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Oxidative stress: Plasma lipoprotein oxidation and the protective role of micronutrients²

1. Introductory remarks: Plasma lipoproteins and cardiovascular diseases

It is now generally accepted that cardiovascular diseases are closely associated with dysregulation of the metabolism of plasma lipoproteins. Older work, the Framingham study, has revealed a statistically significant correlation between the total cholesterol level in blood plasma and the incidence of myocardial infarction [Dawber et al., 1957]. It had been speculated earlier that the dietary intake of cholesterol would be a major risk factor for cardiovascular diseases through favoring atherosclerosis of arterial vessels in heart, brain and extremities. This assumption proved to be an oversimplification. Indeed, the nutrition regimen is crucial for modulation of the risk for atherosclerosis and, in turn, for cardiovascular diseases. Contrary to the previous expectation, however, nutritional factors other than food cholesterol appear to facilitate the development of atherosclerotic lesions. Thus, the composition of fatty acids in food is more important than the content of cholesterol. Intake of saturated fatty acids promotes atherosclerosis, whereas intake of *cis*-unsaturated fatty acids, in particular of polyunsaturated fatty acids, attenuates it. Another important factor is represented by the “micronutrients”, which encompass the antioxidant vitamins C and E, carotenoids, essential trace elements such as selenium, as well as dietary polyphenols. Extensive research has been conducted including cumbersome clinical trials to elucidate the effects and modes of actions of these nutritional ingredients on lipoprotein metabolism and related processes.

Cholesterol is distributed in plasma between various lipoproteins. Two major plasma lipoprotein classes are low-density lipoprotein (LDL) and high-density lipoproteins (HDL species). More detailed investigation of the correlation between plasma cholesterol and risk for cardiovascular diseases revealed opposite roles of LDL and HDL. A high LDL/HDL ratio coincides with a high risk for atherosclerosis, whereas a shift in favor of HDL lowers this risk. This observation led to the practice that e.g. pharmacists designate LDL cholesterol as “bad cholesterol” and HDL cholesterol as “good cholesterol”. Although the estimation of the LDL/HDL ratio proved to be a useful parameter to evaluate the lipoprotein status and, as a consequence, of the health state of an individual, this view is again an oversimplification. Thus, LDL *per se* is not so “bad” as at first sight believed. LDL and HDL exert essential but opposite roles in the cholesterol metabolism of the organism. After generation from its precursor VLDL (very low-density lipoprotein), LDL serves for the transport of cholesterol from the liver to the peripheral organs and tissues via blood stream. These tissues utilize LDL-cholesterol for the biogenesis of plasma membranes, which need cholesterol for a sufficiently high mechanical rigidity. Peripheral tissues must be protected, however, from overloading with excessive water-insoluble cholesterol that would lead to damage to cells. This protection

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is brought about by two kinds of fine-tuned mechanisms. For one, LDL is taken up by the cell via the well-regulated LDL receptor, thus limiting cholesterol uptake. Second, excessive cholesterol is taken up by HDL, which transports cholesterol back to the liver, the central organ of lipoprotein and cholesterol metabolism. By this way, LDL receptor and HDL constitute an anti-atherogenic potential of the organism.

2. Oxidative modification of plasma lipoproteins and its putative role in atherogenesis

What is reason for the development of atherosclerosis? This issue and the mechanistic implications are matter of debate for several decades. Several hypotheses have been proposed, but none of them has been actually proven. The “oxidation hypothesis” has been developed by Daniel Steinberg and his associates around 1989 and is supported by a number of experimental observations [Steinberg, 2009 and refs. therein]. The approach of Steinberg and associates opened a new sight on the modulation of lipoprotein metabolism from normal to pro-atherogenic. The central rationale of this hypothesis is that oxidative modification of plasma lipoproteins changes their roles in affecting endothelial function and modulating atherogenesis (**Fig. 1**). While oxidative modification renders LDL pro-atherogenic, oxidative modification of HDL abolishes its anti-atherogenic property.

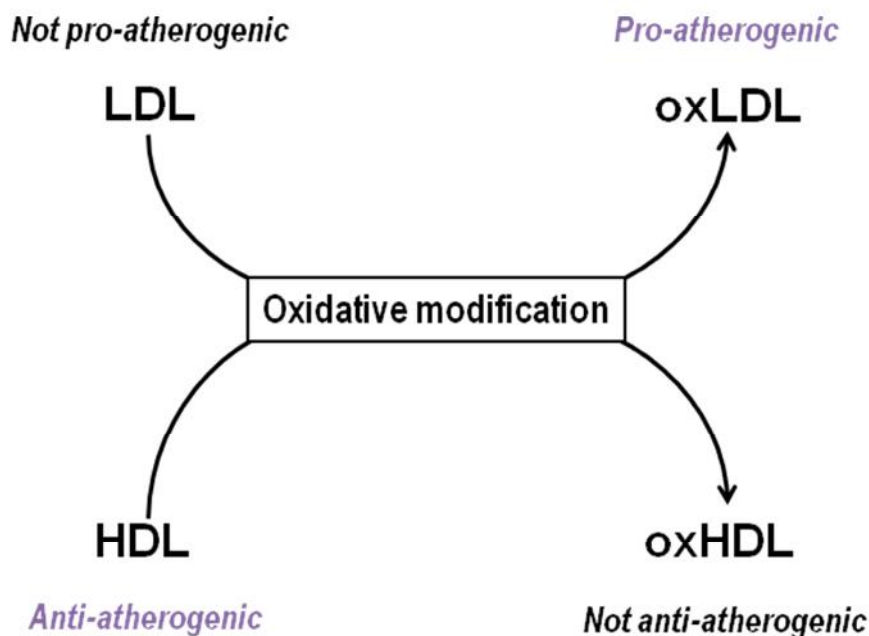


Fig. 1. Oxidative modification changes the roles of plasma lipoproteins.

2.1. Modulation of the biological role of plasma lipoproteins by oxidative modification

The crucial role of oxidatively modified LDL (oxLDL) in the development of cardiovascular diseases has emerged from a number of clinical studies, in which the plasma level of oxLDL was measured and shown to correlate with the incidence of coronary heart diseases [Holvoet et al, 1998; Wallenfeldt et al, 2004; Meisinger et al, 2005; Johnston et al, 2006]. Recently, Van der Zwan and associates have clearly shown that plasma oxLDL related to LDL particle number is a better parameter than LDL cholesterol for risk assessment of cardiovascular diseases. In a cohort study with 624 men and women (50 – 87 years), these authors found that (i) the oxLDL level strongly correlates with that of apoB-100 and (ii) the oxLDL/apoB-100 ratio is negatively related to vascular endothelial function as measured by brachial flow-mediated dilation (FMD), whereas oxLDL or LDL cholesterol are not [Van der Zwan et al, 2009].

ApoB-100 is the single protein moiety of LDL, thus the plasma level of which being a more reliable parameter for the number of LDL particles than LDL cholesterol. Cardiologists apply measurement of FMD as a noninvasive method for assessment of arterial function *in vivo*; lowering of this parameter indicates elevated risk for cardiovascular diseases.

Assay and functional characterization of oxLDL are hampered by a number of difficulties: (i) oxLDL represents a heterogeneous mixture of chemically modified LDL particles of different composition. (ii) It contains a large number of compounds and structural elements belonging to various chemical classes. (iii) The composition varies dependent on degree of modification. (iv) The composition varies dependent on mode of formation. In commercial test kits for oxLDL mostly malondialdehyde-modified LDL is used as standard, which is not representative, however, for all forms of oxLDL that are met in human plasma and atherosclerotic lesions. **Table 1** lists a selection of constituents, which are known to occur in oxLDL.

Constituents of oxLDL (selection)

- Hydro(per)oxy lipids; conjugated dienes
 - PAF-like cleavage products of phospholipids
 - Oxysterols
 - Lysophospholipids
 - Carbonylated and nitrated apoB-100
 - 4-Hydroxynonenal (HNE) and other aldehydes
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Table 1: Compounds and structural elements found in natural oxLDL (e.g. in atherosclerotic lesions or plasma)

The consequences of LDL oxidation are compiled in **Table 2**. Major event is a switch from the LDL receptor pathway to scavenger receptor pathways of LDL metabolism, which initiate, in turn, a number of pro-atherogenic processes.

If LDL oxidation is actually an early key process in atherogenesis, then the issue arises as to the processes leading to oxLDL. Three kinds of such processes may be considered: (i) reactive oxygen and nitrogen species (superoxide, H₂O₂, nitrogen dioxide, peroxynitrite and others) evoked by systemic oxidative stress (such as in smoking, diabetes and inflammatory diseases as well as upon intake of hydroperoxide-rich high-fat diet as given for instance by “fast food”), (ii) Cell-mediated LDL oxidation, which has been demonstrated for monocytes/macrophages, polymorphonuclear neutrophil leukocytes, vascular endothelial cells, platelets and other cells), (iii) actions of oxidant enzymes such as myeloperoxidase or related enzymes, 12/15-lipoxygenase or other mammalian lipoxygenases, and NADPH oxidases. The author’s work in Duesseldorf was mainly concerned with the third group of reactions.

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- Affinity to LDL-receptor is lost.
 - Affinity to scavenger receptors (LOX-1, CD36, SR-A) is acquired.
 - oxLDL-mediated signaling is shut on (endothelium, macrophages, other cells).
 - Dysregulation of cellular lipid metabolism
 - Initiation of oxidative stress and of inflammatory processes
 - Lowering of bioavailability and bioactivity of •NO
 - Endothelial dysfunction
 - Morphological and functional changes in arterial vessels
 - atherosclerosis → coronary heart disease, stroke or other cardiovascular diseases
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Table 2: Consequences of LDL oxidation

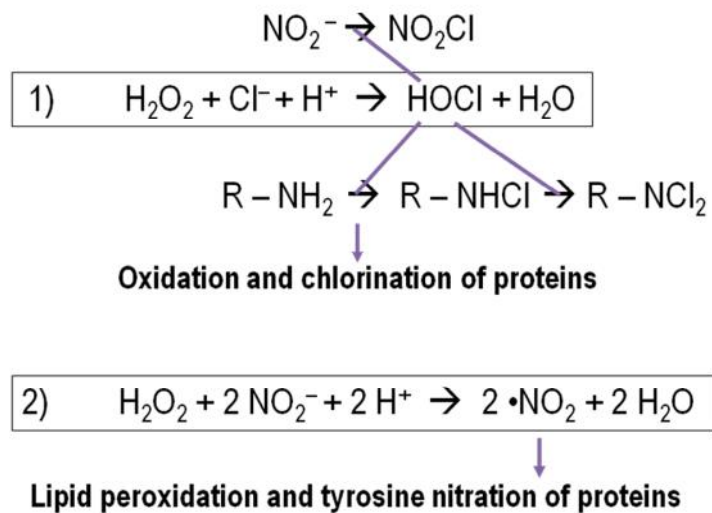
3. Enzymes involved in oxidative modification of plasma lipoproteins

3.1. Myeloperoxidase

Some characteristics of myeloperoxidase (MPO) are compiled in **Table 3**. The biological role of this enzyme appears to be quite broader than originally thought. This is due to the large variety of substrates that are capable of reacting at the level of intermediates of the catalytic cycle (MPO-Compounds I and II), which are formed by reaction of the resting form of MPO with hydrogen peroxide [Arnhold & Flemmig, 2010, and refs. therein]. These substrates include chloride or other halides and pseudohalides, nitrite, nitric oxide, tyrosine in free or protein-bound form as well as numerous phenolics including polyphenols of nutritional importance. For modification of plasma lipoproteins the reactions with chloride, bromide and nitrite deserve special attention.

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- Heme-containing mammalian peroxidase belonging to the same protein superfamily as eosinophil peroxidase, lactoperoxidase, thyroid peroxidase and cyclooxygenases
 - Glycosylated homodimer (150 kDa) linked by a single interchain disulfide bond
 - Present in neutrophil granulocytes and other phagocytes, and released upon activation and degranulation of them
 - Responsible for killing of phagocytosed bacteria, viruses and tumor cells
 - Involved in inflammatory processes
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Table 3: Some characteristics of myeloperoxidase



Basic reactions of myeloperoxidase with chloride and nitrite, and their consequences for lipoprotein modification

As seen from this scheme, two kinds of reactive species are formed, which are capable of modifying LDL or HDL: (i) hypochloric acid or other chlorinating agents (or the corresponding compounds formed from bromide), (ii) nitrogen dioxide, a short-lived highly reactive radical, which belongs to the most reactive inducers of non-enzymatic lipid peroxidation along with the action as a nitrating agent.

By this fashion, plasma lipoproteins are secondary substrates of MPO. Extensive research during the past 15 years revealed a putative involvement of MPO in atherogenesis, the arguments for which are listed in **Table 4**. For details and references the reader is referred to the articles published elsewhere [Schewe & Sies, 2005; Steffen et al, 2006a].

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- MPO is present and active in atherosclerotic lesions but not in uninvolved intima.
 - Reaction products of MPO (3-chloro-tyrosine, 3-nitro-tyrosine, *o,o'*-dityrosine from tyrosyl radicals) are found in atherosclerotic lesions.
 - MPO binds to LDL.
 - MPO renders LDL atherogenic *in vitro*.
 - Plasma levels of MPO are elevated in subjects at risk for major cardiac events.
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Table 4: Indications for a role of myeloperoxidase (MPO) in atherogenesis

In inflamed arterial vessels, MPO is released from recruited and stimulant-activated phagocytes (monocytes/macrophages or neutrophil granulocytes) and acts in a concerted manner along with $\cdot\text{NO}$ synthases and NADPH oxidase isoforms as illustrated in **Fig. 2**. Notably, MPO acts as $\cdot\text{NO}$ oxidase forming nitrite, which in turn is substrate for a consecutive MPO-catalyzed reaction giving rise to nitrogen dioxide radical that attacks LDL together with peroxynitrite, a deleterious $\cdot\text{NO}$ metabolite.

We studied lipid peroxidation in isolated human LDL by a MPO/H₂O₂/nitrite reaction system (**Fig. 3**). In the presence of moderate concentrations of nitrite, there occurred a marked increase in the absorbance at 234 nm, which is indicative of the formation of conjugated

dienes, a typical hallmark of lipid peroxidation. The low-level conjugated diene formation observed in the absence of nitrite is attributed to MPO-generated tyrosyl radicals originating from protein-linked tyrosines in apoB-100. Notably, chloride did not enhance this basal conjugated diene formation suggesting that MPO-derived hypochlorous acid selectively attacks apoB-100 as reported by several authors, but apparently not the lipid moiety of LDL.

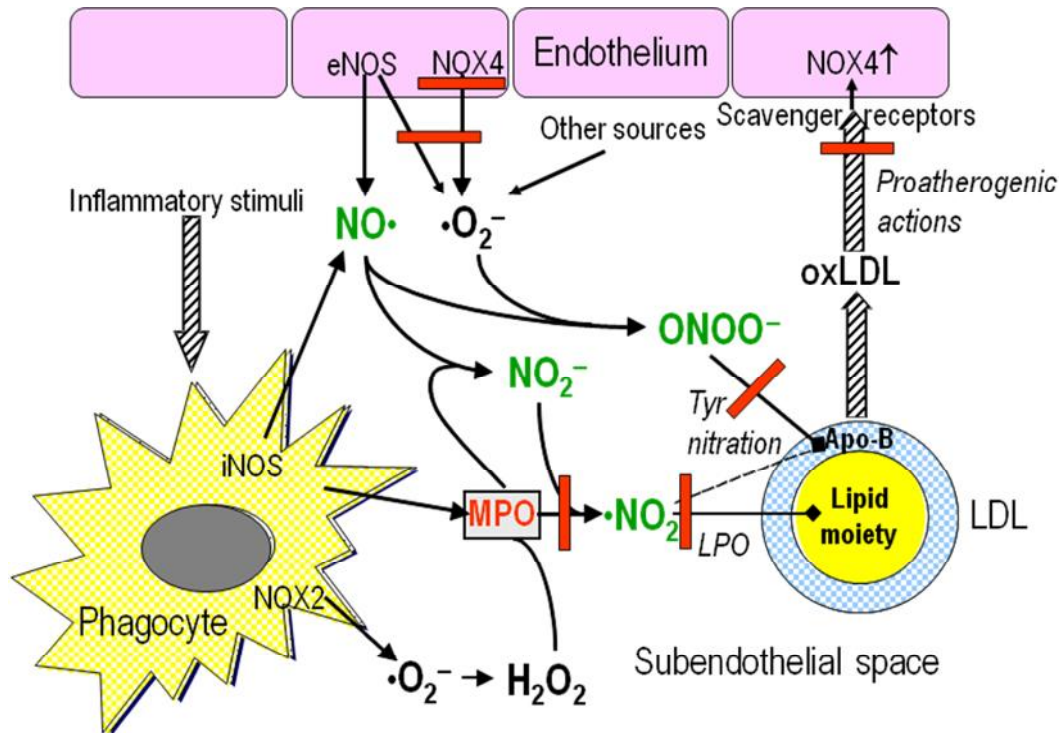


Fig. 2. Putative role of myeloperoxidase (MPO) in endothelial dysfunction.

At inflammatory foci of the subendothelial space, phagocytes are recruited and stimulated by pro-inflammatory mediators leading to a release of MPO as well as up-regulation of inducible •NO synthase (iNOS) and of the NADPH oxidase isoform NOX2. Thus, $\cdot\text{O}_2^-$ and $\cdot\text{NO}$ are generated. Dismutation of superoxide gives rise to hydrogen peroxide, which enables the MPO to react with nitric oxide forming nitrite. $\cdot\text{NO}$ is also generated in endothelial cells by endothelial •NO synthase (eNOS). Nitrite is substrate for a second reaction of MPO generating the highly reactive lipophilic nitrogen dioxide radical that induces lipid peroxidation (LPO) of LDL after passage of which from plasma through the damaged endothelium into the subendothelial space. Nitric oxide and superoxide also avidly react with each other generating peroxynitrite, which causes protein tyrosine nitration of the apoprotein of LDL. The oxLDL thus formed reacts with scavenger receptors of endothelial cells, activating an NADPH oxidase, presumably NOX4, thus reinforcing the oxidative damage to the vascular endothelium. The bars crossing the arrows denote the presumed sites of blockage by (–)-epicatechin (see section 4.2).

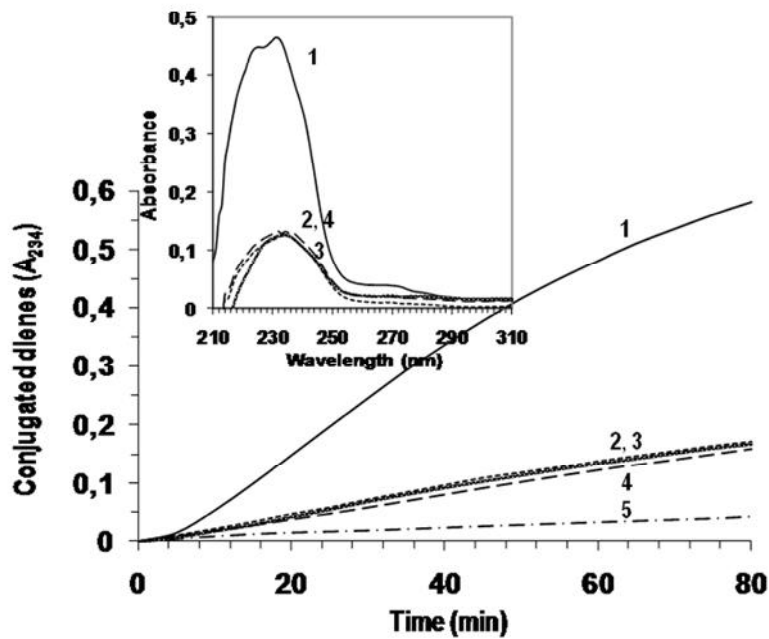


Fig. 3. Myeloperoxidase/nitrite-induced lipid peroxidation of low-density lipoprotein.

Isolated human LDL was treated with MPO plus glucose/glucose oxidase as H_2O_2 -generating system under various conditions for the time indicated. The time-course of the reaction was recorded by the increase in absorbance monitoring formation of conjugated dienes. The inset shows the UV spectra after the reaction was finished. The traces show the reactions in the presence of nitrite (1), with LDL alone (2), in the presence of nitrate (3) or chloride (4), and without MPO (5). Data from Kostyuk et al, 2003.

Our data support the assumption that nitrite plays a crucial role in mediating atherogenesis by MPO. Some experimental observations from the Duesseldorf lab in line with this assumption are summarized in **Table 5**.

It is reasonable to assume that LDL treated with MPO/ H_2O_2 /nitrite is a more appropriate model for oxLDL occurring *in vivo* than oxLDL obtained by Cu^{2+} -catalyzed oxidation of LDL, because free Cu^{2+} ions do not occur in blood plasma. We actually observed a striking difference between MPO/ H_2O_2 /nitrite-oxLDL and Cu^{2+} -oxLDL with respect to the oxysterol composition (Steffen et al, 2006b). The 7β -hydroxycholesterol/7-ketocholesterol ratio was found to be markedly higher in MPO/ H_2O_2 /nitrite-oxLDL and proved to be responsible for the higher cytotoxicity of this kind of oxLDL against vascular endothelial cells.

- Nitrite at physiologically relevant concentrations (~10 μM) modulates the reaction of MPO with LDL.
- Lipid peroxidation (LPO) is a major process of MPO/nitrite-mediated modification of LDL.
- Nitrite is a better co-catalyst than the tyrosyl residues in apo B-100.
- Nitrite suppresses both tyrosine- and chloride-supported reactions.
- Chloride is neither able to support LPO of LDL, nor is its presence required for nitrite-supported LPO.
- At moderate concentrations of nitrite, MPO/nitrite-mediated LPO is not accompanied by protein tyrosine nitration. The latter process requires nitrite above the physiologically relevant concentration range.

Table 5: Role of nitrite in myeloperoxidase-mediated modification of LDL

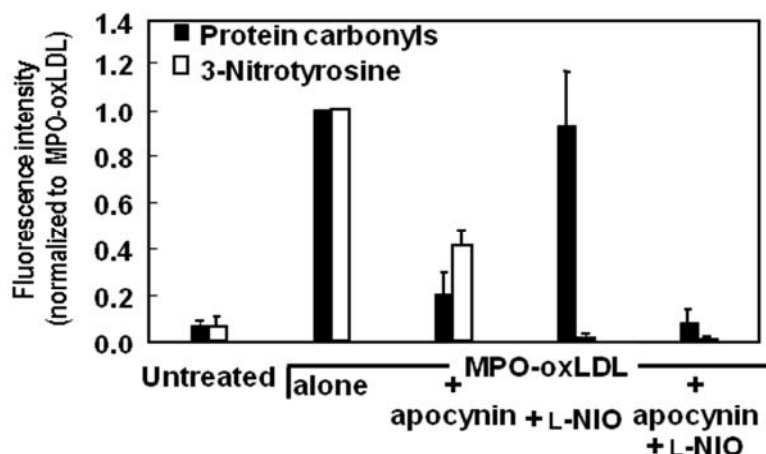


Fig. 4. Evidence for oxidative and nitrating damage to cultured endothelial cells by MPO/nitrite-generated oxLDL.

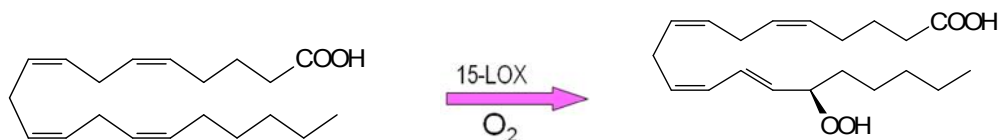
Human umbilical vein endothelial cells were treated with MPO/H₂O₂/nitrite-oxLDL for 24 h without and with pretreatment of the cells with the agents indicated. Formation of protein carbonyls and that of tyrosine-nitrated polypeptides were visualized by immunocytochemical methods and quantified. For further details, see Steffen et al, 2007.

MPO/H₂O₂/nitrite-oxLDL evokes pro-oxidative and nitrating damage to cultured vascular endothelial cells. This was demonstrated by a marked increase in intracellular protein carbonyls as hallmark for oxidation of lysines and other amino acid residues as well as in tyrosine-nitrated polypeptides (Fig. 4). Apocynin, an NADPH oxidase inhibitor, and L-NIO, a selective inhibitor of inducible NO synthase (iNOS), prevented these changes. These data may be interpreted as a consequence of LDL scavenger-pathway-mediated cell signaling involving activation of NADPH oxidase(s) and iNOS (see also Fig. 2).

It must be emphasized that the role of MPO in atherogenesis is still a matter of debate. Conflicting data in experiments with knockout mice evoked some doubt on a pro-atherogenic role of MPO. Therefore, a putative anti-atherogenic role is also discussed.

3.2. Lipoxygenases

Lipoxygenases constitute a family of closely related enzymes, which dioxygenate polyenoic fatty acids forming hydroperoxy derivatives as primary products. In humans at least five lipoxygenases have been identified, which differ among others in the reaction specificity. A classification in 5-, 8-, 12- and 15-lipoxygenases was formerly established according to the carbon atom in arachidonic acid that is dioxygenated. Thus, the reaction of “15-lipoxygenase” is as follows:



It must be stressed, however, that a classification according to the reaction specificity towards arachidonic acid does not mirror the real genetic relatedness of lipoxygenases. One member of this enzyme family, today termed 12/15-lipoxygenase or reticulocyte-type 15-lipoxygenase (15-lipoxygenase-1), was uncovered in 1975 by an East-Berlin group [Schewe et al, 1975], and turned out to be capable of dioxygenating not only free arachidonic and linoleic acids but also phospholipids, cholesterol esters, biomembranes and lipoproteins, thus appearing as a general enzymatic prooxidant or catalyst of enzymatic lipid peroxidation, respectively. A pivotal role of 12/15-lipoxygenase in oxidative modification of low-density lipoprotein and, thus, in early stages of atherosclerosis had been proposed by Steinberg and associates [Parthasarathy et al, 1989]. Detailed investigation in the lab of H. Kühn (Berlin), however, revealed data supporting both pro-atherogenic and anti-atherogenic roles of 12/15-lipoxygenase [Kühn & O'Donnell, 2006, and refs. therein]. It has been speculated that the prominent role may change dependent on the stage of development of atherosclerosis.

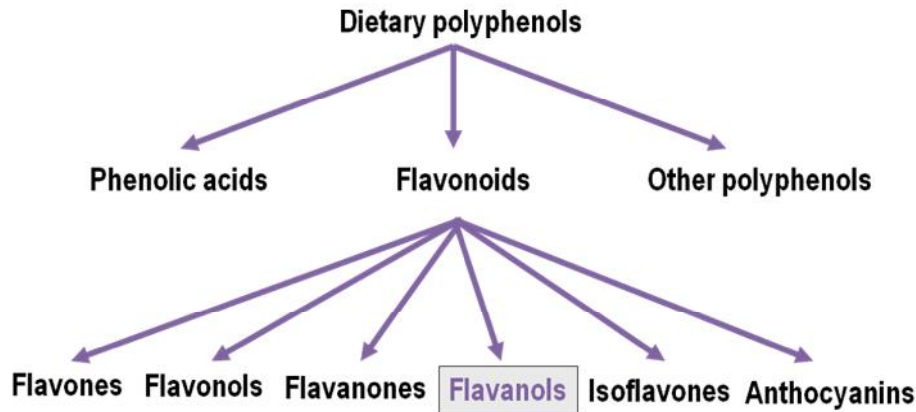
Irrespective of the putative dual role of 12/15-lipoxygenase, LDL can serve as a direct substrate of this enzyme, thus rendering it potentially proatherogenic. The action appears to proceed via two steps, the direct enzyme catalysis followed by a secondary hydroperoxide-triggered non-enzymatic lipid peroxidation. This complexity led to conflicting data in the literature dependent on experimental conditions. Lipoxygenase inhibitors inhibit the whole process, even if they do not possess antioxidant activity. Vitamin E, however, does not inhibit lipoxygenases, but modulates secondary lipid peroxidation.

Habenicht and associates provided strong evidence against any role of 12/15-lipoxygenase in atherogenesis in humans [Spanbroek et al, 2003]. Instead, these authors argue in favor of a proatherogenic role of 5-lipoxygenase, although the latter cannot directly react with lipoproteins. As a consequence of these conflicting data, both types of lipoxygenases should be considered as potential targets of dietary ingredients to attenuate atherogenesis.

4. Protective role of dietary polyphenols against plasma lipoprotein oxidation and cardiovascular diseases

Owing to the high incidence of cardiovascular diseases in industrial countries there is a demand for strategies to prevent atherosclerosis or at least to retard the progress of its pathology. As a cost-saving alternative to drugs, modulation of the nutritional regimen is a promising approach. As mentioned in section 1, intake of dietary polyphenols has attracted special attention during the last decade.

Polyphenols are aromatic compounds having more than one phenolic hydroxyl group. Several thousands of natural polyphenols are known and plenty of them occur in food, in particular in fruits and vegetables as well as in teas, wine and cocoa products. The following scheme provides a simplified survey on the chemical classification of natural polyphenols:



The work in Duesseldorf was focused on flavanols, in particular on those occurring in cocoa. Three major cocoa flavan-3-ols are shown in Fig. 5

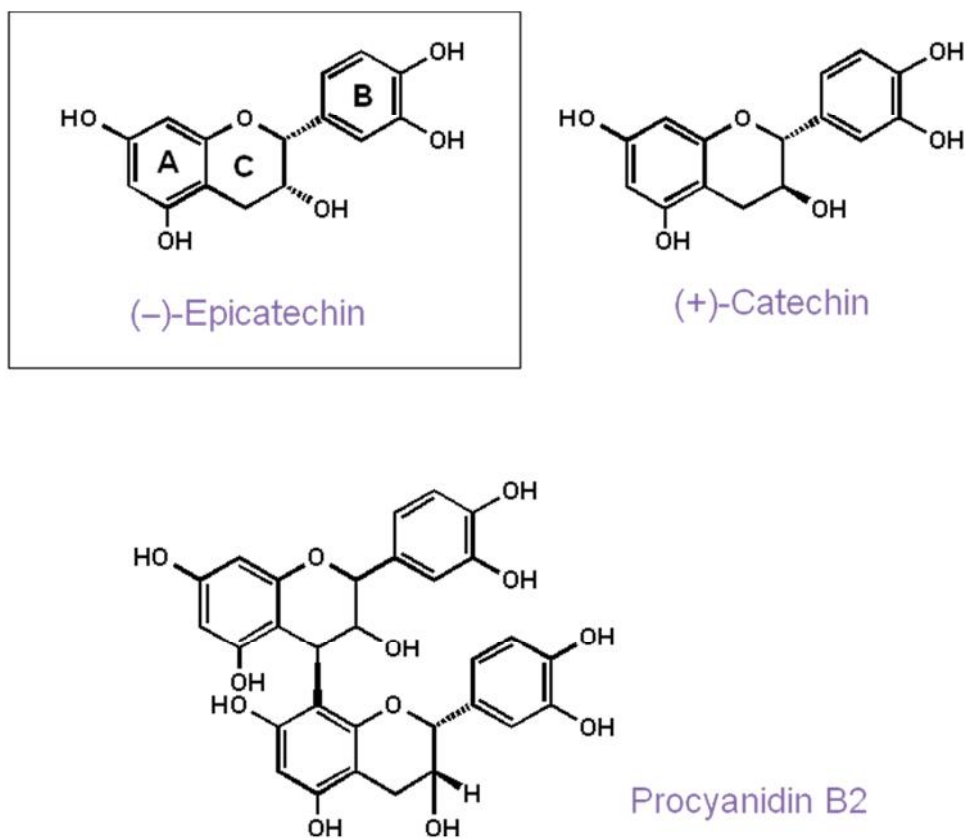


Fig. 5. Chemical structures of the three major flavanols occurring in cocoa products. Procyanidin B2 is an oxidative dimer of (-)-epicatechin

4.1. In vivo studies

Epidemiological evidence for the health-promoting effect of cocoa flavanols was obtained by several research groups. In the “Zutphen Elderly Study” was found that in a cohort of 470 elderly men (average 72 years old) cocoa intake is inversely associated with blood pressure and 15-year cardiovascular and all-cause mortality; compared with the lowest tertile of cocoa intake, the adjusted relative risk for men in the highest tertile was 0.50, which was statistically significant ($P = 0.004$ for trend) [Buijsse et al, 2006]. A similar outcome has been reported for postmenopausal women [Mink et al, 2007].

Moreover, as much as 28 human intervention studies revealed health benefits of intake of high-flavanol cocoa or chocolate (reviewed by Cooper et al, 2008). Among the three major

cocoa flavanols, (–)-epicatechin is most likely the decisive bioactive compound. Therefore we focused on (–)-epicatechin in our *in vitro* studies. The reason for the preference of (–)-epicatechin is as follows: (i) It is abundant not only in cocoa, but also in grapes, red wine, tea, apples and other fruits, and therefore of broader nutritional importance. (ii) The intake leads to plasma levels of metabolites, which are higher than those for other flavanols. (iii) Intake of isolated (–)-epicatechin mimics the biological effects of high-flavanol cocoa in humans [Schroeter et al, 2006]. The latter observation, which will be treated in detail in the companion article, supports the assumption that the health benefit of intake of cocoa or chocolate is most likely due to (–)-epicatechin.

A number of *in vivo* studies indicate that high-flavanol cocoa improves vascular endothelial function via enhancement of bioavailability and bioactivity of •NO. Irrespective of this major action, however, there is some evidence that oxidative modification of LDL is also counteracted. It was recently reported that oral administration of green tea flavanols lowers the plasma level of oxLDL in healthy humans in parallel to improvement of endothelial function as measured by FMD [Inami et al, 2007; Tinahones et al, 2008]. From this observation one may conclude an association between LDL oxidation and bioavailability and bioactivity of •NO. Although (–)-epicatechin contributes to only about one-fifth of the total flavanol content of green tea, recent literature data argue that this flavanol is mainly responsible for the *in vivo* effects rather than epigallocatechin gallate that is contained in green tea with a markedly higher percentage.

4.2. In vitro effects of epicatechin and other flavonoids on oxidative and nitrating modification of LDL

The effect of flavonoids on MPO-mediated lipid peroxidation of human LDL was studied by us for the first time (Kostyuk et al, 2003). MPO-mediated lipid peroxidation in the presence of a H₂O₂-generating system (glucose/glucose oxidase) and nitrite was markedly suppressed by micromolar concentrations of (–)-epicatechin, quercetin, rutin, taxifolin or luteolin. The mode of action of these flavonoids was not yet definitely identified, but a most probable mechanism is scavenging of •NO₂ radicals, the generation of which by the MPO/nitrite reaction system has been demonstrated by other authors. Notably, the well-known free radical scavengers vitamin E and probucol did not inhibit this system at low concentrations. Apparently these agents are capable of selectively scavenging carbon-centered radicals, but not •NO₂ or other nitrogen-based radicals.

Besides the MPO/nitrite system, (–)-epicatechin was found also to protect against a number of other modifications of LDL. The data are summarized in **Table 6**.

Agent	Reaction / criterion	EC (μM) ¹
Myeloperoxidase (nitrite)	Lipid peroxidation, prolongation of lag phase	~2
	Lipid peroxidation, lowering of reaction rate	~5
	Tyrosine nitration	<0.1
Myeloperoxidase (tyrosine)	Lipid peroxidation, extent	~5
Cu^{2+}	Lipid peroxidation, prolongation of lag phase	0.6
Peroxyxynitrite	Tyrosine nitration	<0.1

¹ effective concentration to achieve either 50% inhibition or doubling of lag phase

Table 6. Efficacies of (-)-epicatechin (EC) against oxidative and nitrating modification of human low-density lipoprotein [Sies et al, 2004]

As evident from the strong effects on protein tyrosine nitration, (-)-epicatechin protects not only the lipid moiety of LDL but also the protein component apoB-100. This was also shown by a dose-dependent prevention of MPO-mediated shift in electrophoretic mobility, which is due to oxidative loss of positively charged lysines (Fig. 6). The dual protectant effect of (-)-epicatechin on lipid and protein moieties of LDL is probably due to the solubility characteristics. Since the octanol/water distribution coefficient is not far from unity, this agent may be sufficiently soluble in both lipophilic and hydrophilic environments. For the same reason, (-)-epicatechin and related flavonoids are universal free radical scavengers, detoxifying both lipid and $\bullet\text{NO}_2$ radicals, whereas the antioxidant vitamins C and E do not share this property.

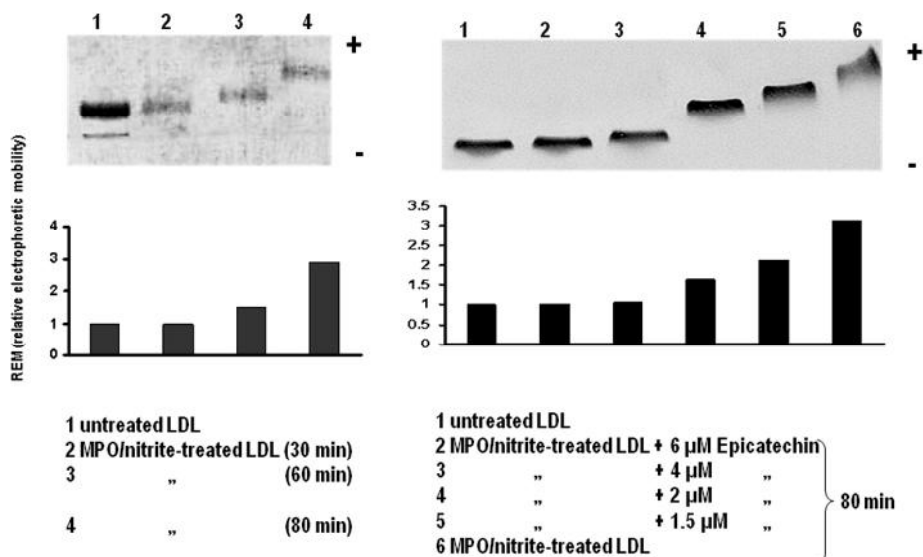


Fig. 6. Epicatechin also protects against oxidative modification of the apoprotein moiety of LDL.

Treatment of LDL with MPO/nitrite leads to oxidative loss of positively charged lysines, so that electrophoretic migration to the anode is accelerated. Pretreatment with (-)-epicatechin prevents this phenomenon. Data from [Steffen et al, 2006a].

The protectant actions of (-)-epicatechin or other flavonoids on MPO-mediated modifications of LDL are accompanied by chemical changes of the polyphenols, as evident from spectral changes [Kostyuk et al, 2003]. The conversion products remain to be identified. In the case of

(-)-epicatechin the formation of a procyanidin B2-like dimer may be hypothesized. This speculation is supported by a report, that MPO converts the phenolic compound apocynin to a dimer, thus rendering it to an NADPH oxidase inhibitor [Ximenes et al, 2007]. Treatment of (-)-epicatechin with MPO also gives rise to a product that inhibits NADPH oxidase, a property, which is shared by procyanidin B2 [Steffen et al, 2008]. Moreover, direct evidence was obtained that (-)-epicatechin is substrate of MPO. It was demonstrated by the use of a multi-mixing stopped-flow technique that (-)-epicatechin is one of the most efficient electron donors for heme peroxidases investigated so far, by reacting with both activated key intermediates of the catalytic cycle, the MPO-Compounds I and II with a high rate each [Spalteholz et al, 2008].

Notably, (-)-epicatechin attenuates not only the generation of oxLDL via multiple mechanisms, but also the deleterious actions of oxLDL on endothelial cells [Steffen et al, 2006b]. The latter complex of actions is directed on oxLDL scavenger pathway-mediated signaling involving inhibition of NADPH oxidase activity. This topic will be treated in the companion article of this series. Summing up, through blunting both generation and actions of oxLDL, (-)-epicatechin turns out to be a pleiotropic protectant for both LDL and endothelial cells.

4.3. Flavonoids as lipoxygenase inhibitors

The plausible, but disputed role of 12/15-lipoxygenase in atherogenesis was discussed in section 3.2. Moreover, this enzyme is located in eosinophil leukocytes and may be involved therefore in certain inflammatory disorders. Also, a role in asthma may be considered, since 12/15-lipoxygenase is over-expressed in several airway tissues of asthmatics. The 5-lipoxygenase deserves attention from several points of view. It is the key enzyme of a special pathway of the arachidonic acid cascade leading to several proinflammatory leukotrienes. An immediate role of 5-lipoxygenase in human atherogenesis has also been proposed [Spanbroek et al, 2003]. Extensive efforts were undertaken to develop drugs based on lipoxygenase inhibition. Apart from this, lipoxygenase inhibition by natural compounds, in particular by those of nutritional importance are of special interest.

We have shown that flavonoids such as quercetin, luteolin, fisetin and a number of flavanols are inhibitors of 12/15-lipoxygenase [Schewe et al, 2001]. A further study addressed the structure-activity relations and the mode of the inhibitory action [Sadik et al, 2003]. Analysis of the inhibition of 12/15-lipoxygenase by quercetin proved to be difficult, since there is an overlap of several components, an instant reversible inhibition, a time-dependent mechanism-based inactivation by the flavonoid as well as suicide inactivation of the lipoxygenase by the substrate fatty acid.

Recombinant human 5-lipoxygenase was shown to be dose-dependently inhibited by (-)-epicatechin up to completeness [Schewe et al, 2002]. From analyses of the metabolites from arachidonic acid it became clear that (-)-epicatechin inhibits both the dioxygenase and the leukotriene A₄ synthase activities of the enzyme, i.e. the first two consecutive steps of the conversion of arachidonic acid into various proinflammatory leukotrienes.

Some data on the inhibition of mammalian lipoxygenases by flavonoids are compiled in **Table 7**. It should be emphasized that flavonoids are direct inhibitors of lipoxygenases, i.e. they are capable of blocking the active site. This is basically different from the action on myeloperoxidase, where secondary processes rather than enzyme activity *per se* are suppressed.

Compound	IC ₅₀ (μM)	
	Human 5-lipoxygenase	Rabbit 12/15-lipoxygenase
(-)-Epicatechin	22	60
(-)-Epigallocatechin gallate	3.1	4
Quercetin	0.6	4

Table 7: Inhibition of mammalian lipoxygenases

Whether inhibition of lipoxygenases also occurs *in vivo*, remains to be clarified. Inhibition of 5-lipoxygenase by (-)-epicatechin may be considered, because it would plausibly explain a literature report that intake of high-flavanol chocolate by volunteers caused a significant decrease in the ratio of the levels of plasma metabolites of cysteinyl leukotrienes and prostacyclin, which coincides with an increase of the plasma level of (-)-epicatechin and its metabolites [Schramm et al, 2001]. Direct evidence for inhibition of 5-lipoxygenase by intake of high-flavanol cocoa or chocolate can be provided by measurement of urinary excretion of leukotriene metabolites during a respective clinical study. Unfortunately such a study has not been carried out so far.

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