-3 PUFAs can suppress cancer cell growth due to increased lipid peroxidation. Results from experiments with human and animal cell lines have shown that ω-3 fatty acids inhibited the growth of lung, prostate and breast cancer cells and the mechanism for this inhibition of growth was through induction of apoptosis, due to increased lipid peroxidation. The same researchers showed that there was no effect in normal human epithelial cells or fibroblasts (Begin M E et al., 1985, Begin M E et al., 1986, Gonzalez M J et al., 1993). Nano et al. showed that incubation of Caco-2 cells with PUFAs strongly increased tumour lipid peroxidation, when saturated and monounsaturated fatty acids had the same effect only at very high concentrations. Preincubation with vitamin E reversed the effect of PUFAs (Nano J L et al., 2003). In another study, where HT-29 colon cancer cells were used, Chen et al. showed that DHA induced apoptosis in a dose-dependent manner, and again this effect was reversed in the presence of antioxidants (Chen Z Y and Istfan N W, 2000). The same results have also been found in animal experiments, when mice were fed with high fish or high corn oil diets. The animals that were in high fish oil diet developed less and smaller tumours than the animals in the corn oil diet. The reason for this was the high level of lipid peroxidation products in the tumour cells of the animals fed fish oil (Gonzalez M J et al., 1991).

All these results suggest that the anticarcinogenic effects of ω-3 fatty acids could be the result of increased lipid peroxidation, and subsequent induction of apoptosis or cell cycle arrest. Ji et al. showed that MDA can cause cell cycle arrest in human cell lines and this effect is dependent on p53 and p21 induction (Ji C et al., 1998).

1.8. Aim, approach and outline of the thesis

Many studies, as have been discussed in this introduction, have shown that both Cox-2 activity and PGE2 production, as well as lipid peroxidation induced oxidative stress play an important role on the carcinogenic or protective effects of ω-6 and ω-3 PUFAs respectively, in colon carcinogenesis. The exact mechanisms by which PUFAs exert their effects are however unclear.

Therefore, the aim of this study is to elucidate the mechanisms of action of PUFAs on colon carcinogenesis, with emphasis on the role of Cox-2 and on the effects of lipid peroxidation on the growth of human colon cancer cells. Moreover, the effect of PUFAs on the expression of genes known to be involved in colorectal carcinogenesis, like PPARγ and Nag-1, was also examined.

Most of the experimental studies published so far focus on the role of PUFAs on colon carcinogenesis during the promotion phase, when little attention is given to the initiation phase or the preventive role of ω-3 fatty acids. Therefore, in this study we used colon cancer cell lines with different levels of Cox-2 expression, which also represent different stages of the colon carcinogenesis process: HCT116 and HCA-7 cells, which represent the carcinoma stage and have no or high expression of Cox-2 respectively; and Caco-2 cells, which represent the stage of adenoma, with low Cox-2 expression. Moreover, a normal human foetal epithelial colon cell line was used, in order to study the cancer preventive or promoting effects of PUFAs.

Different endpoints linked to carcinogenesis, such as proliferation, apoptosis and cytotoxicity, have been checked and the expression of several genes has been analysed.

Our results have shown that ω-6 and ω-3 fatty acids have different effects on the growth of normal and cancer human colon cell lines.

2. Materials and Methods:

2.1. Reagents

Arachidonic (AA), Linoleic (LA), Docosahexaenoic (DHA) and Eicosapentaenoic (EPA) fatty acids, Vitamin E (α-Tocopherol), Actinomycin D, DMSO, Sodium butyrate, Staurosporine and Ethanol were purchased from Sigma-Aldrich. LipofectAMIN Plus reagent was purchased from Invitrogen. Antibodies for Cox-1, mPGES, 15-Lox-1, PPARα, PPARγ, and the specific Cox-2 inhibitor CAY10404 were purchased from Cayman Chemical. The antibody for Cox-2 was purchased from Oxford Biomedical Research and the antibody for β-actin was purchased from Sigma. The secondary antibodies, anti-mouse-HRP and anti-rabbit-HRP, and the ECL Plus reagents for the Western Blotting were purchased from Amersham Biosciences. The anti-Nag-1 antibody was purchased from Upstate Biotechnology and the BCAssay for the protein determination from Uptima Interchim. All fatty acids were diluted in absolute ethanol, at a concentration of 40mM and were stored under argon, at -20^{o}C. The final concentration of ethanol in the treated cells was 0.025%. EMEM and DMEM were purchased from Sigma, DMEM/Ham´s F12 from ATCC, PBS, FBS, trypsin and the antibiotics were purchased from PAA (PAA Laboratories GmbH, Austria), HEPES was purchased from Biochrom (Biochrom, Germany), and all other reagents were purchased from Sigma.

2.2. Cells

HCT 116 (human colorectal carcinoma epithelial cells) were a generous gift from Dr. T. E. Eling (National Institute of Environmental Health Sciences, NIH, USA). Caco2 (human colon adenocarcinoma epithelial cells), HCA-7 colony 29 (human colon carcinoma epithelial cells) and FHC (normal human foetal colon cells) were all purchased from American Type Culture Collection (ATCC-LGC Promochem).

HCT116 were grown in McCoy´s medium supplemented with 10% heat-inactivated FBS and antibiotics (penicillin/streptomycin). Caco-2 cells were grown in Eagle´s minimum essential medium (EMEM) supplemented with 10% heat-inactivated FBS and antibiotics. HCA-7 cells were grown in Dubelcos modified Eagle´s medium (DMEM) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 110mg/L sodium pyruvate and 2%

antibiotics. FHC cells were grown in DMEM 45%/Ham´s F12 45% medium, supplemented with 20% FBS, 25mM HEPES, 10ng/ml cholera toxin, 0.005mg/ml insulin, 0.005mg/ml transferin, 100ng/ml hydrocortisone and antibiotics. All cells were cultured at 5% CO_2 atmosphere at 37^{o}C in T75 flasks (Greiner) and subcultured at a 1:4 ratio, with 0.5g/L Trypsin-0.2g/L EDTA. For the experiments all cancer cells were used between passages 20-35. FHC cells were used until passage 5.

2.3. Treatment with the fatty acids

For the experiments, cells were trypsinised and seeded either in flat-bottomed 6-well or 96-well plates (Greiner). The cells were seeded at a density of $3x10^5$ cells/well in the 6-well plate for all cell lines and $3x10^4$ cells per well in the 96-well plate for HCT116, $2x10^4$ cells per well for Caco-2 and HCA-7, and $4x10^4$ cells per well for FHC cells. 24h after seeding, the medium was removed, the cells were washed with PBS and new medium was added, with serum substitute (diluted from 1000x concentrate, Biochrom) and non-essential amino-acids (diluted from 100x concentrate, Biochrom). In this medium the fatty acids were added at a concentration of 10μM. The cells were treated for 5 days and the medium was refreshed every 48 hours.

2.4. Proliferation assay

Proliferation was examined with the 5-Bromo-2´-deoxy-uridine Labelling and Detection Kit III (Roche Molecular Biochemicals), which is based on the cell ELISA principle. The cells were treated as described. The last 6 hours of the treatment, the cells were incubated with 5-Bromo-2´-deoxy-uridine (BrdU) in a concentration of 10μM. Afterwards, the cells were washed with 10% FBS in PBS and fixed with 70% Ethanol-0,5M HCl for 30min at -20^{o}C. After fixation cells were washed again and incubated with a nucleases solution for 30min at 37^{o}C. After another washing step, cells were incubated with a peroxidase labelled anti-BrdU antibody, diluted in 10mg/ml BSA in PBS at a final concentration of 0.2U/ml. In the final step, the cells were washed with PBS and were incubated with the peroxidase substrate. The peroxidase enzyme catalysed the cleavage of the substrate, yielding a coloured reaction product. The absorbance of the sample was determined using a

microtiter plate reader (Dynatech MR5000). Absorbance was measured at 405nm with a reference wave length at 490nm, and was directly correlated to the level of BrdU incorporated into cellular DNA.

2.5. Cytotoxicity assay

Cytotoxicity was checked with the Cytotox-ONE Homogeneous Membrane Integrity Assay (Promega). The assay is based on the measurement of the Lactate dehydrogenase (LDH) released in the medium from cells with a damaged membrane. After treatment, cells were removed from the incubator and a buffer containing lactate, NAD^+ and resazurin as substrates, in the presence of diaphorase, was added to each sample. The diaphorase enzyme catalyses the conversion of resazurin to resorufin, which is a fluorescent product. The amount of the product is proportional to the amount of LDH. The samples were incubated at 22^{o}C for 10min and the fluorescence was measured with an Ascent Fluoroskan fluorometer (Labsciences) at an excitation wavelength of 544nm and an emission wavelength of 590nm.

2.6. Apoptosis assays

For the apoptosis experiments, a colorimetric assay for Caspase-3 activation was used (BD Biosciences). In particular, after treatment the cells were collected in 200μl of lysis buffer (10mM Tris/HCl, 10mM $NaH_2PO_4/NaHPO_4$, pH7.5, 130mM NaCl, 1% Triton-X-100, 10mM NaPPi), centrifuged at 12,000rpm for 10min at 4^{o}C and kept frozen at -20^{o}C. The floating cells in the medium were also collected and combined with the rest cells of the same sample. For the assay, 100μl of each sample were incubated in 20mM HEPES with 5μg of Ac-DEVD-pNA, at 37^{o}C for 1 hour. Ac-DEVD-pNA was cleaved by active caspase-3 and the released free p-nitro-aniline was measured at 405nm with a microplate reader. The results were corrected for the same protein amount in each sample.

For the detection of apoptosis in the FHC cells, the ApoONE Homogeneous Caspase-3/7 Assay Kit from Promega was used. The ApoONE Homogeneous Caspase-3/7 Buffer combined with the profluorescent caspase-3/7 substrate rhodamine 110, bis-(N-CBZ-L-valyl-L-aspartic acid amide) (Z-DEVD-R110), was added to the treated cells and incubated for 4 hours at room temperature. This buffer allows rapid and sufficient lysis of the cells

and supports optimal caspase-3/7 enzymatic activity. After 4 hours, the cleaved rhodamine 110 emits fluorescent at 521nm. Fluorescence was measured with at an excitation wavelength of 465nm and an emission wavelength of 538nm.

2.7. Cell viability assay

In order to check the viability/cell death in FHC cells, the CellTiter-Blue Cell Viability Assay from Promega was used. This assay was used in combination with the ApoONE Homogeneous Caspase-3/7 Assay. In particular, after treatment of the cells, 20μL of the CellTiter-Blue reagent was added to the cells and incubated for 2 hours at 37^{o}C. The reagent contains resazurin as an indicator of the metabolic capacity of the cells. Metabolically active cells can convert resazurin to resorufin, which emits fluorescence at 590nm. The cells were incubated with the reagent for 2 hours and the fluorescence was measured at an excitation wavelength of 544nm and an emission wavelength of 590nm.

2.8. Western Blotting

For the detection of the different proteins, cell lysates were collected in RIPA buffer: 1% NP-40, 0.1% SDS, 1mM Sodium Vanadate and protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem). The samples were centrifuged at 12,000rpm for 10min at 4^{o}C and the protein concentration of each sample was determined with the BCAssay. 20μg of protein from each sample were loaded on the SDS-Polyacrylamide gel. The proteins were transferred to a Hybond-P PVDF membrane 0.45μm (Amersham Biosciences) and blotted with a semi-dry gel transfer apparatus (Semi-Dry Transfer Cell, Biorad). For the transfer two different buffers were used: the anod buffer (50% of a stock buffer containing 77.26mM Tris, 60mM formic acid, 2mM SDS, and 1.5mM sodium azide, and 20% methanol) and the kathod buffer (50% of a stock buffer containing 34.7mM Tris, 48.1mM taurin, 2mM SDS, and 1.5mM sodium azide, and 20% methanol). The proteins were transfered for 1.5 hours, at 15Volts and 400mA. The membrane was blocked in 5% milk in Western Blotting buffer (0.01M Tris pH 7.2, 0.001% Tween-20, 150mM NaCl) for 1 hour at RT and the membrane was incubated with the primary antibody solution in 1% milk in Western Blotting buffer over night at 4^{o}C. The antibodies for Cox-1, Cox-2, mPGES,

PPARγ, 15-Lox-1 and Nag-1 were used in a 1:1000 dilution. The antibody for PPARα was used diluted 1:125 and the antibody for β-actin was used at a 1:5,000 dilution. All the secondary antibodies were used diluted 1:5,000 in 1% milk in Western Blotting buffer. The membranes were incubated with the secondary antibodies at RT for 1 hour. Between each incubation step the membranes were washed with Western Blotting buffer. Following the last washing step, the membranes were incubated with the ECL reagent and the chemiluminescence was detected with the FluorSMax Multi Imager (Biorad). The intensity of the bands was quantified with the use of Quantity One 4.1.0 software (Biorad).

2.9. Reverse Transcription-Polymerase Chain Reaction

RT-PCR was performed according to standardised protocols of our laboratory. In detail, RNA was isolated with the RNEasy kit for RNA isolation from Qiagen, according to the instructions of the manufacturer. For the reverse transcription 1μg RNA was used. The RNA was incubated with p(DT)15 primer (Roche) and 1mM of dNTP Mix (Invitrogen) for 5min at 65^{o}C, and then the reverse transcription buffer (Invitrogen), the RNAse inhibitor (eppendorf), DTT (0.1M stock, Invitrogen) and reverse transcriptase (M-MLV Reverse Transcriptase, Invitrogen) were added. The mix was incubated at 37^{o}C for 52min. The reaction was stopped by an inactivation step, at 70^{o}C for 15min. For the PCR, 2.5μl of cDNA were mixed with PCR Reaction buffer (Qiagen), dNTP mix at a final concentration of 1.25mM (Invitrogen), Taq Polymerase at a final concentration of 5U/μL (Qiagen) and the primers, at a final concentration of 0.5μM. The following primers were used: for Cox-2 5′-TGACAGTCCACCAACTTACA-3′ (for) and 5′-TGCTTTTTACCTTTGACACC-3′ (rev) (MWG-Biotech AG), for Cox-1 5′-AGTGGCAGACAAACAGACCA-3′ (for) and 5′-CAATAGGGGAGGACAGAGCA-3′ (rev) (MWG-Biotech AG), for mPGES 5′-AGTGAGGCTGCGGAAGAAG-3′ (for) and 5′-GGAAAAGGAAGGGGTAGATGG-3′ (rev) (MWG-Biotech AG), for Nag-1 5′-CGGGACCCTCAGTTGC-3′ (for) and 5′-ATCTGCAACCACTGGATCTGT-3′ (rev) (TIB MolBiol, Berlin), for PPARγ 5′-CCGTGGCCGCAGATT-3′ (for) and 5′-ATCTGCAACCACTGGATCTGT-3′ (rev) (TIB MolBiol, Berlin), and for β-actin 5′-GTGGGGGGCCCCAGGCACCA-3′ (for) and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (rev) (MWG-Biotech AG). The PCR was performed at the following conditions: 4min at 94^{o}C for initiation, 1min at 94^{o}C for

denaturation, or 30sec for mPGES, 1min for annealing at 52^{O}C for Cox-2, mPGES and Nag-1, 1min annealing at 60^{O}C for actin, 1min at 55^{O}C for PPARγ and 1min at 56^{O}C for Cox-1, 1min extension at 72^{O}C for 30 cycles in all cases except actin which was performed in 23 cycles, and a final step of 7min at 72^{O}C. The products were analysed in 1.2% agarose gels.

2.10. Transient transfection

In order to transfect cells to transiently express the proteins of interest, the LipofectAMIN Plus transfection reagent from Invitrogen was used. LipofectAMIN reagent is a liposome formulation of a polycationic and a neutral lipid. Plus reagent has the ability of pre-complexing DNA and thus enhancing cationic lipid-mediated transfection of DNA into the cultured cells. Briefly, cells were seeded the day before transfection in an approximate density of 80% in 12-well plates. Cell numbers were depended on the cell type used. HCT116 cells were seeded at a density of cells/well and HCA-7 at a density of cells/well. After 24 hours cells were transfected according to the instructions of the manufacturer. The transfection vectors for Cox-1, Cox-2, PPARγ and Nag-1 were a generous offer from Dr. T. E. Eling, (National Institute of Environmental Health Sciences, NIH, USA).

2.11. Thiobarbituric acid assay

For the determination of lipid peroxidation, the thiobarbituric acid assay was employed. The assay is based on the detection of Malondialdehyde (MDA), a product formed from the breakdown of polyunsaturated fatty acids. MDA serves as a convenient index for determining the extent of the peroxidation reaction, since it has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535nm. The method was adapted from Buege J. A. and Aust S. D., (Microsomal lipid peroxidation, Methods in Enzymology, 1978; 52; 302-10). After treatment, cells were collected in cold PBS and lysed by three freeze-thaw cycles in liquid nitrogen. Following lysis, the samples were centrifuged for 10min at 12,000rpm at 4^{O}C to get rid of cell debris. 0.5 ml of the supernatant was combined with 1.5ml of TCA-TBA-HCl assay buffer (15%w/v trichloroacetic acid, 0.375% thiobarbituric acid, 0.25N

hydrochloric acid) and heated for 15min in a boiling water bath. After cooling the precipitate was removed by centrifugation at 3000rpm for 10min. The absorbance of the sample was determined at 535nm, against a blank that contained only the assay buffer (no lipid). The MDA concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$: [MDA]= OD x D /ε, were OD is the absorbance at 535nm, D is the dilution of the sample and ε the extinction coefficient. The results were corrected for the same amount of protein in all samples.

3. Results

3.1. Determination of the expression pattern of genes involved in colon carcinogenesis in all the cell lines

In order to study the effect of the treatment of colon cell lines with different PUFAs, we first the expression pattern of the genes of interest on protein level was determined in all our colon cell lines.

For our experiments we used four different cell lines: HCT116 cells, a colorectal carcinoma cell line with no Cox-2 expression, Caco-2 cells, a colon adenocarcinoma epithelial cell line with moderate levels of Cox-2 expression, HCA-7 cells, an epithelial colon carcinoma cell line with high level of Cox-2 expression and FHC cells, a normal foetal colon epithelial cell line. The expression pattern of Cox-2 in each cell line was examined with Western Blotting analysis. The results are shown in figure 3.1.

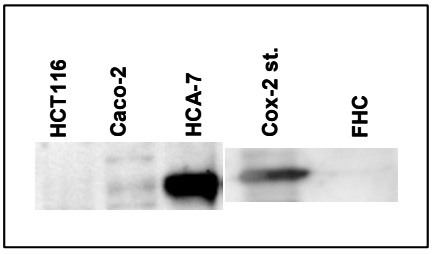


Figure 3.1: Expression pattern of Cox-2 in colon cell lines. 20μg of protein were used for each sample. Cox-2 native protein (50ng) was used as standard.

Mainly, interest was focused on some of the genes that are known to be involved in colon carcinogenesis. These genes were the two Cox enzymes, the microsomal prostaglandin E2 synthase (mPGES), PPARγ, 15-Lox-1 and Nag-1.

Cell lysates for Western Blotting analysis were collected from all the cell lines, without any treatment of the cells. The expression pattern of all of the genes mentioned above is shown in figure 3.2. Cox-1 is expressed in normal cells but it can not be detected in any of the cancer cell lines. mPGES is expressed in all the cancer cell lines but not in normal cells. PPARγ is expressed in all the cell lines and the same is true for 15-Lox-1, whereas Nag-1 is not expressed in any of the cell lines examined here (data not shown).

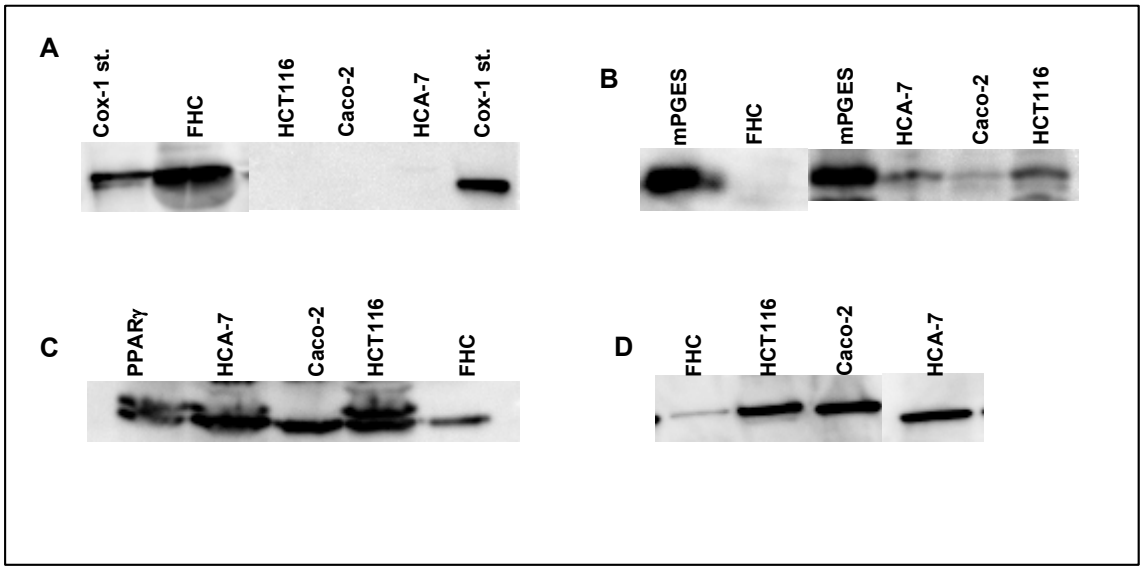


Figure 3.2: Expression pattern of all the genes of interest in all the cell lines examined in this study. **A**: expression pattern of Cox-1, **B**: expression of mPGES, **C**: expression of PPARγ **D**: expression pattern of 15-Lox-1. In the case of Cox-1, native protein was used as control. In the case of PPARγ lysate from cells transfected with the respective plasmid was used and in the case of mPGES, the control sample is lysate from A459 cells, induced with IL-1β to induce mPGES expression.

From these results it was shown that there are differences in the expression of these genes between normal and colon cancer cells. In FHC cells Cox-1 protein is expressed in high levels, whereas in the cancer cell lines Cox-1 expression is too low to be detected in protein level. This result is in agreement with already reported results, showing that Cox-1 is often down regulated in cancer cells (Ikawa H et al., 1999, Kamitani H et al., 1998). In contrast, all the other genes checked were expressed in the cancer cell lines, but not in the normal cells with the exception of PPARγ and 15-Lox-1. These genes have also been already reported to be expressed in normal cells (Lefebre A-M et al., 1999, Ikawa H et al., 1999). The expression pattern of all the genes checked is similar among the cancer cells, with the exception of Cox-2.

These results are summarised on table 1.

	Cox-1	**Cox-2**	**mPGES**	**PPARγ**	**Nag-1**	**15-Lox-1**
FHC	yes	no	no	yes	no	low
HCT116	not detect.	no	yes	yes	no	yes
Caco-2	not detect.	low	yes	yes	no	yes
HCA-7	not detect	high	yes	yes	no	yes

Table 1: Summary of the expression pattern of all genes of interest in all cell lines.

3.2. Effect of PUFAs on Cytotoxicity in colon cell lines

In order to establish our experimental conditions, first our cells were treated with different concentrations of fatty acids for different time periods and checked if there were any cytotoxic effects on the cells. It was found that concentrations of fatty acids higher than 20μM for time periods longer than 24 hours were cytotoxic for the cells. Since we believe that long-time exposure to low concentrations is the situation that resembles better the normal situation in humans concerning the dietary intake of fatty acids, we decided to perform our experiments under such conditions. Cells were treated with 10μM of fatty acids for 120 hours in serum free medium. The medium was refreshed every 48 hours. Cytotoxicity was checked by measuring LDH release from the cells in the medium at the end of the treatment. Our results showed that there was not any cytotoxic effect on the cells under these conditions (figure 3.3), so we decided to establish this procedure as our experimental protocol for all our experiments.

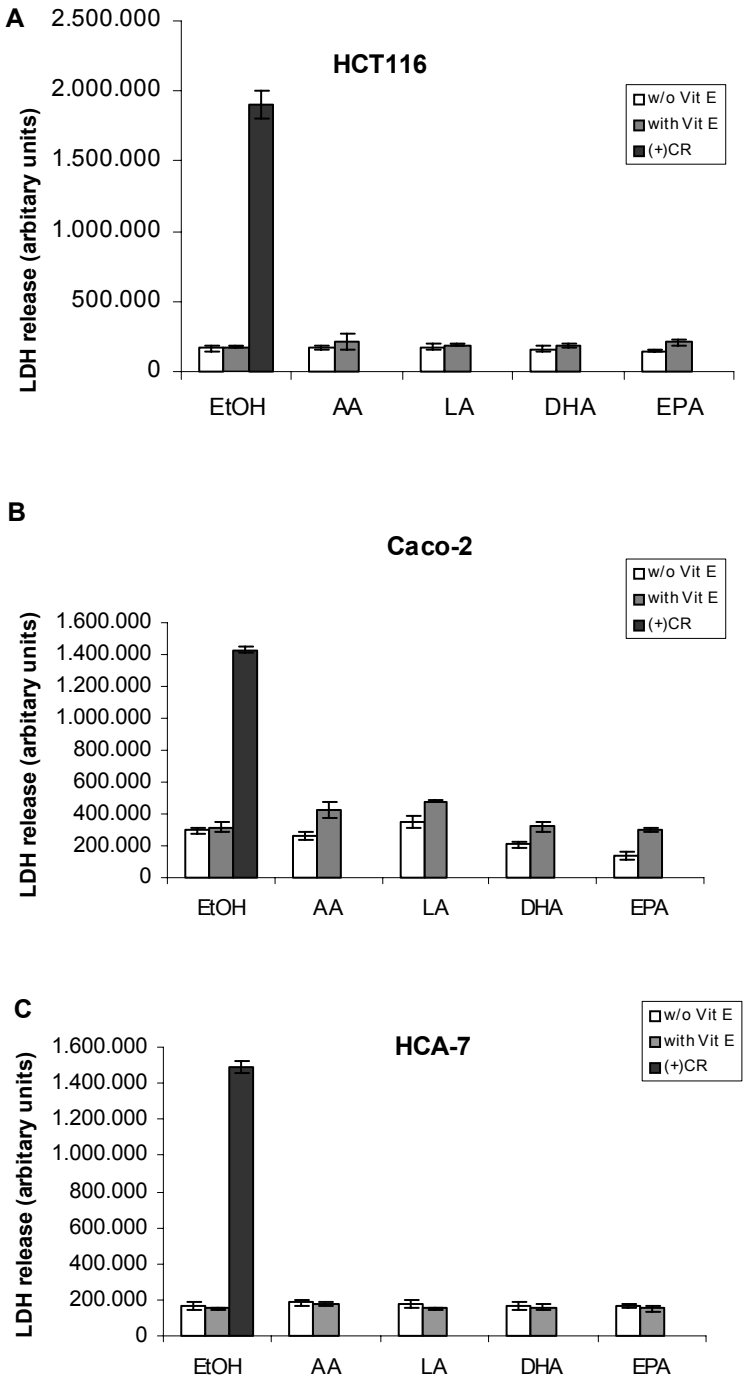


Figure 3.3: Determination of cytotoxicity of fatty acids on HCT116 (**A**), Caco-2 (**B**) and HCA-7 (**C**) cells. Cytotoxicity was determined with the Cytotox-ONE Homogeneous Membrane Integrity Assay, as described in "Materials and Methods". LDH release was measured fluorometrically and compared with control cells (vehicle treated). (+)CR: Lysed cells for determining the maximum LDH release.

3.3. Determination of Cytotoxicity and viability in normal colon (FHC) cells

In FHC cells we had to deal with the restriction of the very slow growth rate. Therefore, we decided to use a multiplex assay, which combines determination of viability and apoptosis in the same samples. In this way we could minimise the cell numbers needed for each experiment.

The CellTiter Blue assay was performed in order to check both cell viability and cytotoxicity, because the components of the medium were interfering with the LDH reaction of the Cytotox-ONE Homogeneous Membrane Integrity Assay.

The results are shown in figure 3.4. There was reduced viability of cells treated with AA but there was no effect in the cells treated with LA, DHA or EPA.

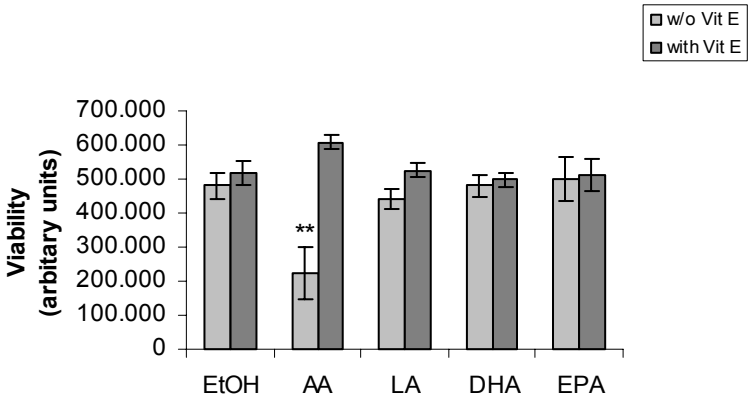


Figure 3.4: Determination of viability in FHC cells after treatment with the different fatty acids. Viability was determined with the CellTiter Blue assay. . **: $p<0.05$ between EtOH and fatty acid-treated samples. Results from one of representative experiments.

This effect of AA on FHC cells could be either due to cytotoxicity or due to increased apoptosis. In order to clarify this, caspase-3/7 activation was checked in these cells, after treatment with the fatty acids, as is shown in the following experiments.

3.4. Effect of the treatment of colon cancer cell lines with ω-3 and ω-6 fatty acids on Cox-2 protein expression

In order to check the effects of the different fatty acids on the expression of Cox-2, cells were treated with 10μM of arachidonic (AA, C20:4ω-6), linoleic (LA, C18:2ω-6), docosahexaenoic (DHA, C22:6ω-6) and eicosapentaenoic (EPA, C20:5ω-3) fatty acids for 5 days (120 hours) in serum free medium. Control cells were treated with the same amount of ethanol. The medium was refreshed every 48 hours. Cell lysates were collected the last day of the treatment, in order to check the expression of Cox-2 gene in protein level.

The results (figure 3.5) showed that there was no influence of any of the fatty acids on the expression of Cox-2 in any of the cell lines.

ω-6 fatty acids failed to induce the expression of Cox-2 in the cells with no or low expression level (HCT116 and Caco-2 respectively) and at the same time ω-3 fatty acids did not down regulate the expression of Cox-2 in HCA-7 cells, which have a high level of expression.

These results suggest that probably the amount of the different fatty acids (ω-6 and ω-3) in the diet has no influence on Cox-2 expression of colon cancer cells in progressed steps of the process of carcinogenesis, like the adenocarcinoma and the carcinoma stages, that our cell lines represent.

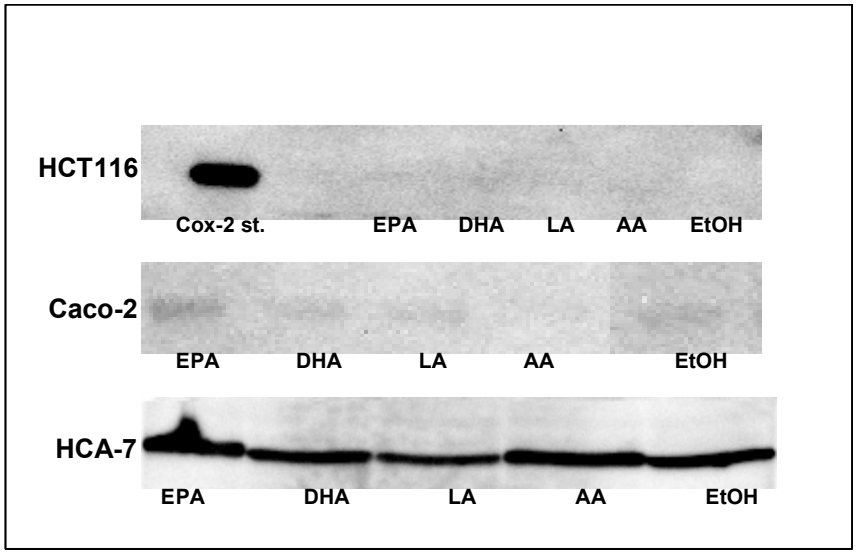


Figure 3.5: Effect of ω-3 and ω-6 fatty acids on the expression of Cox-2 protein in colon cancer cell lines. Cells were treated with 10μM of fatty acids for 120h. Cell lysates were collected and subjected to western blotting analysis. 20μg of protein were used in all samples.

3.5. Effect of ω-3 and ω-6 fatty acids on the expression of mPGES, PPARγ and Nag-1

The same experiments like previously described were performed in order to check the influence of the fatty acid treatment on the expression of the rest of the genes of interest, mPGES, PPARγ and Nag-1. Cell lysates were collected after 120 hours of treatment and analysed by Western Blotting for the expression of these genes. We did not detect any effect on the expression of any of the genes in our cell lines. The result is shown in figure 3.6.

These experiments, like the experiment for Cox-2 expression, were performed in all the cancer cell lines, but not on the normal cells. This was due to the very slow growth rate of FHC and the fact that they can not be cultured for more than five passages, so it was not possible to acquire enough cells for such an experiment.

From these results we concluded that treatment of the cells with ω-3 or ω-6 fatty acids has no effect on the expression pattern of these genes.

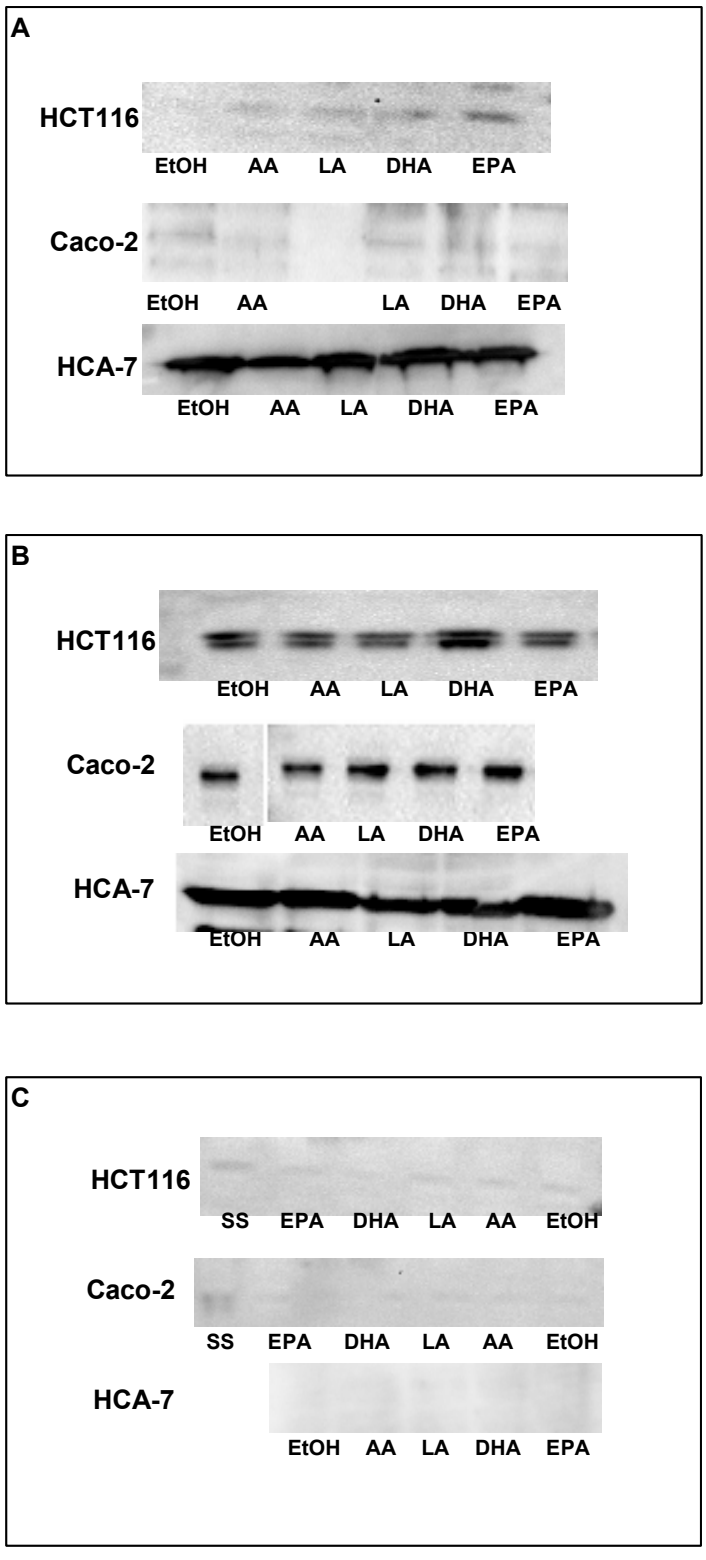


Figure 3.6: Effect of fatty acids on the expression of mPGES (**A**), PPARγ (**B**), and Nag-1 (**C**) in colon cancer cells treated with fatty acids. SS: lysate from HCT116 cells treated with 10μM sulindac sulphide for 24h, in order to induce Nag-1 protein expression. 20mg of protein were used in all cases.

3.6. Effect of ω-3 and ω-6 fatty acids on the expression of 15-Lox-1

In addition to the Cox pathway, another important arachidonate metabolic pathway is the lipoxygenase pathway. There are several reports in the literature showing that 15-Lox-1, one of the three major human lipoxygenases, is involved in colon cancer (reference). In this study we tested the expression of 15-Lox-1 in normal and cancer cell lines. It was found that 15-Lox-1 is expressed in lower levels in normal cells than in cancer cells, in which a high level of expression was found (figure 3.2). When these cancer cells were treated with fatty acids, however, no effect on the expression level of 15-Lox-1 was observed (figure 3.7). Treatment of the cells with 5mM sodium butyrate (NaBT), a known inducer of cell differentiation, for 48 hours, caused a decrease in the level of 15-Lox-1 (figure 3.7). This result is in contrast with previously reported results from other studies, were it is shown that NaBT induces the expression of 15-Lox-1. These results, however, are in agreement with other studies showing that 15-Lox-1 is expressed in tumour tissues in higher levels that in matched adjacent normal tissues. The fact that we cannot detected any effect after the treatment with the fatty acids could be explained either because of the low concentrations of fatty acids used in this study or because there is no effect on the expression level of the lipoxygenase but on the activity of the enzyme. This hypothesis was further examined with experiments on 15-Lox-1 activity in HCT116 and HCA-7 cells after treatment with the fatty acids.

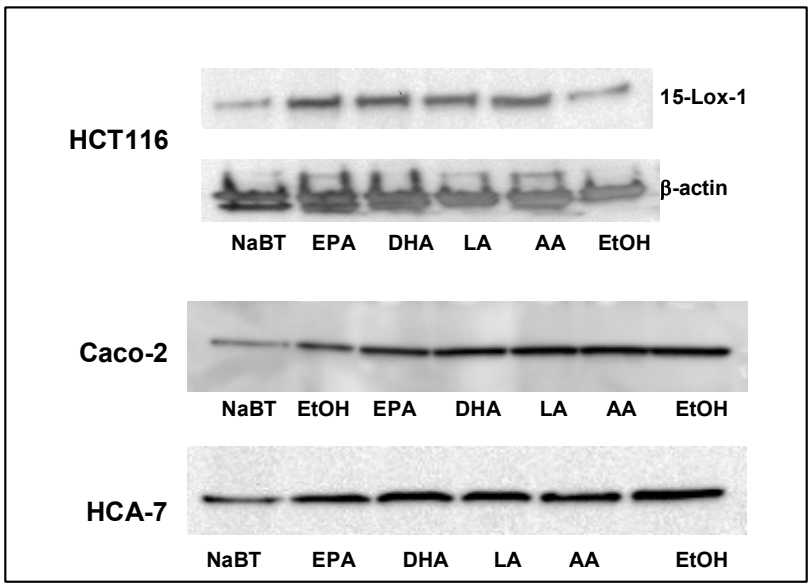


Figure 3.7: Effect of fatty acid treatment on the expression of 15-Lox-1 in the different colon cancer cell lines. NaBT: samples treated with 5mM sodium butyrate for 48 hours. β-Actin was used as loading control.

3.7. Effect of ω-3 and ω-6 fatty acids treatment on the proliferation of colon cancer and normal colon epithelial cells, without or in the presence of vitamin E

An important characteristic of cancer cells is the high rate of uncontrolled proliferation. In the next experiments we decided to check the effect of the exogenously added fatty acids on the proliferation of colon cancer and normal cells. Again, the cells were treated with 10μM of fatty acids for 120 hours in serum free medium, which was refreshed every 48 hours. When the cells were treated with vitamin E, 10μmM of vitamin was also added in the medium. Control cells were treated with the same amount of ethanol. The last 6 hours of the treatment 10μM BrdU was added to the cells and after this time cells were fixed. Proliferation assay was performed as described in the chapter with the materials and methods.

3.7.1. Effect of ω-3 and ω-6 fatty acids treatment on the proliferation of HCT116 cells

In HCT116 cells DHA inhibited proliferation almost completely. EPA (C20:6ω-3) was less effective than DHA, as far as ω-3 fatty acids were concerned. The two ω-6 fatty acids checked, AA and LA, had no effect on the proliferation of HCT116 cells (figure 3.8). In the case of these two fatty acids an induction in the proliferation rate could be expected, according to the literature, but this was not the case in our experiments.

Since it is known that lipid peroxidation products can cause cell death, we decided to check if this growth inhibition effect of DHA was due to lipid peroxidation. In this case, the presence of an antioxidant, like Vitamin E, would protect the cells. For this reason, the experiment was repeated in the same conditions like before, except that 10μM of vitamin E was also added to the medium with the fatty acids. Indeed, the growth inhibitory effect of DHA on HCT116 cells was completely reversed in the presence of vitamin E.

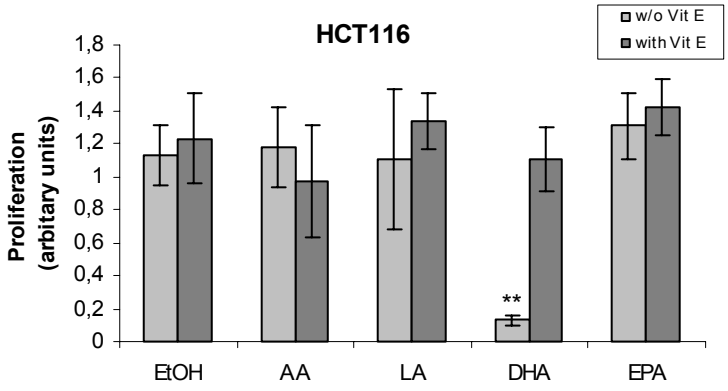


Figure 3.8: Effect of fatty acids on the proliferation of HCT116 cells. **: $p<0.05$, *: $p<0.01$ between EtOH and fatty acid-treated samples. Results from one of representative experiments.

3.7.2. Effect of ω-3 and ω-6 fatty acids treatment on the proliferation of Caco-2 cells

In the case of Caco-2 cells, DHA again was proven very potent in inhibiting proliferation, but in these cells EPA also had a strong inhibitory effect. In these cells there was also an almost 4-fold inhibition of the proliferation after treatment of the cells with AA (figure 3.9). Although a growth inhibitory effect of AA was not expected, similar results have already been published. It is known that a high concentration of AA in the membrane of the cells leads to high intracellular concentrations, which lead to growth inhibition and cell death (reference).

In the presence of Vitamin E, however, the growth inhibitory effect of all the fatty acids was again reversed almost completely.

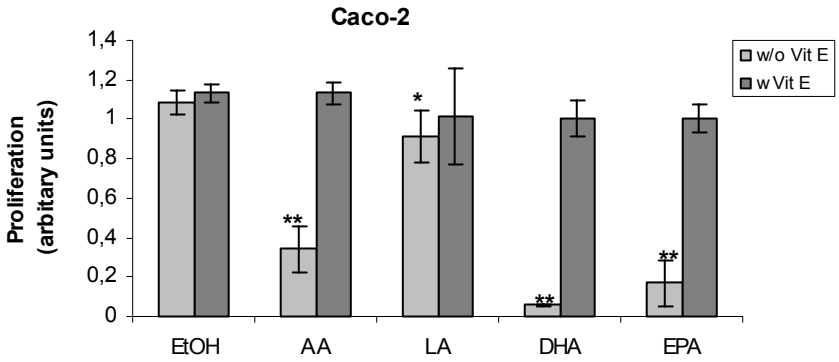


Figure 3.9: Effect of fatty acids on the proliferation of Caco-2 cells. **: $p<0.05$, *: $p<0.01$ between EtOH and fatty acid-treated samples. Results from one of representative experiments.

3.7.3. Effect of ω-3 and ω-6 fatty acids treatment on the proliferation of HCA-7 cells

In HCA-7 cells, once more DHA had the strongest effect on growth inhibition, but in these cells this effect was not so prominent like in the other two cell lines. In the case of HCA-7 cells there was no effect of EPA or LA, but again there was a moderate inhibition of proliferation by AA (figure 3.10). This difference between HCA-7 and Caco-2 cells concerning the effect of AA could be explained by the fact that since HCA-7 cells express more Cox-2, AA is more rapidly metabolised to prostaglandins than in Caco-2 cells. This results in a lower intracellular concentration of AA. This hypothesis however, can not explain why AA has no effect on HCT116 cells. A possible explanation could be the fact that other enzymes involved in lipid metabolism, like 15-Lox, could be responsible for the metabolism of AA in HCT116 cells.

The growth inhibitory effect of DHA on HCA-7 cells could not be completely reversed in the presence of vitamin E, in contrast to what was observed in the other two cell lines. In the case of AA and EPA however, there was a stronger protective effect of vitamin E on the growth of the cells, than in the case of DHA. Nevertheless, the growth inhibitory effect of AA, DHA and EPA could not be completely reversed in these cells, despite the presence of the antioxidant.

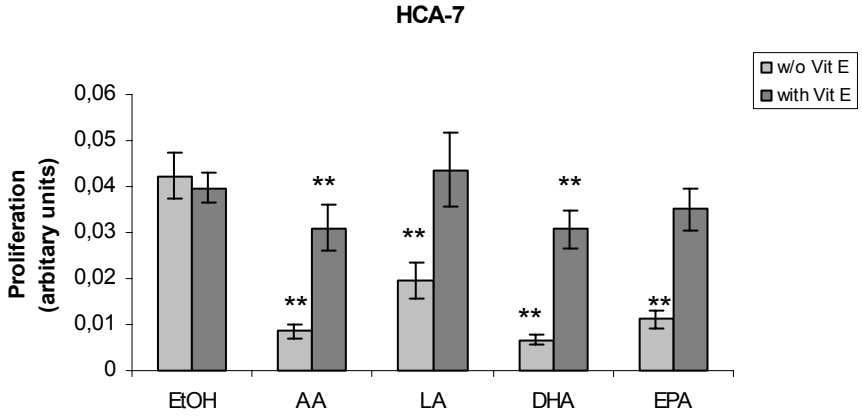


Figure 3.10: Effect of fatty acids on the proliferation of HCA-7 cells. **: $p<0.05$, *: $p<0.01$ between EtOH and fatty acid-treated samples. Results from one of representative experiments.

3.7.4. Effect of ω-3 and ω-6 fatty acids treatment on the proliferation of FHC cells

Interestingly, treatment of FHC cells with the fatty acids resulted in inhibition of proliferation in these cells too (figure 3.11). There was a significant inhibition of proliferation after treatment with AA, DHA and EPA. LA seemed to induce proliferation, but this effect was less significant than the inhibitory effect of AA, DHA and EPA.

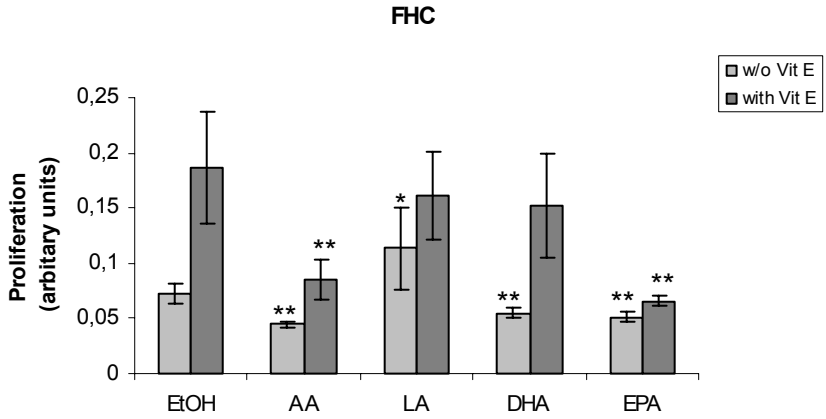


Figure 3.11: Effect of fatty acids on the proliferation of FHC cells. **: $p<0.05$, *: $p<0.01$ between EtOH and fatty acids-treated samples. Results from one of representative experiments.

The results from these experiments showed that there was a strong inhibition of the proliferation in all cancer cell lines after treatment with DHA (C22:6ω-3). This inhibition in proliferation was especially prominent in HCT116 cells. Since it is known that during lipid metabolism, lipid peroxidation takes place, we checked if this growth inhibitory effect of DHA was due to lipid peroxidation products and if the presence of vitamin E could protect the cells. Indeed, the growth inhibitory effect of DHA on HCT116 and Caco-2 cells was completely reversed in the presence of vitamin E. This was not the case with HCA-7 cells, where vitamin E did have a protective effect, but the proliferation rate of these cells was not completely recovered. This could be the result of the high lipid peroxidation rate, due to the high levels of Cox-2 in these cells.

In the normal cells AA, DHA and EPA again were able to cause an inhibition in proliferation, when LA in contrast could cause a small induction. In order to investigate this effect of the fatty acids on normal cells, apoptosis experiments were performed in combination with the viability experiments. Unfortunately, it has not been possible to perform the thiobarbituric acid assay in these cells, due to the problem of the slow growth rate, as has already been mentioned.

3.8. Effect of DHA on the proliferation of HCT116 cells expressing Cox-2

From our results on the effect of DHA on the proliferation of the different colon cancer cell lines, it was concluded that DHA is a potent inhibitor of proliferation in all cell lines, independently of Cox-2 expression. In order to further prove this result, some control experiments were performed, in which we could rule out the difference between the three cancer cell lines, namely the difference in Cox-2 expression.

For this reason, first some experiments were performed in which HCT116 cells were transiently transfected to express Cox-2. After transfection the cells were treated with DHA either in the presence of vitamin E or not, for 120 hours. The level of Cox-2 expression was checked every 24 hours, in order to confirm that there was still enough protein expressed even after 5 days from the time of the transfection. Indeed, the protein was still expressed in sufficiently high levels, as is shown in figure 3.12.

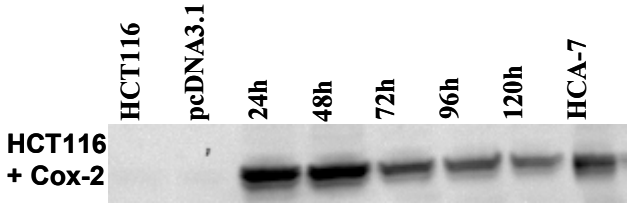


Figure 3.12: Expression of Cox-2 in HCT116 cells transfected with a Cox-2 expression vector (pcDNA3.1-Cox-2) for 120 hours. HCT116: not transfected cells, pcDNA3.1: cells transfected with the empty vector. HCA-7: sample from HCA-7 cells was used as control.

The results from the proliferation assay showed that there was no difference in the effect of DHA in HCT116 cells after transfection with Cox-2 compared to not transfected cells.

DHA inhibited the proliferation of the cells in the absence of vitamin E, although the effect was less dramatic than in not transfected cells. This effect could be reversed in the presence of the antioxidant (figure 3.13).

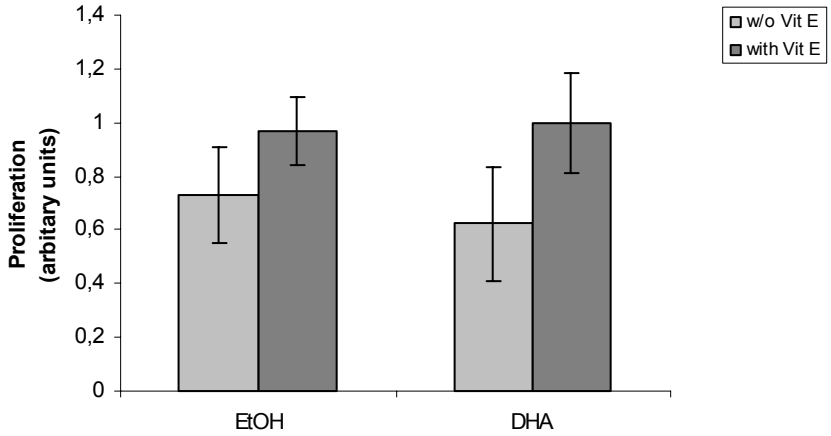


Figure 3.13: Effect of DHA treatment on the proliferation of HCT116 cells transfected with the Cox-2 expression vector. 24 hours after transfection, cells were treated with 10μM DHA for 120h.

3.9. Effect of DHA on the proliferation in HCA-7 cells in the presence of a specific Cox-2 inhibitor

In the next experiment HCA-7 cells were again treated with DHA for 120 hours either in the presence or in the absence of vitamin E. In this case 5nM of CAY10404, a highly specific Cox-2 inhibitor, was also added in the medium.

The presence of the inhibitor had no effect on the level of the protein expression as is shown in figure 16B. The inhibitor itself showed no effect on the proliferation of HCA-7 cells.

Once more, as is shown in figure 3.14, DHA inhibited the proliferation of HCA-7 cells and this effect could not be reversed in the presence of vitamin E. This result is in agreement with the previous results of the effect of DHA on the proliferation of HCA-7 cells, in the absence of the Cox-2 inhibitor (figure 3.10).

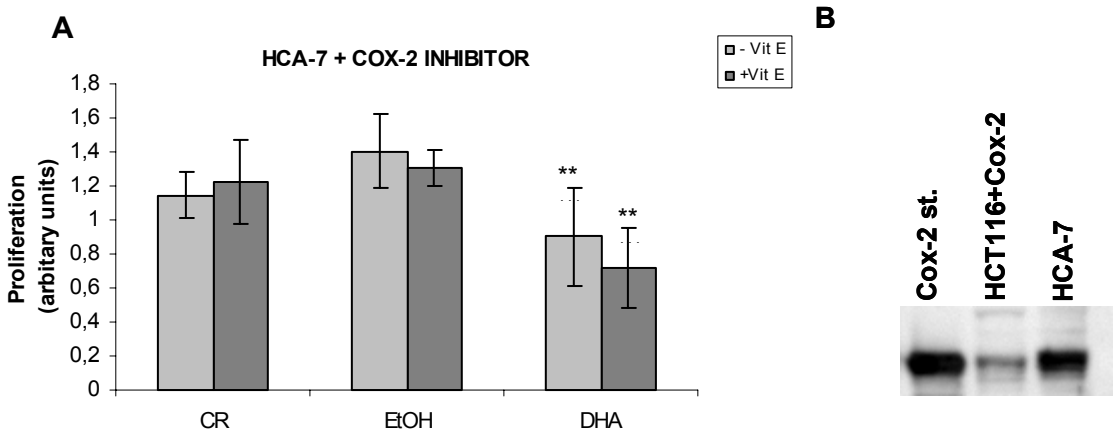


Figure 3.14: **A**: Effect of DHA treatment on the proliferation of HCA-7 cells in the presence of 5nM CAY10404, a highly specific Cox-2 inhibitor. Cells were co-treated with 10µM DHA and 5nM CAY10404 for 120h. **B**: Expression of Cox-2 in HCA-7 cells in the presence of CAY10404 after 120 hours. **: $p<0.05$ between EtOH and fatty acids-treated samples.

The results from this experiment, as well as from the experiment with HCT116 cells transfected with the Cox-2 expression vector, clearly show that the growth inhibitory effect of DHA on the cancer cell lines is independent of Cox-2 expression in the cells.

We can therefore conclude that DHA is a potent inhibitor of proliferation of cancer cells and this effect is due to enhanced lipid peroxidation. This effect is independent of Cox-2 expression. DHA also inhibits proliferation of normal colon cells, but in this case there is no effect on the viability of the cells. There was no induction of apoptosis in the cancer cells after treatment with DHA or any of the other fatty acids.

3.10. PUFAs and apoptosis

From the experiments on the effects of the different types of fatty acids on the proliferation in different colon cancer cell lines, we were able to show that there was an inhibition of proliferation in these cells, especially after the treatment with DHA. In the next step we wanted to clarify the mechanism of this inhibition of proliferation and cell death. For this reason, apoptosis experiments were performed after the treatment of the cells with 10μM DHA. Cells were treated exactly the same way as already described in previous experiments, and on the fifth day cell lysates from the attached and floating cells were collected and combined. The lysates were checked for caspase-3 activity with a synthetic substrate, as described in "Materials and Methods".

Caspase-3 is a frequently activated death protease. Another known caspase-3-like protease is caspace-7, which has analogous *in vitro* substrate preference like caspase-3 (DEVDG).

The results of these experiments showed that there was no caspase-3 activation after treatment of the cells with DHA (figure 3.15). Neither the two ω-3 fatty acids, nor AA were successful in inducing apoptosis in any of the cell lines (data not shown). In order to verify this result, cell lysates were also collected every 24 hours of the treatment and analysed for caspase-3 activation. Again, we could not detect caspase-3 activation at any time point in any cell line (data not shown).

From these results we concluded that the growth inhibitory effect of the fatty acids, mainly DHA, on the colon cancer cells is not due to increased apoptosis.

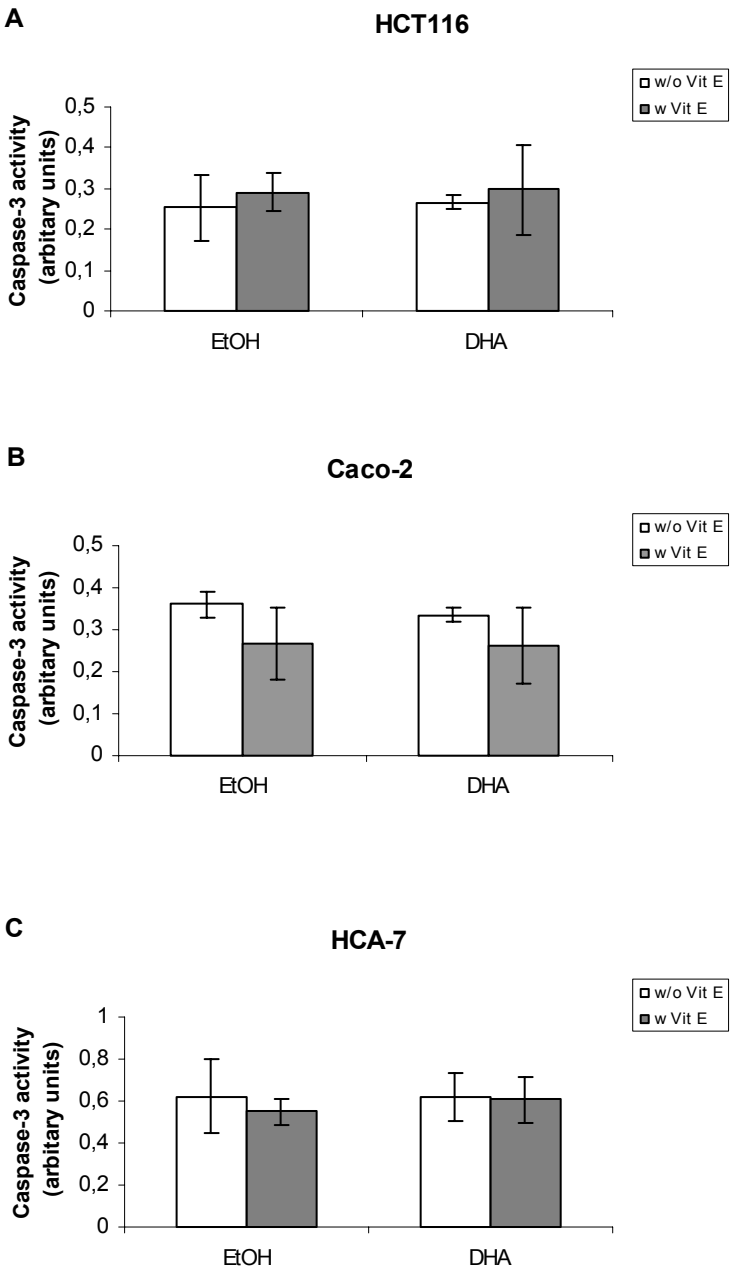


Figure 3.15: Determination of apoptosis in colon cancer cells treated with fatty acids. **A**: HCT116 cells, **B**: Caco-2 cells and **C**: HCA-7 cells. Apoptosis was measured after treatment of the cells with 10μM DHA for 120h. Samples were collected at 120h. Results from one of representative experiments.

In FHC cells in contrast, we could detect a high activation of caspases 3 and 7 in the cells treated with AA (figure 3.16), which explains very well the reduced viability of these cells, as is shown in figure 10. In contrast, DHA and EPA did not induce apoptosis in FHC cells, despite their inhibitory effect on the proliferation, as described before (figure 3.11). In the case of the normal cells we can therefore conclude that AA induces apoptosis.

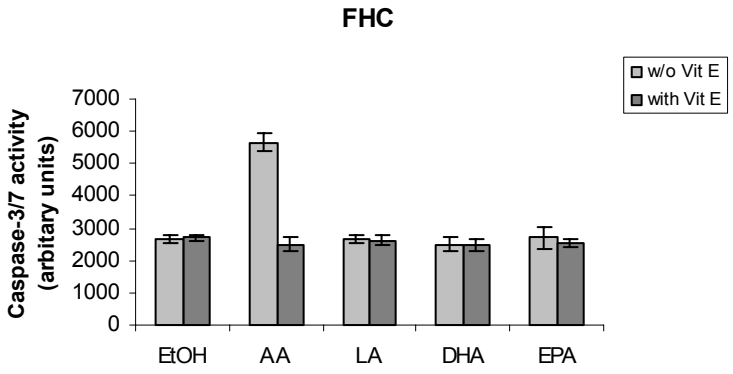


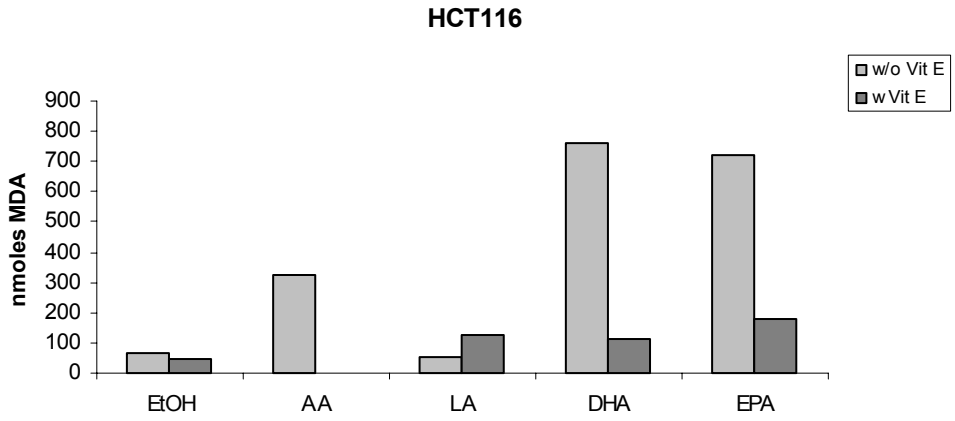
Figure 3.16: Determination of apoptosis in FHC cells treated with the different fatty acids. Cells were treated with 10mM of fatty acids and caspase-3/7 activation was determined after 120h with the ApoONE Homogeneous Caspase-3/7 Assay. Results from one of representative experiments.

3.11. Stimulation of Lipid Peroxidation by PUFAs

In order to clarify if the inhibitory effect of DHA on the proliferation of the colon cancer cell lines was due to oxidation products, we performed the thiobarbituric acid assay. With this assay the levels of intracellular MDA formation were measured after treatment of the cells with DHA. MDA is a by-product formed during the metabolism of the fatty acids by cyclooxygenases, but can also be formed during non-enzymatic lipid peroxidation. Non-enzymatic lipid peroxidation can be initiated by free radicals, needs oxygen and has the characteristics of a chain reaction. This assay is based on the detection of MDA formed from the breakdown of polyunsaturated fatty acids, after its reaction with thiobarbituric acid, to give a red species absorbing at 535nm.

Indeed, our results showed that there was a very high amount of MDA produced in the cells treated with DHA, which was reduced in the presence of vitamin E (figure 3.17). This reduction was really pronounced in HCT116 cells. The fact that there are still high amounts of MDA in HCA-7 cells treated with DHA in the presence of vitamin E, explain the results from the experiments on the proliferation, where it was shown that vitamin E could not reverse completely the inhibition of proliferation caused by DHA. Again, this experiment was not performed in FHC cells because as it has already been mentioned it was not possible to acquire enough cells from that cell line.

A



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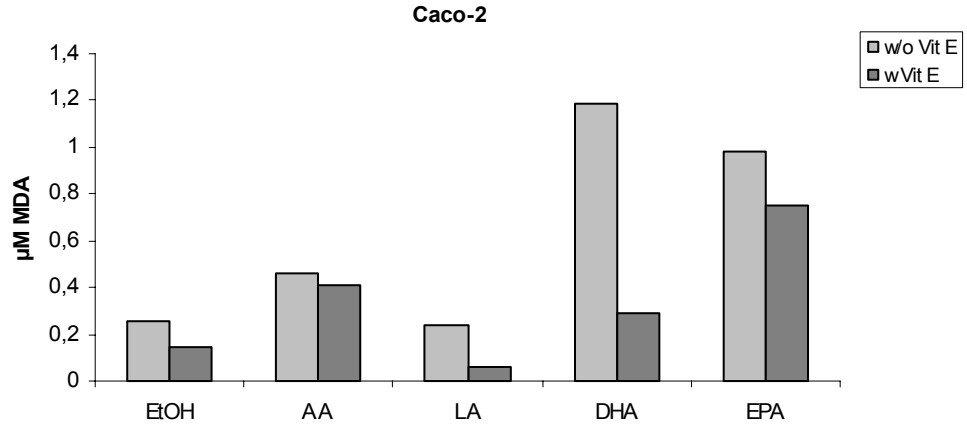

C

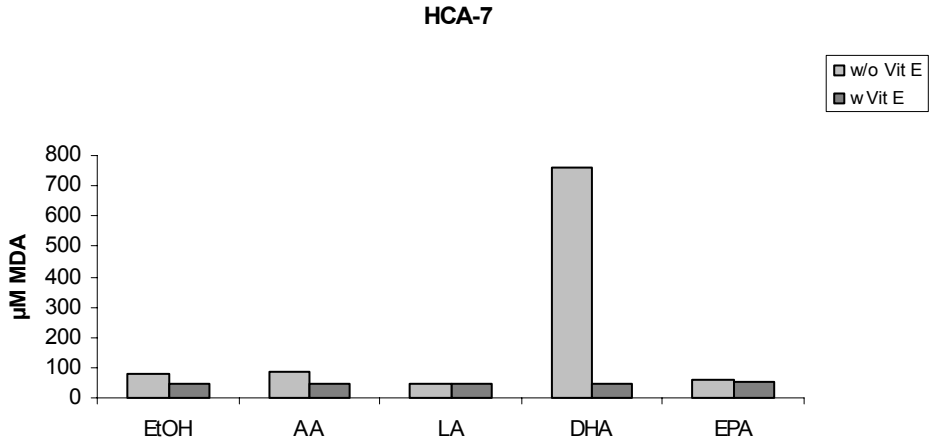

Figure 3.17: Thiobarbituric acid reactive species (TBARS) formation (MDA) after treatment of HCT116 (**A**), Caco-2 (**B**) and HCA-7 (**C**) cells with fatty acids, with or without Vitamin E. Results from one of three representative experiments.

4. Discussion

4.1 Effect of the different polyunsaturated fatty acids on the gene expression of colon cancer cells

4.1.1. PUFAs and Cox-2

Several genes have been found to be involved in colon carcinogenesis, some of them functioning as tumour suppressor genes and others functioning as oncogenes. Among the oncogenes, Cox-2 has been found to play a major role in colon carcinogenesis and has attracted much attention. The Cox-2 pathway has been under intense investigation as a target for both treatment and prevention of colon cancer. The exact role of the enzyme in colonic cell transformation and tumour development, however, has not been fully elucidated yet.

Studies with AOM-induced colon carcinogenesis in rats have revealed upregulation of many genes, including Cox-2. Cox-2 has been shown to be expressed in aberrant crypt foci (ACF), but is clearly increased in the stromal elements of adenomas and adenocarcinomas. In epithelial cells, however, there is no Cox-2 expression in normal mucosa or ACF, slight in adenomas but frequent in adenocarcinomas. In large carcinomas Cox-2 expression is particularly high, suggesting the growth promoting effects of this protein (Takahashi M and Wakabayashi K, 2004).

In this study the effect of different PUFAs on colon cancer cell lines with different levels of Cox-2 expression was examined. The results showed that there was no effect of the different fatty acids on the expression of the enzyme under the conditions the experiments were performed. The same was true for the expression of mPGES, the terminal enzyme in the production of PGE_2 from AA through the Cox-2 pathway. mPGES has been shown to be functionally linked with Cox-2.

These results imply that treatment with ω-6 or ω-3 fatty acids has no effect either on the protein expression or on the activity of Cox-2. This result was the same in all cell lines, independently of Cox-2 expression level.

Many studies have shown that one of the mechanisms by which ω-3 fatty acids exert their anticarcinogenic effects is through downregulation of Cox-2 expression and PGE_2 production. Calvilello et al. showed that ω-3 fatty acids can inhibit VGEF production in HT-29 colon cancer cells, through downregulation of Cox-2 expression and ERK1/2

activation (Calviello G et al., 2004). In other studies, Murray et al., found that ω-3 fatty acids exert their growth inhibitory effects on colon cancer through downregulation of PKCβII and upregulation of the TGFβ pathway, and these effects are mediated through Cox-2 downregulation (Murray N R et al., 2002, Yu W et al., 2003). In Caco-2 and HT-29 cells it has been shown that ω-3 fatty acids reduce both Cox-2 and Bcl-2 expression, resulting in enhanced apoptosis (Llor X et al., 2003). The same inhibitory effect of ω-3 fatty acids on Cox-2 expression has been also found in other cell types, like endothelial cells (Ait-Said F et al., 2003), monocytes (Lee J Y et al., 2002) and hepatoma cells (Vecchini A et al., 2003). In these studies the effect of the fatty acids on Cox-2 expression was studied either *in vivo*, in animals were tumour formation was induced with AOM (Calviello et al., Murray et al.), or in different colon cell lines than the ones used in this study. In the cases where Caco-2 cells were used (Llor et al.) the investigators had used higher concentrations and different time points. Moreover, in many cases different cell models were used like monocytes, brain melanoma cells or rat intestinal cells (Lee et al., Vecchini et al., Yu et al.)

On the other hand, in many studies it was found that ω-3 fatty acids have the same growth inhibitory effects on cells which do not express Cox-2. In a study of Boudreau et al., they show that ω-3 fatty acids suppress the growth of HCT116 cells both in culture or after injection in nude mice, and this effect was the same even when cells were transfected with a Cox-2 plasmid (Boudreau M et al., 2001). Moreover, Chen and Istfan have shown that indomethacin could not block the DHA-induced apoptosis in HT-29 cells, which express high levels of Cox-2, whereas this effect was reduced in the presence of an antioxidant (Chen Z Y and Istfan N W, 2000).

Although the anticarcinogenic effect of ω-3 fatty acids is well established, the role of Cox-2 in ω-3 fatty acids-mediated effects is not clear yet. It is very possible that the effects of ω-3 fatty acids on cancer development are mediated by both Cox-2-dependent and Cox-2-independent pathways. Narayanan et al have shown that treatment of Caco-2 cells with DHA results in upregulation of many genes involved in growth arrest and apoptosis and down regulation of genes involved in inflammatory responses like iNOS and NFκB (Narayanan B A et al., 2003). These genes are also involved in the regulation of Cox-2 expression, so it is very possible that the effects of ω-3 fatty acids on Cox-2 are more complicated than we know so far.

4.1.2. PUFAs and PPARγ

The first report of dietary fat affecting gene transcription via a nuclear transcription factor came from Gottlicher et al. in 1992 (reviewed in Sampath H and Ntambi J M, 2004). Since then PUFAs have been established as endogenous ligands for PPARs. Kliewer et al. in 1997 showed that AA, LA and their metabolites competed for binding to PPARγ in the 2-20μM range. These values are within the range of concentrations found in human serum (Kliewer S A et al., 1997). PPARγ is expressed in normal colon epithelium and the expression increases from the crypts, where cell division takes place, to the villous tips, where cells are highly differentiated. This suggests that PPARγ expression correlates with and possibly influences differentiation (Mansen A et al., 1996). In Caco-2 cells, it has been shown that PPARγ expression is increasing when the cells are acquiring a higher degree of differentiation (Lefebvre A-M et al., 1999). The role of PPARγ in colon cancer development is under investigation.

Talking all these data into account, it was decided to check if the exogenously added PUFAs had any effect on the expression level of the receptor in our colon cancer cell lines. For this, first we examined the basal expression pattern of the receptor in the cancer cell lines and also in the normal colon epithelial cells. The results showed that the receptor is expressed in all cancer cell lines, in agreement with previously published reports. The same result was acquired for the normal cells.

Treatment with the different fatty acids however, had no effect on the expression of the receptor. This result was the same in all cell lines, independently of Cox-2 expression. Although a Cox-2-independent mechanism of action has already been described for PPARγ (Lefebvre A-M et al., 1998), it is very probable that PUFAs have no effect on the level of the expression but on the inhibition or activation of the receptor. In most of the studies mentioned above, PPARγ was activated by synthetic ligands, like triglitazone. The inhibition or activation of the receptor was checked by examining the phosphorylation status of the receptor with Western Blotting analysis or with DNA microarrays. In our experiments there were no bands of phosporylated PPARγ. Even in HCT116 cells, where two bands could be detected, they were the same in all treatments. This result is in contradiction with the results of Narayanan et al., who show that 15μM of DHA for 48 hours induced the expression of PPARγ by 2-fold in Caco-2, in a DNA microarray study (Narayanan B et al., 2001, Narayanan B et al, 2003). A plausible explanation for this result could be the fact that the concentration of fatty acids used in this study was too low

to detect an effect on the receptor. Another reason for this discrepancy could be that different lines were used in this study as well as different time points.

4.1.3. PUFAs and 15-Lox-1

Currently, some controversy exists regarding the precise role of 15-Lox-1 in colorectal carcinogenesis and other aspects of cancer biology. Mainly, there are two different notions in the literature. The anti-tumorigenic role of 15-Lox-1 is supported by the group of Lippman and some studies from the group of Eling. In many of their studies the two groups support that 15-Lox upregulation results in induction of differentiation and apoptosis in colon, oesophageal and prostate cancer cells. They have shown that treatment of Caco-2 cells with sodium butyrate (NaBT), a known inducer of cell differentiation or apoptosis by the mechanism of histone hyperacetylation, induces the expression of 15-Lox-1 and at the same time downregulates the expression of Cox-2, resulting in increased apoptosis (Kawajiri H et al., 2002). In some studies of the Lippman group, it has been shown that the antitumorigenic activity of NSAIDs is mediated via upregulation of 15-Lox-1 expression and apoptosis in oesophageal cancer cells (Shureiqi I et al., 2001) and also in colorectal cancer cells. In the latter case the effect was shown to be Cox-2-independent (Shureiqi et al., 2000). In another study they have shown that the 15-Lox-1 product 13-S hydroxyoctadecadienoic acid (13-S-HODE), a PPARδ ligand, downregulates PPARδ activity, resulting in apoptosis (Shureiqi I et al., 2003). The anti-tumorigenic effects of 15-Lox-1 have also been described by another group, who showed that 15-Lox-1 mediates cyclooxygenase-2 inhibitor-induced apoptosis in gastric cancer (Wu J et al., 2003).

The carcinogenic effects of 15-Lox-1 on the other hand are mainly supported by the group of Eling, who have shown that there is elevated expression of 15-Lox-1 in human colorectal cancer tissues, compared to normal adjacent tissue (Ikawa H et al., 1999) and that overexpression of the enzyme in a human prostate carcinoma cell line increases tumorigenesis (Kelavkar U et al., 2001). In a study with HC116 cells, the same group showed that 15-Lox-1 metabolites of LA downregulate PPARγ activity via the MAPK signalling pathway (Hsi L et al., 2001). The picture becomes even more complicated in a study of the same group, which shows that although 15-Lox-1 expression is induced during

differentiation of Caco-2 cells and after treatment with NaBT, inhibitors of 15-Lox-1 can enhance the NaBT induced apoptosis in these cells (Kamitani H et al., 1998).

Some light on this complicated picture is shed by a study of Hsi et al., who show that there are different, and possibly opposite effects between the metabolites of the two 15-Lox isoforms. The investigators show that the 15-Lox-1 metabolite, 13-S-HODE acts as an inhibitor of PPARγ, when the 15-Lox-2 metabolite 15-S-HETE activates the receptor, resulting in inhibition of proliferation of prostate cancer cells (Hsi L et al., 2002). So it seems that the two isoforms of the enzyme can exert different biological functions.

In this study we first checked the basal level of expression of 15-Lox-1 in our colon cancer cells. We found high levels of expression in the cancer cell lines and reduced expression in the normal cells, in agreement with the studies showing upregulation of the enzyme in cancer tissues. Treatment of the cancer cell lines with the different fatty acids, however, had no effect on the level of the expression. The preferred substrates for the two isoforms of 15-lox are LA for 15-Lox-1 and AA for 15-Lox-2. Treatment with these two fatty acids had no effect on the expression of the 15-Lox-1 isoform. It is very possible however, that the activity of the enzyme was affected, but no experiments on the enzymatic activity were performed in this study, neither on the different metabolites of the fatty acids used in the different cell lines. To our knowledge there is no reference so far showing a direct effect of ω-3 fatty acids on the level of expression of 15-Lox-1. Experiments on the activity of the enzyme and the metabolites produced should also be performed to clarify this question.

An interesting finding of this study, however, was the fact that in all our cancer cells, where we have high expression of 15-Lox-1, treatment with NaBT resulted in down regulation of the expression and inhibition of cell growth and induction of apoptosis. This result is in controversy with the studies mentioned above, which all claim an upregulation of 15-Lox-1 expression after NaBT treatment. This result seems very plausible, however, if we bear in mind the tumorigenic effects of 15-Lox-1 and the antitumorigenic effect of NaBT. It is also in agreement with the study of Hsi et al., who showed that inhibition of 15-Lox-1 enhanced the NaBT-induced apoptosis.

In conclusion, the role of 15-Lox-1 in the process of carcinogenesis is a very promising field of research, since the data we have to date are still controversial. As far as the role of the enzyme in relation to the effects of the different PUFAs is concerned, more and more specific studies are necessary, in order to draw any conclusion.

4.1.4. PUFAs and Nag-1

Nag-1 is a recently identified member of the TGFβ transcription factors family. The gene was discovered by the group of T. Eling, after treatment of colon cancer cells with NSAIDs (Baek S-J et al., 2001a). Since then, many studies of the same group have shown that Nag-1 protein expression is induced by NSAIDs (Baek S-J et al., 2002), and the expression of the protein leads to induction of apoptosis (Baek S-J et al., 2001b). Except of NSAIDs, also dietary flavonoids have been shown to induce the expression of the gene (Baek S-J et al, 2002 Baek S-J et al., 2004, Wilson SC et al., 2003, Bottone FG et al., 2001), and the protein was shown to induce apoptosis not only in colon cancer cells, but also in prostate, ovarian, oral cavity and gastric cancers (Shim M and Eling TE, 2005, Kim JS et al., 2005, Kim KS et al., 2004, Jang TJ et al., 2004).
In this study it was checked if treatment of the colon cancer cell lines with PUFAs had any effect on the expression of Nag-1. The gene was not expressed in any of the cells under normal conditions (no treatment) and the expression could be induced in HCT116 cells after treatment with sulindac sulphide, a well known NSAID. PUFAs, however, had no effect on the expression of the gene in any of the cell lines. So far there has been no reference in the literature about PUFAs-mediated effects on Nag-1 induction, so it is very possible that the expression of Nag-1 is not regulated by PUFAs.

4.2. Effect of the different PUFAs on the proliferation and apoptosis of normal and cancer colon cells

One of the hallmarks of cancer cells is their high rate of proliferation, even in the absence of growth signals on one hand, and their insensitivity to anti-growth signals on the other. In this study the effect of the different PUFAs on the growth of our cancer and normal colon cells was examined. References in the literature have already shown that ω-3 fatty acids have a suppressive effect on the growth of cancer cells. We found similar results in this study. DHA was proven a potent inhibitor of cell growth in all cell lines. In HCT116 cells, DHA inhibited growth almost completely, and it also had a strong growth inhibitory effect on Caco-2 and HCA-7 cells. These results were confirmed in HCT116 cells transfected with a Cox-2 expression vector as well as in HCA-7 cells in the presence of a highly specific Cox-2 inhibitor. DHA inhibited proliferation in both cases. DHA treatment

resulted also in inhibition of proliferation in FHC cells. When the cells were treated with the different fatty acids in the presence of Vitamin E, the growth inhibitory effect of DHA was reversed completely in HCT116 and Caco-2 cells. In HCA-7 and FHC cells the effect of DHA could not be completely reversed. A very important conclusion of these results is the fact that although all cell lines behave the same in DHA treatment, they do not behave the same in the treatment with Vitamin E. HCT116 cells were the most sensitive in DHA treatment but also reacted better in the addition of vitamin E. This could probably be attributed to the fact that they are a carcinoma cell line, the most undifferentiated and with the highest rate of proliferation. HCA-7 cells in contrast are differentiated and have a slower growth rate, so it is very possible that these cells cannot overcome the damage induced by DHA even in the presence of the antioxidant.

In the cancer cell lines EPA also had a strong inhibitory effect, although not so strong like DHA. Again, EPA did not show the same potency in all cell lines. In HCT116 cells EPA had no effect on proliferation, whereas in Caco-2 and HCA-7 cells there was an inhibition of proliferation, although not so strong like with DHA. In normal cells, however, EPA was equally effective like DHA. So we cannot generalise the effects of all PUFAs in all different cell lines, since obviously there are more factors influencing the behaviour of each cell. In any case, the fact that EPA was as effective as DHA in normal cells proves the growth inhibitory effect of ω-3 fatty acids.

As far as the ω-6 fatty acids are concerned, AA had a growth inhibitory effect on Caco-2 and HCA-7 but not on HCT116. The same results were acquired also from the normal colon cells. AA inhibited growth of FHC cells strongly. This growth inhibitory effect of AA is not surprising, since there are already references in the literature showing the same effect. Intracellular unesterified AA is known to induce apoptosis in colon cancer and other cell lines (Cao Y et al., 2000). Although it has been suggested that enzymes that utilise AA, like Cox-2 and fatty acid-CoA ligase can prevent apoptosis by lowering the levels of free AA (Cao Y et al., 2000), in this case AA had no effect in the cells that do not express Cox-2 (HCT116). The explanation for this result could be the fact that AA is not metabolised only through Cox-2, but also through the Lox pathway of eicosanoid metabolism, through the monooxygenases (P450) metabolic pathways and also through non-enzymatic oxidation (Brash A R, 2001). AA is also highly unsaturated (four double bonds) and is subjected to spontaneous lipid peroxidation. It is possible that other enzymes than Cox-2, are involved in the effects of AA in our cell lines. For example in HCT116 cells AA could be metabolised by lipoxygenases or P450 enzymes, so there is not enough

AA free to exert growth inhibitory effects in these cells. On the other hand, it is possible that in the Cox-2 expressing cell lines AA serves as substrate not only for Cox-2 but also for non-enzymatic lipid peroxidation, leading to oxidative stress. However, it is also possible that HCT116 are less sensitive to AA due to their highly undifferentiated status.

LA treatment had no effect on the proliferation of the cancer cells, except a slight effect on HCA-7, but in general this fatty acid was the less potent. There was no case of induction of proliferation after LA treatment in any of the cancer cell lines. In FHC cells in contrast, there was a slight induction of proliferation after treatment with LA. This is another clue of the fact that ω-6 fatty acids can induce proliferation in normal colon cells.

From these results the following conclusions can be drawn: An important finding is that the effect of DHA on the growth of colon cancer cells is Cox-2 independent. DHA was the most potent inhibitor of proliferation in all cell lines, normal and cancer ones, and it had the same effect even in HCT116 cells expressing Cox-2, or when Cox-2 in HCA-7 cells was inhibited with a highly specific receptor. This result implies that the eicosanoid metabolism pathway is not important for the effects of DHA. Since many studies have already shown that DHA induces apoptosis in cancer cells we also checked if this growth inhibitory effect of DHA was due to induction of apoptosis. We could not detect any caspase-3 activation in our cells after treatment with 10μM DHA for 120 hours. The same result was acquired even when cells were checked for caspase-3 activity every 25 hours. There are two possibilities to explain this result. Although it is already mentioned in the literature that DHA can induce apoptosis in colon cancer cells (Chen Z Y and Istfan N W, 2000, Sanders L M et al., 2004), some other studies show that the growth inhibitory effect of n-3 fatty acids is due to growth arrest and induction of differentiation (Chamras H 2002). However, the possibility that in our case apoptosis could not be detected due to the experimental approach that we used, cannot be excluded. Cells were treated with a low concentration of fatty acids and for a long period of time. It is very possible that a small percentage of cells were undergoing apoptosis each day of the treatment, but this percentage was too small to detect enough active caspase-3.

The most important finding of this study was the fact that the effects of the fatty acids on the cell growth were different between normal and cancer cells. In FHC cells, a normal foetal colon epithelial cell line, it was found that DHA had no effect on apoptosis or viability of the cells. It has already been published that n-3 PUFAs can selectively kill breast, lung and prostate cancer cells at concentrations that have no adverse effects on normal cells (Begin M E et al., 1985). In this study it is shown for the first time that DHA

selectively kills colon cancer cells at a concentration that has no effect on normal colon cells. The same is true for EPA. Still, the fact that there was no cytotoxicity or apoptosis on FHC cells, proves that the growth inhibitory effect of DHA due to lipid peroxidation is specific for cancer cells. The inhibition of proliferation in FHC cells could be regarded as a favourable effect, since high rates of proliferation can lead to mutations and neoplastic transformation of cells, whereas differentiation is connected to slow or no proliferation.
The fact that the growth inhibitory effect of DHA on the proliferation of all cells could be reversed in the presence of Vitamin E implies that the main mechanism for the growth inhibitory effect of DHA is the induction of oxidative stress, via lipid peroxidation. This was confirmed with the experiments on MDA production, as will be discussed later.

4.3. PUFAs and lipid peroxidation

Long chain highly polyunsaturated fatty acids are known to be very sensitive to lipid peroxidation. Although the beneficial effects of ω-3 PUFAs have been well documented, the undesirable effects of high fish oil intake have nevertheless been a concern, since ω-3 PUFAs are more readily oxidized under atmospheric conditions. Previous studies have shown that a diet containing high levels of fish oil enhances lipid peroxidation in the organs, blood and urine of experimental animals and humans, as has already been mentioned in the introduction. Since DHA is a highly polyunsaturated fatty acid, and based on the fact that in the presence of Vitamin E the inhibitory effect of this fatty acid on the proliferation of all cells could be reversed, the production of MDA in the cells after treatment with the fatty acids was checked.
The results showed that there was a high production of MDA in the cells treated with DHA in all cases. This means that after treatment of cancer cells with n-3 fatty acids lipid peroxidation is enhanced. This is not the case with n-6 fatty acids. This result is in agreement with previously reported results showing increased lipid peroxidation products in cells treated with n-3 fatty acids, or in the serum of individuals that consume a lot of fish in their diet (Ito Y et al., 1999). It has already been shown that treatment of cells with DHA results in increased lipid peroxidation and finally increased apoptosis or cell death (Begin M E et al., 1986, Nano J L et al., 2003). So it is very possible that the growth inhibitory and anticarcinogenic effect of n-3 fatty acids is attributed to their high susceptibility to lipid peroxidation. It is known that the more unsaturated bonds in a fatty acid chain, the

highest the susceptibility to lipid peroxidation. DHA, with 6 double bonds, is the most unsaturated fatty acid used in this study.

The hypothesis that ω-3 fatty acids exert their favourable effects on cancer prevention through increased lipid peroxidation seems a very challenging one. Although it is still speculative, more and more studies are published supporting it. In a review of Dianzani (Dianzani MU, 1989) it is stated that in cancer (hepatoma) cells the loss of lipid peroxidation is proportional to the degree of de-differentiation. Moreover, that during carcinogenesis, the loss is already evident at the stage of pre-neoplastic nodules. The reason for this decline in lipid peroxidation could be either low activity of the monooxygenase microsomal enzymes or the change in the lipid composition of the membranes. This hypothesis is in a good agreement with the anti-carcinogenic effects of the ω-3 fatty acids and their susceptibility to lipid peroxidation.

Recent studies have also shown that lipid peroxidation products, like MDA, can cause cell cycle arrest through downregulation of cyclin-E and cyclin-B dependent kinase activity and upregulation of p21 (Ji C et al., 1998). The same authors have shown that 3-hydroxynonenal, another product of the lipid peroxidation pathway, induces apoptosis in human colorectal carcinoma cells, through the mitochondrial pathway, involving cytochrome c release (Ji C et al., 2001).

Although it was not possible to perform the same experiments with the normal cells, it is very plausible to hypothesise that DHA causes cell death to colon cancer cells due to high lipid peroxidation. The mechanism of this effect could be either due to growth arrest, apoptosis or necrosis. Normal cells seem to be more resistant to this effect, since there was no increased cell death in these cells after treatment with DHA.

Aw proposed a model in order to explain the molecular and cellular responses to oxidative stress in the intestine. He proposed that low levels of oxidative stress are required for the normal tissue homeostasis. Intermediate levels could affect selective activation of genes and protein expression, whereas high levels are cytotoxic. A schematic representation of the model is shown in figure 4.1 (Aw T Y, 1999). This model can also support these results.

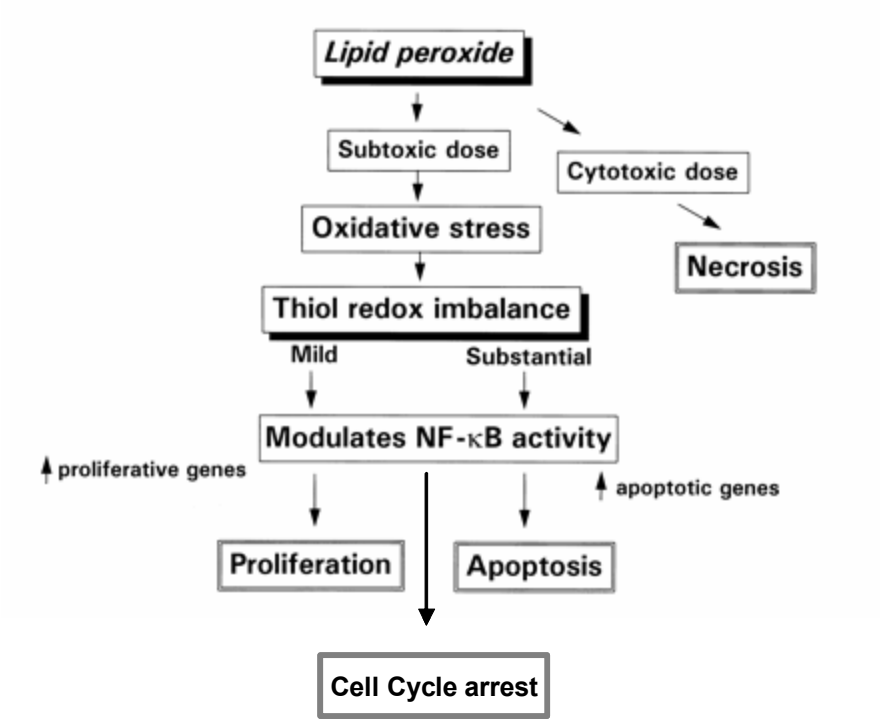

Figure 4.1: Working hypothesis of lipid peroxide-induced cell proliferation or apoptosis. Adapted and modified from Aw T Y, 1999.

In conclusion, our results indicate that lipid peroxidation-induced inhibition of cell proliferation could be an important pathway for the anti-carcinogenic effects of the ω-3 PUFAs.

4.4. General conclusions (Summary)

Although life style, genetic and many other factors have been linked to the risk of colon cancer, much of the variation in colon cancer incidence across countries and cultures remains unexplained. The wide geographic variation spurred interest in the role of the diet, leading to extensive research on fat intake. In 1986 Reddy et al. first reported that ω-3 PUFAs had diminished colon tumour promoting effects relative to ω-6 PUFAs (reviewed in Cave W T, 1991). Since then many studies have proven the beneficial effects of ω-3 fatty acids in colon cancer prevention. Many mechanisms have been proposed, but important gaps still remain.

One of the proposed mechanisms is related to the alterations of the eicosanoids metabolic pathways that are caused by ω-3 fatty acids. In this study we were able to show that the effect of DHA, the most potent ω-3 fatty acid concerning growth inhibition of cancer cells, was Cox-2 independent. Moreover, in normal cells, AA, the precursor of eicosanoid metabolism, could induce apoptosis, implying that the production of prostaglandins through this metabolic pathway is not sufficient to induce proliferation or inhibit cell death in these cells. This is very important since the products of the AA metabolic pathway are in general considered pro-inflammatory and growth stimulating. Although the protective effects of inhibitors of this pathway, namely Cox-2 inhibitors (NSAIDs) have been well documented, recent studies are showing Cox-2-independent mechanisms of action of these compounds. The same is true for PUFAs, since their effects on the inhibition of growth of cancer cells were Cox-2 independent.

In this study we were able to show that one important mechanism for the anti-tumorigenic effects of ω-3 fatty acids is the inhibition of proliferation through lipid peroxidation. More important, we were able to show that cancer cells are more sensitive to the growth inhibitory effects of ω-3 fatty acids than normal cells. The implications of this finding are many and important.

The higher sensitivity of cancer cells to DHA-induced growth arrest makes it an ideal candidate not only for cancer prevention, but also for cancer treatment. Indeed, recent studies have shown that when DHA is combined with anticancer drugs results in an additive growth inhibitory effect. This effect was shown in colon cancer cell lines with 5-fluorouracil (Jordan A and Stein J, 2003, Calviello G et al., 2004), but also in mammary tumour cells and radioactivity (Colas S et al., 2004). From these studies it may be concluded that pharmacological-dietary combination can improve the anti-tumour efficacy

over either dietary or pharmacological intervention alone. These findings bring new prospects for the use of DHA as an anticancer agent and open new fields for future research.

While the exact mechanisms by which PUFAs exert their effects are not fully understood, PUFAs have been shown to modulate gene transcription by interacting with at least three nuclear receptors, among them PPARs, and by regulating transcription factors, like the regulatory element-binding proteins. These transcription factors regulate the transcription of genes involved in cholesterol, triglyceride and fatty acid synthesis (reviewed in Sampath H and Ntambi J M, 2004).

It is clear that much work has to be done to understand the mechanisms by which PUFAs exert their effects. While there is no recommended allowance for fat, it is widely recognised that the amount and type of fat ingested can have far reaching consequences on health. Understanding the molecular mechanisms by which ω-3 and ω-6 PUFAs function could pave the way to find novel targets for pharmacologic treatment for various chronic diseases, among them cancer. Furthermore, such knowledge will prove invaluable to educators and policy makers in setting recommendations for reaching optimal health through good nutrition.

5. Abstract

Introduction: The aim of this study was to analyse the mechanisms by which dietary factors can initiate and promote colon cancer development. The attention was focused on the way n-3 and n-6 PUFAs influence colon cell proliferation and apoptosis in relation to Cox-2. In order to explore the mechanisms by which fatty acids exert their effects in relation to Cox-2 expression, PPARγ, 15-Lox-1 and Nag-1 expression was also analysed.

Materials and Methods: Four human colon cell lines were used: HCT116, a colorectal carcinoma cell line with no Cox-2 expression, Caco-2 a colon adenocarcinoma cell line with low Cox-2 expression, HCA-7 a colon carcinoma cell line with high Cox-2 expression and FHC, a normal foetal colon cell line. Proliferation was studied after treatment of all cell lines with 10μM of n-6 (arachidonic and linoleic) and n-3 (docosahexaenoic and eicosapentaenoic) fatty acids for 120 hours, BrdU labelling and immunodetection of the incorporated BrdU. For apoptosis, a colorimetric assay for Caspace-3 activation using pNA as substrate was used. Cytotoxicity was checked with the CytoTox-ONE Homogeneous Membrane Integrity Assay. Lipid peroxidation is detected with the Thiobarbituric acid reactive species assay (TBARS). The expression of Cox-2, PPARg, 15-Lox-1 and Nag-1 was analysed by Western Blotting.

Results: There was no effect on Cox-2, Nag-1 1-Lox-1, or PPARγ protein expression after treatment with 10μM of fatty acids in all cell lines. Treatment of cancer cells with 10μM of n-3 and n-6 fatty acids for 5 days caused inhibition of proliferation in the cells treated with docosahexaenoic acid. Cox-2 deficient cells (HCT116) are more sensitive to docosahexaenoic acid treatment than the Cox-2 expressing cells (HCA-7, Caco-2). Cox-2 expressing cells (Caco-2 and HCA-7) are more sensitive to arachidonic acid. The growth inhibitory effect of docosahexaenoic acid in all cells is not due to cytotoxicity, but due to lipid peroxidation induced cell death. In FHC cells, however, docosahexaenoic acid also inhibited cell growth, without inducing cell death, whereas treatment with arachidonic acid resulted in increased apoptosis. Addition of Vitamin E could reverse the growth inhibitory effect of docosahexaenoic acid in HCT116 and Caco-2 but not in HCA-7 cells. In FHC cells, Vitamin E could reverse the arachidonic acid induced apoptosis.

Discussion: These results show that docosahexaenoic acid can inhibit cancer cell proliferation through induction of lipid peroxidation. Moreover, cancer cells are more sensitive to the growth inhibitory effect of docosahexaenoic acid than normal cells. Therefore, docosahexaenoic acid can be effectively used to prevent or inhibit selectively cancer cell growth in the colon, without affecting normal cells.

6. Literature

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7. Abbreviations

AA	Arachidonic acid
ACF	Aberrant crypt foci
BrdU	5´-Bromo-deoxy-Uridine
AOM	azoxymethane
Cox-1	Cyclooxygenase-1
Cox-2	Cyclooxygenase-2
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ERK	Extracellular signal regulated kinase
EtOH	Ethanol
15-S-HETE	12- Hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid
13-S-HODE	13-S-Hydroxy-9Z,11E-octadecadienoic acid
iNOS	inducible Nitric oxide synthase
LA	Linoleic acid
LDH	Lactate dehydrogenase
LNA	Linolenic acid
MAPK	Mitogen activated protein kinases
MDA	Malondihaldehyde
mPGES	microsomal Prostaglandin E Synthase
NaBT	Sodium Butyrate
Nag-1	NSAID-activated gene-1
NFκB	Nuclear factor κB
NSAIDs	Nonsteroidal anti-inflammatory drugs

PCNA	Proliferating cell nuclear antigen
PGE	Prostaglandin E
PGG	Prostaglandin G
PGH	Prostaglandin H
PKCbII	Protein kinace C bII
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferatior responsive element
PUFA	Polyunsaturated fatty acids
RXR	Retinoid X receptor
TBARS	Thiobarbituric acid reactive species
TGFb	Tumour growth factor b
VEGF	Vascular endothelial growth factor

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2. "DHA induced growth inhibition of colon cancer cell lines is mediated by lipid peroxidation"
   Soufi M and Abel J (in preparation).

3. "The cytoplasm is a new target for UVB signaling: evidence for the AhR being the central molecule"
   Fritsche E, Shaefer C, Huebenthal U, **Soufi M**, Hanenberg H, Krutmann J and Abel J (in preparation).

ABSTRACTS

1. "Detection of numerical abnormalities of chromosome 17 and c-erbB-2 amplification in benign, borderline and malignant epithelial breast lesions by dual color fluorescence in situ hybridization"
   Bai M.C., Demou A A., **Soufi M.**, Kamina S.A., Agnantis N.J.
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2. "Detection of numerical abnormalities of chromosome 17 and c-erbB-2 amplification in hyperplastic and malignant breast lesions by dual color fluorescence in situ hybridization"
   Demou A., **Soufi M.,** Zagorianakou P., Bai M., Kamina S., Agnanti N. J.
   Proceedings 7th Congress of the Hellenic Society of Pathological Anatomy, p. 442 (2000)

3. "Targeted expression of RhoD in the epidermis of transgenic mice: alterations in proliferation and adhesion"
   Maria Soufi, Angelika Giner, Maria Bai, Cecilia Bucci, Ekaterini Panopoulou, Eleni Bagli, Elizabeth Hatzi, Giuseppina Cantalupo, Vera Roberti, Theodore Fotsis, Marino Zerial & Carol Murphy
   Proceedings 53rd Meeting of Hellenic Society of Biochemistry and Molecular Biology, p. 376, vol. 48 (2001)

4. "The influence of dietary compounds like polyunsaturated fatty acids in colon cancer development: roles for PGHS2 and PPARgamma"
   Maria Soufi and Josef Abel
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5. "The influence of dietary compounds like polyunsaturated fatty acids in colon cancer development: roles for PGHS2 and PPARs"
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6. "PUFAs mediate cell growth inhibition in human colon cancer cells"
   M. Soufi and J. Abel.
   Proceedings 10th International Congress of Toxicology, Toxicology and Applied Pharmacology p. 253 Volume 197, Number 3 (2004)

7. "Role of PUFAs on the proliferation of different colon cancer cell lines"
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Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit von mir selbständig und ohne fremde Hilfe angefertigt wurde. Keine anderen als die angegebenen Quellen und Hilfsmittel wurden verwandt. Zitate wurden kenntlich gemacht.

Düsseldorf, October 2005