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Review

The relation of lipid peroxidation processes with atherogenesis: A new theory on atherogenesis

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The extremely high sensitivity of polyunsaturated fatty acids (PUFAs) to oxygen is apparently used by nature to induce stepwise appropriate cell responses. It is hypothesized that any alteration in the cell membrane structure induces influx of Ca^{2+} ions. Ca^{2+} ions are required to activate degrading enzymes, such as phospholipases and lipoxygenases (LOX) that transform PUFAs bound to membrane phospholipids to lipidhydroperoxides (LOOHs). Enzymatic reduction products of LOOHs seem to serve as ligands of proteins, which induce gene activation to initiate a physiological response. Increasing external impact on cells is connected with deactivation of LOX, liberation of the iron ion in its active center followed by cleavage of LOOH molecules to $LO^{\bullet}$ radicals. $LO^{\bullet}$ radicals induce a second set of responses leading to generation of unsaturated aldehydic phospholipids and unsaturated epoxyhydroxy acids that contribute to induction of apoptosis. Finally peroxyl radicals are generated by attack of $LO^{\bullet}$ radicals on phospholipids. The latter attack nearly all types of cell constituents: Amino- and hydroxyl groups are oxidized to carbonyl functions, sugars and proteins are cleaved, molecules containing double bonds such as unsaturated fatty acids or cholesterol suffer epoxidation. LOOH molecules and iron ions at the cell wall of an injured cell are in tight contact with phospholipids of neighboring cells and transfer to these reactive radicals. Thus, the damaging processes proceed and cause finally necrosis except the chain reaction is stopped by scavengers, such as glutathione. Consequently, PUFAs incorporated into phospholipids of the cell wall are apparently equally important for the fate of a single organism as the DNA in the nucleus for conservation of the species. This review intends to demonstrate the connection of cell alteration reactions with induction of lipid peroxidation (LPO) processes and their relation to inflammatory diseases, especially atherosclerosis and a possible involvement of food. Previously it was deduced that food rich in cholesterol and saturated fatty acids is atherogenic, while food rich in n-3 PUFAs was recognized to be protective against vascular diseases. These deductions are in contradiction to the fact that saturated fatty acids withstand oxidation while n-3 PUFAs are subjected to LPO like all other PUFAs. Considering the influence of minor food constituents a new theory about atherogenesis and the influence of n-3 PUFAs is represented that might resolve the contradictory results of feeding experiments and chemical experiences. Cholesterol-PUFA esters are minor constituents of mammalian derived food, but main components of low density lipoprotein (LDL). The PUFA part of these esters occasionally suffers oxidation by heating or storage of mammalian derived food. There are indications that these oxidized cholesterol esters are directly incorporated into lipoproteins and transferred *via* the LDL into endothelial cells where they induce damage and start the sequence of events outlined above. The deduction that consumption of n-3 PUFAs protects against vascular diseases is based on the observation that people living on a fish diet have a low incidence to be affected by vascular diseases. Fish are rich in n-3 PUFAs; thus, it was deduced that the protective properties of a fish diet are due to n-3 PUFAs. Fish, fish oils, and vegetab-

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Abbreviations: **AA**, arachidonic acid; **ALA**, linolenic acid; **DHA**, docosahexaenoic acid; **EPA**, eicosapentaenoic acid; **F-acid**, furan fatty acid; **4-HNE**, 4-hydroxynonenal; **L$^{\bullet}$**, radical derived by removal of a hydrogen from a PUFA; **LA**, linoleic acid; **LDL**, low density lipoprotein; **LO$^{\bullet}$**, radical derived by cleavage of an **LOOH** molecule; **LOO$^{\bullet}$**, peroxyl radical; **LOOH**, lipidhydroperoxide; **LOX**, lipoxygenase; **LPO**, lipid peroxidation; **OA**, oleic acid; **PL**, phospholipase; **PPAR**, peroxisome proliferator activated receptor protein; **PUFA**, polyunsaturated fatty acid

les contain besides n-3 PUFAs as minor constituents furan fatty acids (F-acids). These are radical scavengers and are incorporated after consumption of these nutrients into human phospholipids, leading to the assumption that not n-3 PUFAs, but F-acids are responsible for the beneficial efficiency of a fish diet.

Keywords: Atherosclerosis / Cholesterol / Furan fatty acids / Lipid peroxidation / n-3 fatty acids / Polyunsaturated fatty acids

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1 Introduction

Atherosclerosis is closely connected with oxidation of the low density lipoprotein (LDL) [1, 2]. LDL is a large particle with a molecular weight of about 2.5 million. Its core is constructed by approximately 170 trigyceride molecules and 1600 cholesterol ester molecules [3]. The majority of the core cholesterol molecules is esterified with polyunsaturated fatty acids (PUFAs), mainly with linoleic acid (LA) and to a lower extent with arachidonic acid (AA) (Fig. 1) [3]. PUFAs are characterized by the presence of the structural element: –CH=CH–CH_2–CH=CH–.

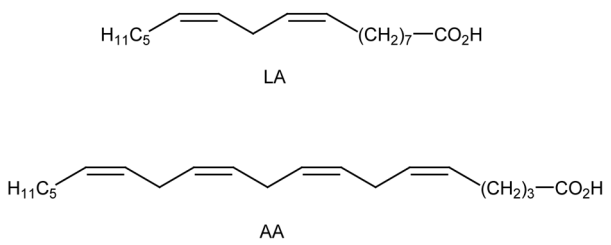

Figure 1. Main PUFAs in LDL: LA and AA.

The core of LDL is surrounded by a layer of approximately 700 phospholipid molecules and 600 molecules of free cholesterol [4]. Embedded in this layer is a protein, apolipoprotein B. This protein enables LDL recognition by endothelial cells *via* lysyl groups. Blocking of free amino groups of apolipoprotein B lysyl residues renders LDL atherogenic [4–6].

PUFAs belong to the most oxygen sensitive molecules in nature, exemplified by the observation that fats containing PUFAs become rancid just by storage [7].

2 Oxidation products of LDL

In order to prove the ability of PUFAs in LDL to undergo oxidation, we oxidized LDL artificially with copper ions [8], as originally detected by Steinberg *et al.* [1], and investigated LDL samples in the time course of oxidation. The GC of the acid fraction obtained from LDL samples derived from healthy young volunteers after saponification and derivatization reflected the content of acids in LDL: Main acids are LA and oleic acid (OA), while AA is only a minor compound, other PUFAs occur only in traces. First oxidation products were recognized about 3 h after inducing lipid peroxidation (LPO). Their amounts increased exponentially and reached maximum values after 6 h [8].

The pattern of oxidation products obtained after 6 h resembled qualitatively closely that of oxidation products obtained after an artificial induced oxidation of pure LA [9]. The pattern obtained after 3 h resembled that obtained by storage of LDL for several weeks and that of LDL derived from atherosclerotic patients [10].

These observations explain the findings of Berliner *et al.* [11] that LDL becomes atherogenic after prolonged storage time. This LDL is still accepted by the LDL receptor, indicating that the oxidation of the protein, which prevents the uptake of LDL, but induces its uptake by macrophages [4], occurs after oxidation of PUFAs.

Because the oxidation products of LDL contain toxic components like those of pure LA [9], such as 2,4-decadienal [12, 13] or 4-hydroxynonenal (4-HNE) [14], it is very likely that these toxic LPO products are introduced into cells.

3 Dramatic differences in the products derived either from enzymatic or nonenzymatic LPO processes

LPO reactions are induced either by enzymes called lipoxygenases (LOX) [15] or by radicals [16].

Mammalian LOX have in their active centers iron ions which are in the resting state present in form of Fe^{2+} ions. Exemplified with a plant-derived 13-LOX it was shown that stimulation induces the transformation of the iron ion to Fe^{3+}. In this activated state the enzyme is able to remove a hydrogen radical from the activated CH_2-group of a PUFA, the iron ion takes up an electron from the hydrogen radical forming a proton and is therefore transformed back to a Fe^{2+}-ion. The generated PUFA radical reacts with oxygen by formation of a chiral lipidhydroperoxide (LOOH) radical. The Fe^{2+} transfers in the next step one electron to the peroxide radical forming an anion which unifies with the proton formed in the proceeding step to a hydroperoxy acid

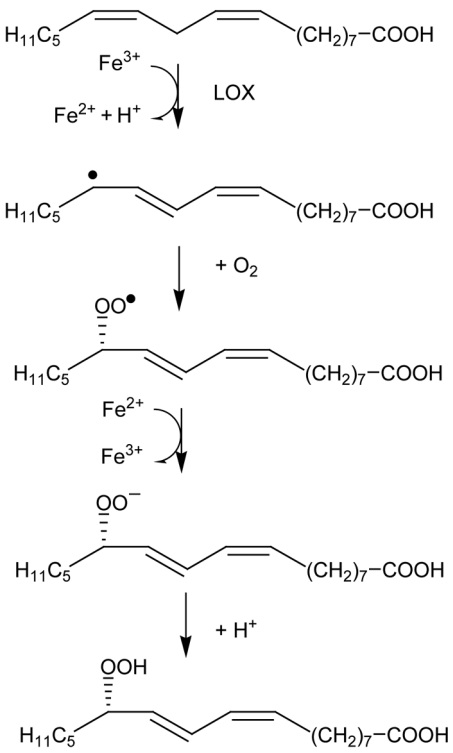

Figure 2. LPO of LA by 13-LOX.

[17]. All these reactions occur within the enzyme complex preventing the escape of radicals before the reaction is finished (Fig. 2).

Most of the generated LOOH molecules are quickly reduced by enzymes, such as hydroperoxide glutathione peroxidase [18, 19], and reducing agents, for instance glutathione, to corresponding hydroxydienoic acids. Some of these, such as 13-hydroxy-9,11-octadecadienoic acid (13-HODE) (Fig. 3), were found to serve as ligands of proteins, such as the peroxisome proliferator activated receptor proteins (PPARγ) [20], that are linked to monocyte/macrophage differentiation [21].

The second path by which LOOHs are generated requires presence of radicals. Radicals are generated, for instance, by radiation [22], but probably in most cases in tissue by a

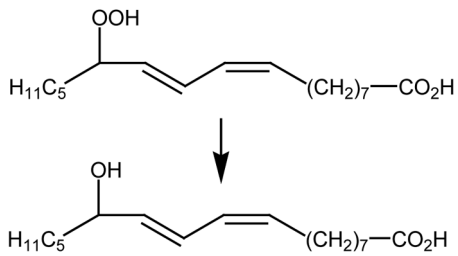

Figure 3. Generation of 13-HODE.

so-called "Fenton reaction": LOOH molecules derived from either free or esterified (*e.g.*, phospholipids, cholesterolesters) PUFAs are easily cleaved by Fe^{2+} ions to generate LO$^\bullet$ radicals [23] (Fig. 4).

LO$^\bullet$ radicals remove a hydrogen from other PUFAs (in free or esterified form). The thus generated L$^\bullet$ radicals are stabilized by mesomerism. L$^\bullet$ radicals react readily with oxygen to regio- and stereoisomeric peroxyl radicals (LOO$^\bullet$) [16] (Fig. 4) which in turn subtract from the closest available PUFA a hydrogen to form another LOOH molecule, while the generated new L$^\bullet$ radical reacts with oxygen to a new LOO$^\bullet$ radical, thus initiating a chain reaction.

In the course of this nonenzymatic LPO reactions not a single pure enantiomer is generated, as in enzymatic LPO reactions, but all possible regio- and stereoisomers. In contrast to LOO$^\bullet$s generated within LOX, LOO$^\bullet$s produced in nonenzymatic reactions are not trapped. LO$^\bullet$ and LOO$^\bullet$ radicals react not only with activated C–H bonds of free PUFAs and their esters, but also with activated C–H bonds of proteins, sugars, and nucleic acids [24]; they abstract hydrogens even from allylic C–H groups, *e.g.*, of cholesterol [25]. In addition, LOO$^\bullet$ attack single double bonds, for instance, of OA [26] or of cholesterol [25] by epoxidation. Therefore, they cause severe cell damage.

The enormous differences between enzymatic and nonenzymatic LPO reactions has apparently not been sufficiently considered in the past. Only nonenzymatic reactions are responsible for the severe tissue degradation typical for atherosclerosis and other inflammatory diseases [27].

4 LPO processes are involved in cell damage

PUFAs are constituents of the phospholipid membranes of plant and mammalian cells. When tissue is subjected to homogenation [28–30], LPO products are generated. Because LA is the main PUFA in endothelial phospholipids and LDL [8], its LPO product pattern closely resembles that of LPO of pure LA [9] and of artificial oxidized LDL [8]. The qualitative similarity in the product patterns of homogenized tissue and that of oxidized LDL raises the suspicion that PUFA oxidation products are generated in both cases according to the same mechanism. Since it is much easier to study LPO reactions after homogenation instead to investigate LDL samples, containing a bulk of different compounds, it seemed useful to investigate the events occurring by homogenation in more detail.

If tissue is boiled before homogenation, no oxidation products are obtained. Boiling destroys enzymes. This observation led to the conclusion that the generation of oxidation products is induced by enzymes that are destroyed by boil-

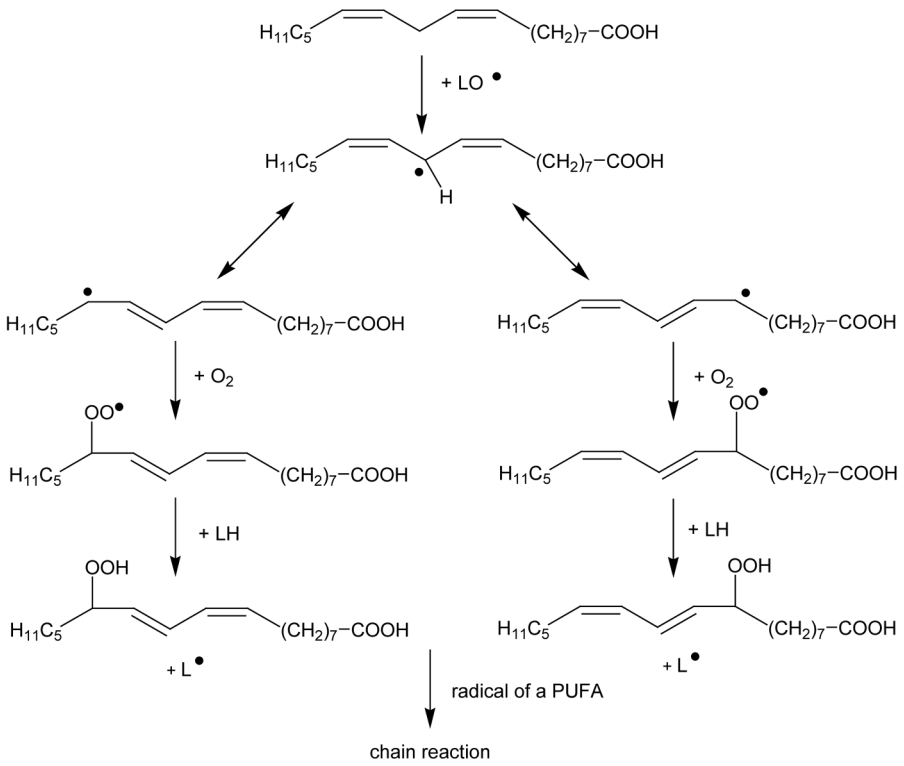

Figure 4. Nonenzymatic LPO.

ing. Nevertheless, the product pattern after homogenation resembles a nonenzymatic LPO (generation of stereo-isomeric LPO products). As a consequence, oxidation of PUFAs in tissue, and probably also in LDL, is a two-step process induced by LOX followed by a switch from enzymatic to nonenzymatic LPO [29, 30].

This switch requires, as outlined above, the presence of bivalent metal ions, such as iron ions to induce a Fenton reaction (Fig. 5). Iron ions are abundant constituents in hemoglobin and other iron-containing blood constituents. Therefore, heme is regarded to be the main source of iron ions inducing radical reactions in mammals [31]. Due to the extreme reactivity of bivalent metal ions in Fenton-type reactions iron and other bivalent metal ions are extremely well shielded in nature by complexation, like in heme or enzymes which catalyze oxidation reactions, such as NADPH oxidases, xanthine oxidases, catalases, myeloperoxidases, and also LOXs. This complexation is destroyed by homogenation of tissue, but usually not in course of physiological processes. Generation of large amounts of LPO products is observed not only by homogenation of mammalian [28, 30], but also by homogenation of plant tissue [32, 33]. This process is preceded by induction of an enzymatic LPO reaction [32] which switches apparently to a nonenzymatic one [34]. In contrast to mammalians (where the main

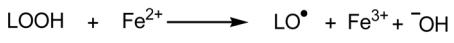

Figure 5. Generation of LO• radicals by a Fenton reaction.

source of radical-inducing iron ions is regarded to be released from hemoglobin) hemoglobin is missing in plants. Therefore, the required iron ions to induce nonenzymatic LPO processes in plants must be generated from an alternative source: Bivalent metal ions, able to induce a Fenton reaction, are constituents of the above-mentioned enzymes including LOX [17]. By homogenation of plant tissue phospholipases (PLs) are activated, which cleave phospholipids [32]. Thus, large amounts of free PUFAs are generated which are substrates of LOX, activated by cell disruption, too [32]. LOX suffer self-deactivation if the substrate-free LA–surmounts a certain limit [35]. This limit concentration is always reached by severe cell-damaging reactions, such as homogenation. The self-deactivation is connected with the destruction of the shielding protein cover of the iron ions resulting in a partly liberation of the iron ions [36]. Liberation of iron ions was also observed to occur with mammalian 15-LOX after treatment with its products, LOOHs [37].

Evidence for liberation of iron ions from LOX was obtained even in absence of tissue: Although enzymes are usually known to be highly specific, very different oxidation reactions seem to be induced by soy bean LOX in presence of LA, for instance, cholesterol is oxidized to isomeric 7-hydroxycholesterols and 6,7-epoxycholesterols [25] while furan fatty acids (F-acids, Fig. 6) are transformed with the identical reagents to corresponding dioxoenes [38]: The presence of the cosubstrate LA turned out to be essential in

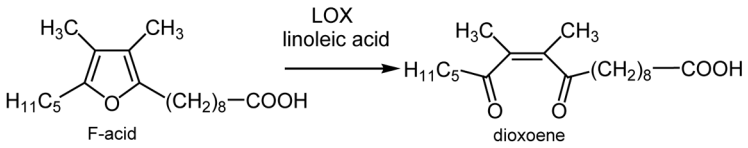

Figure 6. Generation of dioxoenes requires the presence of LOX as source of iron ions.

both reactions. This observation raises the suspicion that in fact LA does not serve as cosubstrate but is actively involved in the reactions. Indeed, involvement of radicals was proven by conversion of cholesterol into its oxidation products: hydroperoxylinoleates were recognized to be intermediates, but if cholesterol was reacted with hydroperoxylinoleate alone, the reaction proceeded only very slowly, a strong hint for contribution of radicals in the transformation reaction [25]. Radical generation from LOOHs is very fast by addition of iron ions. Thus, the fast reaction of cholesterol with LOX and LA [25] indicates a possible liberation of iron ions during the course of the reaction.

Convincing evidence for the liberation of iron ions from LOX was obtained by conversion of F-acids to corresponding dioxoenes (Fig. 6) [38].

A detailed study [39] of this reaction revealed that it was inhibited if an iron chelator (EDTA) was added. The only available source of iron ions in this experiment was LOX, consequently proving the decomposition of LOX in the course of the reaction.

The reaction of LA with LOX generates LOOH molecules (Fig. 7). The LOXs are destroyed after some cycles due to the high content of LA and this liberates the iron ions required for cleavage of LOOH molecules. The thus generated $LO^{\bullet}$ radicals then attack the F-acids to convert these into dioxoenes and serve therefore as radical scavengers [39, 40].

The above-outlined experiments raise the suspicion of a possible participation of iron ions liberated from LOX also in mammalian cell-damaging processes. This suspicion is enforced by considering that induction of radical chain reactions requires theoretically only a single iron ion. If this

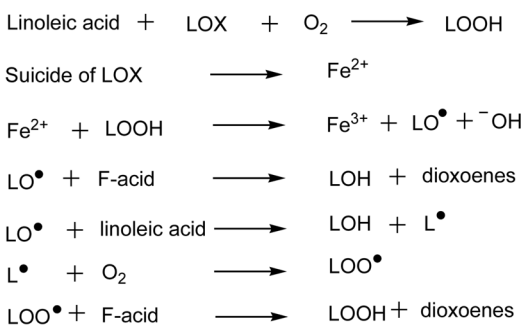

Figure 7. Sequence of events leading to generation of dioxoenes.

iron ion is produced by decomposition of LOX, it will always find LOOH molecules in its close surrounding generated in previous cycles. In contrast, after liberation of iron ions from heme these might be far away from LOOH molecules, localized at a different site, usually too far away where the iron ion is produced. As a consequence, the involvement of iron ions derived from LOX in LPO reactions seems likely.

It is tempting to speculate that also the enzymes that are known to induce atherogenesis, such as NADPH oxidase or catalase, release their bivalent metal ions, like LOX, if confronted with large amounts of their substrates and thus induce nonenzymatic LPO reactions.

5 Does the alteration of cell membranes induce LPO reactions?

LPO reactions are involved in atherosclerosis and other inflammatory diseases, raising the question which events induce LPO. The fastest method to generate plaques in animals is mechanical injury [41]. This process is comparable with homogenation, where a great number of cells are damaged at the same time. Attack of bacteria [42] or chlamydia [43–45] is combined with a more selective damage, but is equally deleterious for a single cell as homogenation for a whole organ. Likewise mechanical injury of cells cannot be avoided completely by organ transplantation [46], or by coronary artery balloon angioplasty and related procedures [47]. All these events represent risk factors of atherosclerosis. In addition, inflammation [48], observed in inflammatory diseases, has been recognized to be a risk factor of atherosclerosis, such as diabetes [49, 50], kidney diseases combined with renal failure [51], or rheumatoid arthritis [52, 53]. Typical for all these diseases is an increase in "oxidative stress" and LPO products [54, 55]. Increased amounts of LPO products were also detected in other inflammatory diseases, such as liver fibrosis [56], pancreatitis [57], psoriasis [58], or HIV [59]. A high increase in LPO products is further reported to occur by reperfusion injury [60, 64].

The most serious risk factor of atherosclerosis is aging, apparently not involved directly with inflammatory events, but known to be combined with a dramatic increase in LPO products [62–64]. The atherosclerotic risk factors hypertension [65], hyperlipidemia [66, 67], and obesity [68] are combined with LPO, too. In addition, an increase in LPO

processes is observed in smokers [69] and habitual alcohol drinkers [70].

Increases in oxidation products derived from proteins, apparently induced by preceding LPO processes (see later), were observed in individuals suffering from neurodegenerative diseases [71], such as Alzheimer's disease [72]. Peroxidation processes are even involved in muscle contractions [73].

LPO is further induced by environmental toxicants and xenobiotics, such as carbon tetrachloride [74], pesticides [75], organic compounds [76], such as antibiotics (*e.g.*, adriamycin) [77], the liver damaging aflatoxin [78, 79], or detergents [80], inorganic compounds [76], for instance, cisplatin [81], heavy metal ions, such as nickel ions [82] or ionophors [83]. On the other hand, the ionophore A 23187A has been used to stimulate tissue [84, 85] and to initiate tissue to generate prostaglandins [85] by induction of cyclooxygenases [86]. Cyclooxygenases produce peroxides like LOX.

The great number of organic and inorganic reagents that initiate generation of identical LPO products seems to have a common source. This source is visualized to be an alteration in cell structure [27, 87]: The cell structure is changed by attack of microorganisms, by inflammation, by pressure such as hypertension, by addition of organic and inorganic compounds which influence the cell structure, such as complexing agents (act on proteins) or surfactants and ionophors (act on phospholipids).

The hypothesis that any change in cell structure induces LPO reactions is corroborated by the observation of similar responses to alterations in cell structure in the plant kingdom [34]: LPO products are generated by germination [88, 89], but also in apoptosis and necrosis, depending only on the size of the exerted impact [90]. Large amounts of LPO products are produced by homogenation [32, 33] or wounding of plant tissue; lower amounts are formed during stress situations, induced by salt [91, 92], chill [93, 94], heat [94], or heavy metal ions [95]. The generation of eicosanoids in mammals according to a mild stress is paralleled in plants by production of jasmonic acid [96].

Probably LPO reactions were invented by nature to defend against enemies: A microorganism attacks a cell first at its surface, the cell membrane. This causes a readjustment of cell constituents, such as phospholipids in the attacked cell membrane. These rearrangements of constituents certainly influence the channels passing the cell membrane. Change of cell channels induces influx of Ca^{2+}ions into the cell, observed, for instance, after treatment of cells with the ionophore A23187 [97] or substance P released after "stimulation" from sensory nerves which induces expression of adhesion molecules ICAM-1 and VCAM-1 [98] involved in atherogenesis. The deduction that changes in the cell membrane are connected with influx of Ca^{2} ions into cells is further supported by an investigation of Ca^{2+} homeostasis after a temperature stress on grape mesophyll cells which showed a shift of Ca^{2+} ions [94].

Ca^{2+} ions in turn are required to activate degrading enzymes, such as PLs [99, 100], LOXs, and myeloperoxidase [101]. Activation of PL is indicated, for instance, by the observation that the amount of lysophospholipids generated by enzymatic cleavage of phospholipids is strongly increased in LDL of atherosclerotic patients [3]. The generated free PUFAs are substrates of most LOX which need free PUFAs as substrates, not available in resting healthy cells, but are enriched in LDL of atheroscleroic patients, together with lysophospholipids [3, 4]. The author of this paper suspects that iron ions are liberated from LOX in tissue, like shown with 13-LOX in *in vitro* experiments, if the amount of free LOX surmounts a certain limit in tissue. These iron ions should be generated close to the formed LOOH molecules and therefore induce a Fenton reaction producing $LO^{\bullet}$ radicals.

The $LO^{\bullet}$ radicals abstract a hydrogen from the closest available activated C–H bond. In most cases this is a hydrogen of a PUFA in the cell membrane. Usually a preferential attack on AA is assumed, based on model experiments in which equal amounts of LA and AA were reacted with iron ions [102]. These experiments revealed a preferential oxidation of AA. In contrast, in biological samples the situation is different since only a few iron ions are available. The subsequently generated few $LO^{\bullet}$ radicals cannot select a special PUFA like in the experiment above where AA and LA were available in excess, enabling a selection, but attack the closest located PUFA and this is statistically an LA containing phospholipid.

Because $LO^{\bullet}$ radicals are produced at the surface of cells they may attack PUFAs of adjacent cells, too. Thus the reaction may proceed from cell to cell like an infection.

Generated $LOO^{\bullet}$s do not only attack PUFAs bound to phospholipids but nearly all other biological molecules, causing severe cell damage as outlined below. These damaging reactions are stopped by radical scavengers such as glutathione [103] or other antioxidants. Due to the increased stability of glutathione radicals, their lifetime seems sufficient to combine by dimerization. Thus, the proceeding of the radical chain reaction is interrupted (Fig. 8).

6 Study of LPO reactions and their products by model reactions

Radical reactions generate a tremendous variety of products which are buried under the unchanged constituents of healthy tissue. Therefore, the elucidation of their structures

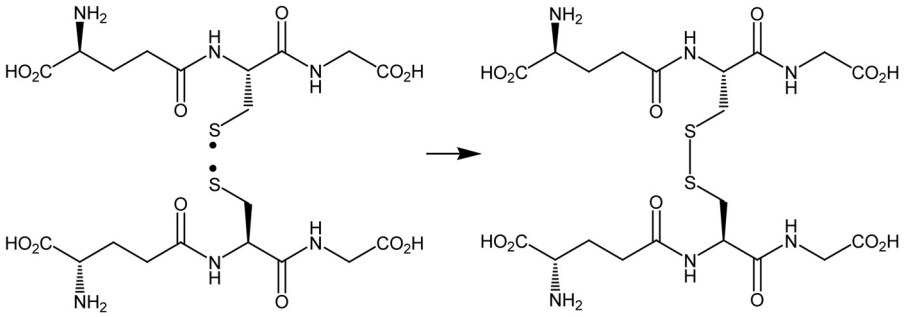

Figure 8. Glutathione stops radical reactions by dimerization.

and physiological properties seems to be an unresolvable problem. Nevertheless, there is a way to overcome these difficulties: Considering that all LPO reactions are determined by the presence of the structural element –CH=CH–CH_2–CH=CH– it seems reasonable to investigate first which products are generated by LPO of the simplest PUFA, LA.

All PUFAs are decomposed according to identical mechanisms, although the reaction products are different in most cases. Therefore, the obtained informations about degradation processes and products of LA can be applied to deduce the structures of the degradation products of higher unsaturated PUFAs, saving the efforts to separate their much more complicated mixtures in comparison to the oxidation products of LA, due to the increase of isomeric products. Moreover, the knowledge about degradation paths of LA LPO allows to deduce the structures of more complex products derived by degradation of phospholipids esterified with PUFAs or cholesterol-PUFA esters.

Many primarily generated LPO products are highly reactive. They react with other biological compounds in a natural mixture. In order to detect these minor products in the biological samples it is necessary to react a pure LPO product, such as 4-HNE, with a single compound present in LDL, such as lysine, to elucidate which products are generated. Similar reactions are expected to occur with 4-HNE and apo B. The knowledge of molecular weights of the expected products enables finally to search for these products in biological mixtures with the highly sensitive method LC/MS and to confirm their presence by comparison with synthetic products. These may serve finally for the elucidation of their physiological properties.

7 Products of LPO reactions

Already artificial oxidation of LA produces a great number of products [9, 104, 105] due to secondary and tertiary decomposition reactions of the highly reactive primarily generated compounds. Nevertheless, these products can be separated after appropriate derivatization by GC. The investigation of these products revealed that the majority of nonenzymatic LPO processes is started by decomposition of LOOHs. Generated $LO^{\bullet}$ radicals decompose either by cleavage of an adjacent bond to 2,3-unsaturated aldehydes [9, 106, 107] (Fig. 9) or by formation of unsaturated epoxy-hydroxy fatty acids [9, 107] (Fig. 10), or generate $LOO^{\bullet}$s (Fig. 4) that are responsible for degradation of nearly all types of biological molecules.

The degradation paths of LOOHs derived from LA are paralleled by similar reactions of phospholipids containing LA. Synthetic phospholipids with LA were subjected to LPO to study physiological properties. These studies revealed that aldehydic degradation products of phospholipids serve as ligands to proteins. The complexes between the phospholipid ligands and the proteins activate the genes that in turn activate the macrophage scavenger receptor CD36 [108, 109].

Moreover, many primarily generated LPO products, such as 2,3-unsaturated aldehydes (Fig. 9) and unsaturated epoxy-hydroxy acids (Fig. 10), are highly reactive and are therefore prone to add nucleophilis, such as compounds with basic amino groups, for instance, present in form of lysyl residues of proteins. At present, a study of these reactions by model compounds is still missing.

The deductions about the ability of LPO products to serve as ligands of proteins which induce gene expression might explain why cells incorporate PUFAs in their outer sphere: Phospholipids esterified with PUFAs serve apparently as stores of signaling molecules that are generated in response to external events to induce an appropriate reaction of the cell and probably not only to give the cell fluidity, as usually

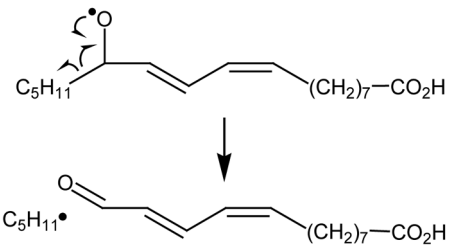

Figure 9. Decomposition of $LO^{\bullet}$ radicals to aldehydic compounds.

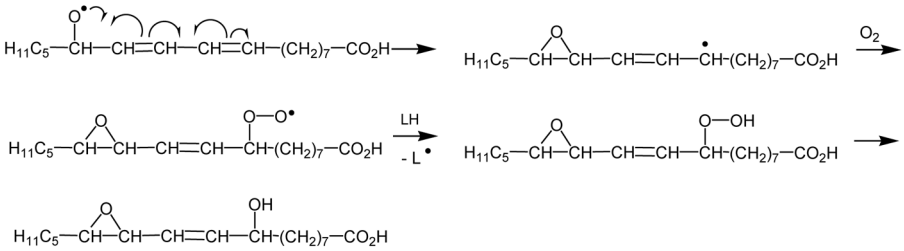

Figure 10. Decomposition of LO• radicals to unsaturated epoxyhydroxy acids.

assumed [110]. In dependence from the extent of an external impact a variety of different LPO products are generated that serve apparently as ligands to proteins which in turn activate genes. These respond in dependence on the size of the impact by generation of signals that either cause cell reinforcement or apoptosis or necrosis [111, 112]. For instance, a very early step after an injury of cells is the activation of PL A_2. This enzyme cleaves a phospholipid in position 2 of the glycerol backbone. The resulting lysophospholipids are signals which induce cell reinforcement [113].

If the size of the impact increases and a nonenzymatic LPO is started by generation of radicals, aldehydic derivatives of phospholipids are produced [108, 109], which induce the above-mentioned conversion of monocytes to macrophages, necessary to remove toxic LPO products or to avoid the next step of LPO-induced reactions, apoptosis. When the production of LPO products increases more and more degrading enzymes, such as esterases and proteases are activated to such an extent that the nonprogrammed cell death is induced [112].

While the physiological properties of aldehydic products have been investigated to some extent, as outlined above, those of unsaturated epoxyhydroxy acids, surmounting aldehydes considerably, have been studied only in rare cases, apparently because their extreme high reactivity, exceeding those of aldehydic compounds, has prevented a direct investigation, so far. Some of the secondary degradation products of unsaturated epoxyhydroxy acids, such as unsaturated epoxyoxo acids, develop unexpected physiological properties, they stimulate adrenal steroid genesis [114].

Epoxides are generated not only by an intramolecular attack of the radical site of LO• particles to the adjacent double bond (see Fig. 10) but also by reaction of LOO•s with double bonds of neighbouring molecules [25]: As mentioned above LOO•s epoxidize all compounds containing double bonds, not only PUFAs forming leukotoxins (Fig. 11) [115], but also compounds containing an isolated double bond like OA [26] or cholesterol [25]. Cholesterol epoxidation is achieved probably by an intramolecular reaction [116]: When the LA part of cholesterol linoleate is oxidized by a nonenzymatic LPO reaction a peroxylradical is generated. The closest available double bond for this intermediate is the double bond of cholesterol resulting in production of a cholesterolepoxide (Fig. 12) [116].

Cholesterol triols, generated by hydrolysis of cholesterol epoxides, are common constituents of atherosclerotic plaques [117]. Plaques are also rich in 7-oxocholesterol. This compound is generated by intramolecular attack of the LOO• at the allylically activated hydrogens of cholesterol in position 7 [25].

In addition peroxylradicals attack alcohols by removing the hydrogen from the activated carbon carrying the hydroxylic group, for instance, of a sugar or a HODE molecule, thus finally generating a superoxide anion and an oxo derivative of the starting molecule [22] (Fig. 13).

Previously "leakage" of mitochondria was assumed to be a prominent source of superoxide. The structural similarities of lysophospholipids and isopropanol suggest that superoxide might be produced as a consequence of a preceding LPO reaction: Mitochondria are separated from their surroundings by a layer of phospholipids containing PUFAs. Release of superoxide requires stimulation–addition of reagents which alter the structure of the phospholipid layer (see above), typical prerequisites to induce LPO reactions.

The above-outlined reaction of sugar with LOO•s accompanied by generation of superoxide seems to be also the essential step of Maillard reaction. The Maillard products are apparently formed by reaction of dicarbonyl compounds or their equivalent Schiff bases with proteins [49]. The finally produced advanced glycation end products (AGEs) are enriched in diabetic patients [49].

Peroxylradicals are able to oxidize free amino groups in a similar reaction as pointed out above for secondary alcohols. Proteins containing lysyl residues are therefore oxidized at the basic amino group to aminoadipic semialdehydes [118] which undergo further oxidation to aminoadipic acid derivatives. These reactions contribute probably more to the "blocking" of the lysyl residues of apolipopro-

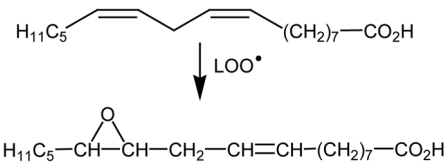

Figure 11. Generation of 12,13-epoxy-9-octadecenoic acid, a leukotoxin, by epoxidation of LA with an LOO•.

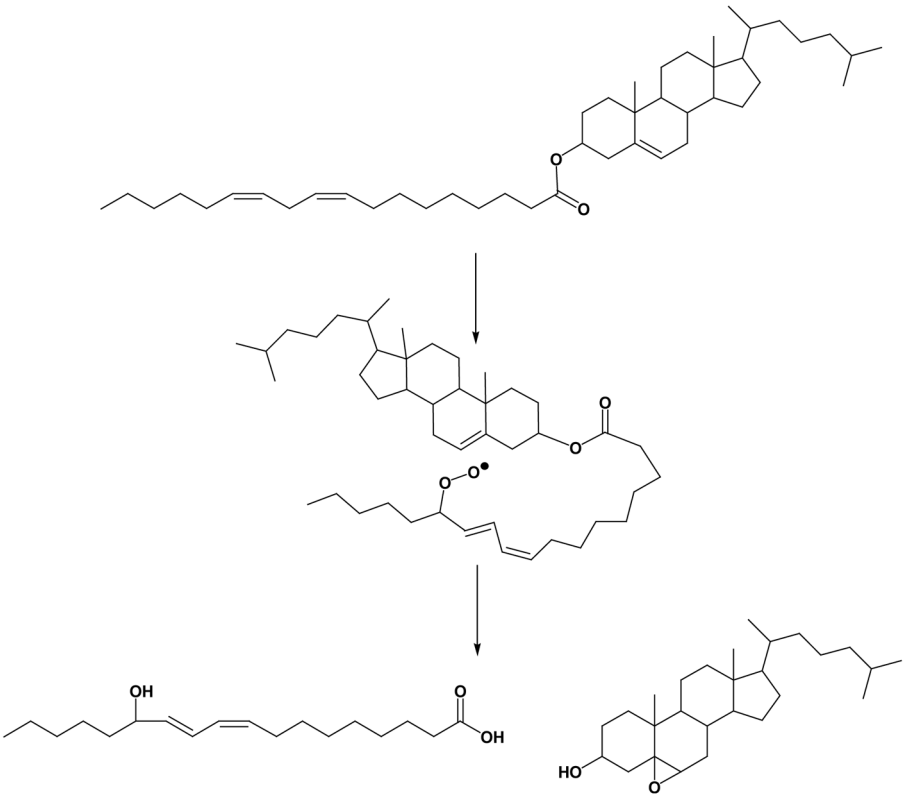

Figure 12. Generation of cholesterolepoxide in an intramolecular reaction.

tein B than the originally postulated condensation with 4-hydroxy-2,3-nonanal to Schiff bases [5]. In fact, free amino groups react preferentially by a 1,4-Michael addition with 2,3-unsaturated aldehydes [119].

Moreover, methionine-containing proteins suffer with LOO•s oxidation at the sulfur atom [120].

8 Consumption of mammalian derived food induces cell damage from inside

The above-outlined hypothesis that any change in cell membrane structure induces LPO and thus initiates peroxidation of LDL seems to reduce the many risk factors of atherosclerosis to only one event: cell membrane alteration. Nevertheless, one prominent group of atherogenic risk factors seems to be not related to the alteration of cell membranes, the induction of atherosclerosis by food intake: It

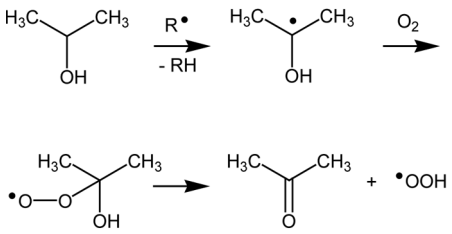

Figure 13. Generation of superoxide.

has been deduced not only from many animal feeding experiments, but also by cohort studies of man that atherosclerosis is paralleled by an increase in the cholesterol level in the LDLs. Moreover, food rich in saturated fatty acids was found to be a risk factor [121, 122], while consumption of fish rich in n-3 PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA, Fig. 14), was recognized to be antiatherogenic [123, 124].

As outlined above and confirmed experimentally [8] atherosclerosis is combined with the oxidation of PUFAs. Saturated fatty acids withstand oxidation; all compounds containing the structural element –CH=CH–CH_2–CH=CH– characteristic of PUFAs undergo LPO reactions. Thus, the deductions derived from feeding experiments contradict chemical experiences.

These puzzling differences between the results of feeding experiments and chemistry become less mysterious by considering that not only main fatty acid in food may contribute to deleterious or positive effects but also minor ones exemplified by the dominant role of the minor food component cholesterol in plaque formation.

Considering this possibility it seems important to remember that mainly esterified cholesterol and not free cholesterol is transferred *via* LDL to endiothelial cells [4]. Cells use readily biosynthetic precursor molecules for incorporation into lipoproteins, such as cholesterol and cholesterol esters. Ideal precursor molecules for incorporation into lipopro-

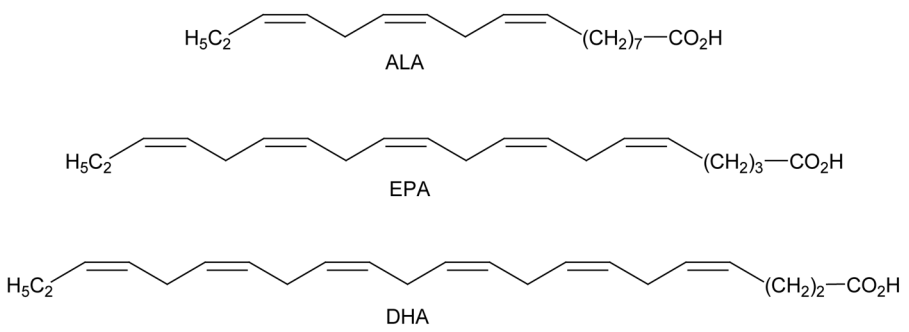

Figure 14. n-3 PUFAs: ALA, EPA and DHA.

teins are therefore cholesterol esters, present besides free cholesterol in mammalian, but not in plant-derived food. Cholesterol itself is less suited for incorporation, because this would require an energy-consuming esterification step. Even more energy would be required to use mammalian derived cholesterol esters, if they are saponified and reesterificated for incorporation. Although fats (glycerol esters) are readily saponified in the intestine, cholesterol esters derived from nutritional sources withstand saponification [125]. Therefore, we have to assume that cholesterol esters are directly and readily incorporated into lipoproteins. Because all compounds containing PUFAs suffer LPO by storage or heating [126], mammalian derived food always contains cholesterol-PUFA esters, peroxidized in the acidic part and further decomposition products thereof. As demonstrated by Staprans *et al.* [127] 5,6-epoxycholesterol is incorporated into LDL. Therefore, we have to assume that oxidized cholesterol esters which contain as minor constituents 5,6-epoxycholesterol esters, present in nutrition, are transferred to LDL as easy as unchanged cholesterol esters. This assumption is corroborated by considering that cholesterol esters with a great number of different acidic residues are incorporated into LDL, like cholesterol esterified to stearic acid, palmitinic acid, OA as well as to LA and AA. At least some of the oxidized cholesterol-PUFA esters are toxic [12, 14]. If the LDL containing toxic products are introduced into cells, the toxic oxidation products are liberated and should induce cell damage from inside of cells, as indicated by fatty streaks which are generated underneath a healthy cell surface [128]. Thus, it seems that merely one factor induces atherosclerosis, an alteration of cell membranes caused by either external or internal events leading to cell destruction. The induction of atherosclerosis by injurious processes has been hypothesized already three decades ago by Ross [48, 129].

9 Are pro- and antiatherogenic effects of food related merely to main constituents?

The above-outlined deduction indicate that previously not considered constituents of nutrition, peroxidized PUFA-cholesterol esters, are involved in atherogenesis, raising the question if it might be sufficient to consider only the presence of main products in food by deducing conclusions about pro- or antiatherogenic properties of food. Cholesterol-PUFA esters are typical constituents of mammalian fats, although they represent only a minor fraction of these fats. PUFA cholesterol esters are nearly absent in plants which contain plant sterols instead. In spite of the close structural relationship between cholesterol and plant sterols, plant sterols and their esters are not accepted for incorporation into lipoproteins, indicating that the side chain in position 17 of the sterol part is very important for incorporation into LDL in contrast to the acid part of cholesterol esters.

The main fatty acid in animal derived fats, butter, and lard, often used in animal feeding experiments, is palmitinic acid. Because the most abundant constituent in butter or lard and other mammalian derived food were saturated fatty acids, it was concluded that fats rich in saturated fatty acids are atherogenic, although feeding experiments with plant-derived fats rich in saturated fatty acids remained without any atherogenic effect [130]. Butter and other mammalian derived fats always contain PUFA-cholesterol esters, prone to LPO. Their presence in mammalian derived food may be an alternative way to explain the plaque generation after a feeding time for several weeks or months. This would be in agreement with the fact that saturated fatty acids withstand oxidation.

The deductions about the protective properties of n-3 PUFAs against cardiovascular diseases were based on the findings that people living mainly on fish are less subjected to atherosclerosis [131]. Fish and fish oils are relatively rich in n-3 fatty acids. Therefore, it was deduced that n-3 fatty acids are protective [132], although the mechanism of their action remained obscure. Many efforts were made to reveal the reasons for their positive effects [124]. Besides an increase in fluidity [110] it is assumed that these positive effects are caused by the alteration of the n-3/n-6 fatty acids ratio in favor of n-3 fatty acids [133]: Increased EPA/AA ratios decreases prostaglandin formation by cyclooxygenases and reduces therefore the probability to induce inflammatory processes. This reasoning does not consider

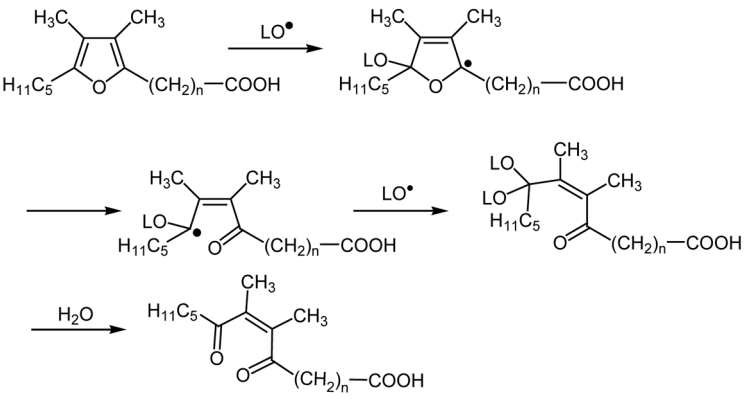

Figure 15. Scavenging properties of F-acids.

that damaging processes in vascular diseases are induced, as pointed out above, by nonenzymatic LPO reactions, while the activation of cyclooxygenase is still an enzymatic physiological process. The generation of radicals requires, besides LOOH molecules, iron ions and thus the switch from enzymatic to nonenzymatic LPO; consequently, the fish diet should reduce the generation of radicals. This is not achieved by changing the n-3/n-6 fatty acid ratio or a decrease in prostaglandin production. Moreover, n-3 fatty acids are equally well oxidized as n-6 fatty acids demonstrated experimentally [124]; feeding a fish oil diet even increased the oxidizability of LDL [134]. The shift in the PUFA ratio can only slightly influence the generation of radicals. Moreover, plant-derived oils, often richer than fish in n-3 PUFAs linolenic acid (ALA) had no or even an reverse effect [135–137]. These and other findings raised the question if the positive effects of fish consumption might be caused by presence of unknown constituents [134].

Fish contain, besides n-3 PUFAs, a group of unusual fatty acids, so-called F-acids, detected about 30 years ago by Schlenk *et al.* [138 (Fig. 5)]. F-acids occur in fish like other fatty acids in esterified form [139]. Due to their fat character they are not removed by preparation of fish oil and are therefore, for instance, constituents of cod liver oil [140]. F-acids are metabolized in the body to so-called urofuran fatty acids [141]. These were detected enriched to three- to six-fold amounts after consumption of fish oils [142], also indicating that fish oils are enriched in F-acids.

F-acids closely resemble n-3 PUFAs in their physical properties. It is rather difficult to separate F-acids from n-3 PUFAs. Therefore, F-acids accumulate by isolation of PUFAs in the same fractions as n-3 PUFAs [138]. F-acids are not generated by fish, but are derived from algae [143], other marine products, and plants [144]. Labeling experiments proved that algae are able to transform LA resp. 9,12-hexadecadienoic acid to the corresponding F-acids [143].

F-acids are potent scavengers of LOO•s [145]. They react with radicals by ring opening forming finally rather stable radicals. The lifetime of these radicals is apparently sufficient for scavenging a second LO• radical, producing finally a dioxoene (Fig. 15). Therefore, we assume that F-acids are in fact the protecting compounds in fish and not n-3 PUFAs.

If fish are consumed, F-acid is incorporated into phospholipids of human blood [146], placing the protecting compounds in close vicinity to the radical producing sites of cells enhancing their protective abilities.

The enhancement of protective properties by incorporation of scavenger molecules into tissue [146] close to the site where radicals are generated indicates that scavenging compounds have to be divided into two classes: those that are incorporated into tissue, such as F-acids, and those which are transformed into the body by nutrition, such as phenolic compounds. The latter are not bound to endothelial tissue and therefore in emergency cases not close to the sites where radicals are generated and are therefore probably less effective. As a consequence, considering all these facts it was hypothesized [116] that F-acids are the effective compounds in fish and fish oil and not the n-3 fatty acids.

10 Nutritional derived PUFAs suffer oxidation at the cell wall

The above-outlined deductions seem to indicate that consumption of plant-derived PUFAs might be less deleterious than consumption of mammalian derived fats because these always contain oxidized cholesterol-PUFA esters. In order to clarify this question volunteers were feeded for only a 24 h period with a fat-free diet consisting only of pure rice and water.

In these experiments we used 9-hydroxy-10,12-octadecadienoic acids (9-HODEs) and 13-HODEs as marker compounds: LA represents the most abundant PUFA in LDL. This acid can only be transformed to two regio-isomeric hydroperoxides which are readily enzymatically reduced to the corresponding HODEs. In contrast higher unsaturated

PUFAs generate an increasing number of LOOHs dependent on the number of double bonds. Therefore, HODEs represent the most abundant LPO products in LDL [147], facilitating the detection of LPO processes compared to other LPO marker compounds, such as isoprostanes [148, 149], surmounting these for several orders of magnitude.

The collected LDL samples derived from blood of volunteers after a 1 day diet, consisting only of rice and water, were subjected to saponification and derivatization and finally analyzed by GC/MS [150]. Then, the volunteers consumed a single meal consisting of rice and an overload either of sunflower oil (rich in LA) or of olive oil (rich in OA). Blood samples were withdrawn in time intervals. Samples removed before the feeding experiment showed only low levels of HODEs. Twelve to 16 h after consumption of the fat load the HODE content in LDL had increased in young volunteers for approximately one half, in old individuals for about several hundred percent. Then, the HODE levels dropped slowly to the original values.

A diet rich in OA showed similar increases and decreases of LA oxidation products in the time course of the experiment, except that the overall increase in oxidation products was reduced to about 50% reflecting the lower amount of LA in an OA-rich fat. During all the diet experiments the cholesterol levels remained unchanged.

These experiments confirm the results of previous investigations which showed that a diet rich in OA transformed the acid content in LDL *versus* OA and reduced its ability to undergo oxidation considerably [151].

Moreover, the feeding experiment demonstrates that diets rich in OA cannot prevent LDL oxidation, because all fats contain PUFAs.

The most important conclusion drawn from these experiments is the finding that already one meal rich in fat increases the level of peroxidation products in LDL dramatically while the level of cholesterol remains constant. This is a strong hint that an increase of cholesterol is only a side effect of a long-lasting damaging process caused by LPO. This deduction is further corroborated by considering that two thirds of the cholesterol are produced in the body while only one-third is derived from nutrition [4]. Usually the body does not respond to large variations of the amount of a nutritional compound in the diet; it seems therefore not justified to assume that the relative little change in the amount of cholesterol in food should be directly involved in atherogenesis.

The extreme high differences in HODE values between young and old individuals confirms the fact that the risk to become atherosclerotic increases dramatically with increasing age. It is also remarkable that the maximum HODE values were reached only about 12–16 h after the intake of the fat-rich meal, indicating that the LDL production is a long-lasting process. It is assumed that the LDL oxidation occurs at the cell walls containing the reagents which induce LPO, iron ions, and LOOHs, sufficient to peroxidize bypassing LDL molecules. Apparently the endothelium of old individuals contains much higher amounts of damaged cells compared to young people.

Therefore, it seems that atheroscleosis is a two-step process: at first oxidized cholesterol esters are introduced into cells and cause their cell damage. This process might last for decades. When damaged cells cannot be replaced sufficiently fast, the second step in atherosclerosis becomes more and more pronounced, the oxidation of the phospholipid layer of LDL at the cell walls of injured cells by a transfer of the radicals produced at the injured sites. A diet rich in OA may delete the onset of atherosclerosis but is not able to prevent it. Otherwise progression of atherosclerosis is delayed probably efficiently by incorporation of radical-scavenging compounds, such as F-acids, in tissue samples. The increase of oxidation products with increasing age is a strong hint that atherosclerosis cannot be avoided, only delayed.

11 Conclusions

1. It is hypothesized that any change in the cell membrane structure, caused by external or internal events, is combined with initiation of LPO processes.
2. It seems that a great number of the generated LPO products serve as ligands of proteins which in turn activate gene expression. Thus gene expression is apparently a consequence of LPO. LPO products seem to link events outside and inside the cell with an appropriate cell response.
3. All inflammatory diseases are connected with LPO processes.
4. Atherosclerosis seems to be not a multifactorial disease, but be induced by a sequence of enzymatic and nonenzymatic peroxidation processes. LPO precedes all other reactions, including alteration of proteins.
5. Mammalian cholesterol-PUFA ester suffer peroxidation in the PUFA part by storage and heating. These oxidized cholesterol-PUFA esters are apparently incorporated into lipoproteins and transferred by LDL into cells where they induce cell destruction.
6. Cell destruction generates LOOHs and iron ions. These get in contact with neighbouring cells and transfer to their membrane phospholipids the LPO reaction. Thus the reaction spreads like an infection from cell to cell.

7. Feeding experiments indicate that an LDL oxidation is a direct consequence of PUFA oxidation caused by contact with injured endothelial cells. Increase of PUFA oxidation occurs within 12–16 h. In contrast the cholesterol level remains unchanged, indicating that the increase of cholesterol levels in atherosclerosis is a secondary effect.
8. Saturated fatty acids are stable against oxidation. Therefore, they cannot be atherogenic as assumed previously. n-3 PUFAs suffer oxidation like all PUFAs, they seem therefore not to protect against attack of radicals in cardiovascular diseases.
9. The antiatherogenic action of a fish diet seems to be caused by presence of F-acids which are radical scavengers.

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