Myeloperoxidase/nitrite-mediated lipid peroxidation of low-density lipoprotein as modulated by flavonoids

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Abstract In the presence of a H_2O_2-generating system, myeloperoxidase (MPO) caused conjugated diene formation in low-density lipoprotein (LDL), indicating lipid peroxidation which was dependent on nitrite but not on chloride. The oxidation of LDL was inhibited by micromolar concentrations of flavonoids such as (−)-epicatechin, quercetin, rutin, taxifolin and luteolin, presumably via scavenging of the MPO-derived NO_2 radical. The flavonoids served as substrates of MPO leading to products with distinct absorbance spectra. The MPO-catalyzed oxidation of flavonoids was accelerated in the presence of nitrite.
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Key words: Atherosclerosis; Flavanol; Flavonol; Oxidized LDL; Peroxidase; Reactive nitrogen species

1. Introduction

Oxidative modification of low-density lipoprotein (LDL) appears to play a pivotal role in atherosclerosis [1]. A number of endogenous factors have been reported to oxidize LDL in vitro [2]. Myeloperoxidase (MPO) is a candidate to participate in this process also in vivo [2,3]. Its pro-oxidant action on LDL was originally believed to be due to the formation of hypochlorous acid. In later studies, however, it became evident that the MPO is also closely connected with the metabolism of NO. Nitrite, its oxidation product, serves as substrate of MPO-forming reactive nitrogen species [4–6]. Recent studies demonstrated that this reaction is involved in protein nitration both in vitro [7] and in vivo [8,9]. These findings prompted us to study the reaction of the MPO/nitrite system with human LDL. In particular, we examined whether flavonoids counteract MPO-induced oxidation of LDL. Since epidemiologic studies have revealed an inverse correlation between intake of dietary flavonoids and mortality from coronary artery disease [10,11], blunting of MPO-induced oxidation of LDL by flavonoids may contribute to their supposed beneficial effects.

2. Materials and methods

2.1. Enzymes and chemicals

Human MPO was purchased from Calbiochem and glucose oxidase (GOD) from Roche Molecular Biochemicals. All other chemicals and solvents were purchased from Sigma-Aldrich or Merck.

2.2. Preparation of LDL

LDL was prepared from blood serum of healthy volunteers according to Kleinveld et al. [12] with minor modifications and stored in the presence of 4 mM EDTA at 4°C in the dark up to 8 days. Under these conditions no measurable lipid peroxidation occurred during storage. The concentration of LDL was estimated by determination of total cholesterol assuming a molecular mass of 2.5 MDa for LDL and a cholesterol content of 31.6%. LDL cholesterol was determined with Cholesterol reagent (Roche Diagnostics).

2.3. Lipid peroxidation of LDL

Reactions were carried out at 37°C in 0.1 M potassium phosphate buffer, pH 7.4, supplemented with 0.1 mM diethylenetriaminepentaacetic acid. Unless stated otherwise, the reaction mixtures contained 0.1 μM LDL, 50 μM nitrite, 53 nM MPO ($\varepsilon_{430} = 170$ mM^{-1} cm^{-1} [5]), 310 ng/ml (1.29 nkat/ml) GOD and 0.56 mM D-glucose (final concentrations). In blank samples MPO was omitted. Flavonoids were added to the reaction mixture dissolved in 2-methoxyethanol. The final concentration of solvent was kept constant in all samples (1% by volume). The oxidation of both LDL and flavonoids was followed by repetitive (every 5 min) scanning of the absorbance spectrum between 220 and 600 nm.

After 80 min incubation, the oxidation of LDL was terminated by reduction of lipid hydroperoxides with sodium borohydride followed by lipid extraction according to Bligh and Dyer [13]. After evaporation of the organic phase, the lipids were dissolved in *n*-heptane/propan-2-ol (1/1) for recording of the absorbance spectrum between 200 and 350 nm. The difference in absorbance at 234 nm vs. blank was estimated for calculation of the amount of conjugated dienes ($\varepsilon_{234} = 25\,000$ M^{-1} cm^{-1}).

3. Results

3.1. MPO-induced lipid peroxidation of LDL requires nitrite

Incubation of human LDL with MPO and a hydrogen peroxide-generating system (glucose+GOD) for 80 min resulted in only slow formation of conjugated dienes as judged from a linear time-dependent increase in the absorbance at 234 nm in both whole mixture and lipid extract (Fig. 1A,B, traces 2). In the presence of 50 μM nitrite, however, the rate of lipid peroxidation was enhanced about four-fold after a short lag phase (traces 1), whereas this stimulation did not occur in the presence of either 50 μM nitrate or 100 mM chloride (traces 3 and 4), demonstrating a specific requirement for

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Abbreviations: GOD, glucose oxidase; LDL, low-density lipoprotein; MPO, myeloperoxidase

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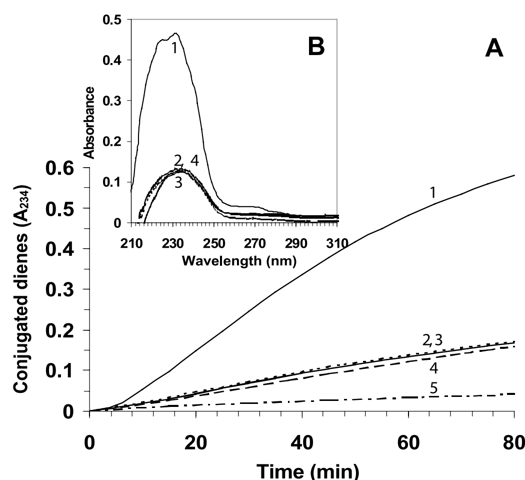

Fig. 1. Lipid peroxidation of LDL by MPO in the presence of GOD/glucose. A: Time course. B: UV difference spectra of lipid extracts after 80 min oxidation versus blank. 1, full line, 50 µM nitrite; 2, dotted line, no addition; 3, full line, 50 µM nitrate; 4, dashed line, 100 mM chloride; 5, dot-dashed line, 50 µM nitrite, MPO omitted (blank).

nitrite as MPO substrate. In Table 1 the reaction parameters under these conditions estimated in the presence and absence of nitrite are compiled.

3.2. *Flavonoids suppress MPO/nitrite-induced lipid peroxidation of LDL*

Seven selected flavonoids suppressed the MPO/nitrite-induced lipid peroxidation of LDL by affecting both the lag phase and the propagation phase. Representative examples are shown in Fig. 2A. The lag time τ_i was increased in a dose-dependent manner. At 10 µM the catechol-bearing flavonoids caused nearly complete inhibition of conjugated diene formation over a reaction period of 80 min. Except for kaempferol, the flavonoids also diminished the reaction rate of the linear part of the propagation phase in a dose-dependent manner. In Table 2 the potencies of the flavonoids are compiled, showing that flavonoids bearing a catechol arrangement in the B-ring proved to be more potent than those lacking this feature (morin, kaempferol).

Interestingly, moderate concentrations of kaempferol (5 and 10 µM) markedly increased rather than decreased the reaction rate of the propagation phase by 50–70%, in addition to a three- to seven-fold prolongation of the lag time (Fig. 2B, traces 2 and 3). This behavior implies both antioxidant and pro-oxidant effects of one parent compound in the same system. Pro-oxidant effects by flavonoids not containing a catechol arrangement have recently been described to be mediated by phenoxyl radicals [14].

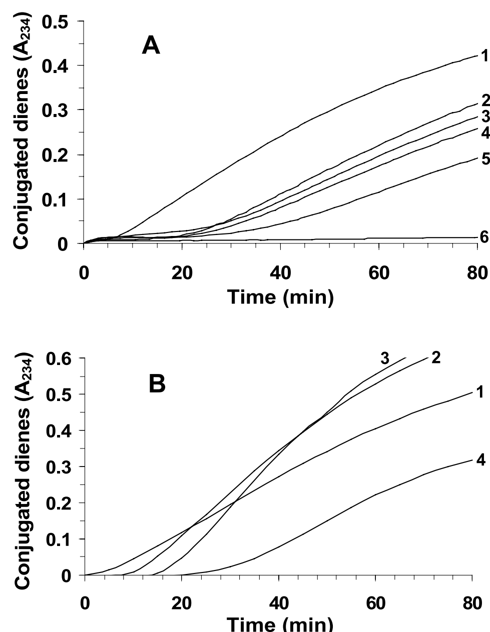

Fig. 2. Effect of flavonoids on lag phase and rate of lipid peroxidation of LDL by MPO in the presence of GOD/glucose/nitrite. A: 1, control; 2, rutin, 2 µM; 3, taxifolin, 2 µM; 4, epicatechin, 2 µM; 5, quercetin, 2 µM; 6, blank without MPO. B: 1, control; 2, kaempferol, 5 µM; 3, kaempferol, 10 µM; 4, kaempferol, 20 µM.

3.3. *Flavonoids are oxidized during MPO/nitrite-induced lipid peroxidation of LDL*

Repetitive scanning of the absorbance spectra under the reaction conditions shown in Fig. 2 revealed time-dependent loss of all seven flavonoids studied. Examples are shown in Fig. 3. In the case of quercetin, morin and kaempferol, the formation of new products with pronounced absorbance maxima at 325–335 nm was observed. From the time courses of the spectral changes (Fig. 4), it can be concluded that the quercetin intermediate(s) were potent inhibitors of LDL oxidation as well (panel 3). By contrast, the kaempferol intermediate(s) revealed a marked pro-oxidant effect (Fig. 4, panel 4), whereas for morin the time after the conversion of the primary flavonoid had completed coincided with the end of the lag phase (Fig. 4, panel 2). Collectively, one may conclude, therefore, that the lag phase is brought about by potent inhibition of lipid peroxidation by the parent flavonoid, whereas the conversion product(s) (except quercetin intermediates) which peak during the propagation phase are only modest inhibitors or even pro-oxidants. This interpretation implies

Table 1
Parameters of MPO-induced lipid peroxidation of LDL in the presence and absence of nitrite

Parameter	Nitrite (50 µM)	Control
Lag time τ (min)	6.6 ± 1.9 (n = 20)[a]	none
Rate of lipid peroxidation during propagation phase (mol conjugated dienes/mol LDL per min)	3.6 ± 1.1 (n = 20)[a]	1.1 ± 0.25 (n = 3)
Total amount of conjugated dienes formed after 80 min (mol/mol LDL)	150 ± 43 (n = 20)[a]	44.0 ± 4.0 (n = 3)

Conditions as described in Section 2.3. The number of independent experiments using different batches of LDL is indicated in parentheses.
[a]Different LDL preparations.

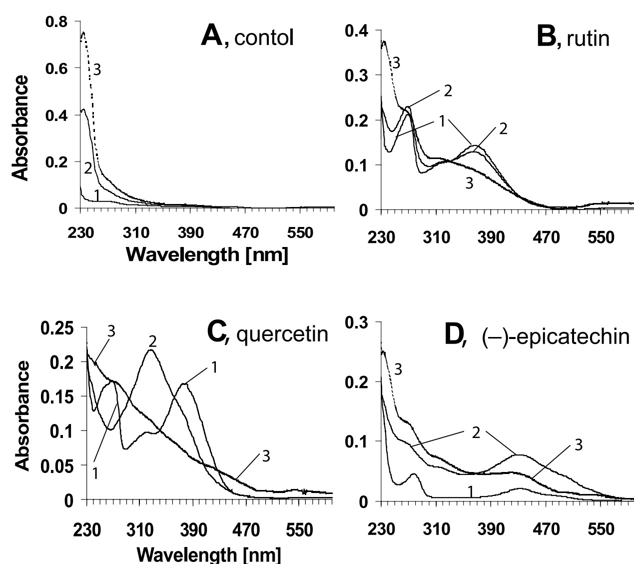

Fig. 3. Spectral changes during MPO/nitrite-induced lipid peroxidation of LDL without flavonoids (A) and with 10 μM rutin (B), quercetin (C), or (−)-epicatechin (D). 1, Before reaction; 2, after 10 min for (B) and (C), and 40 min for (A) and (D), respectively; 3, after 80 min.

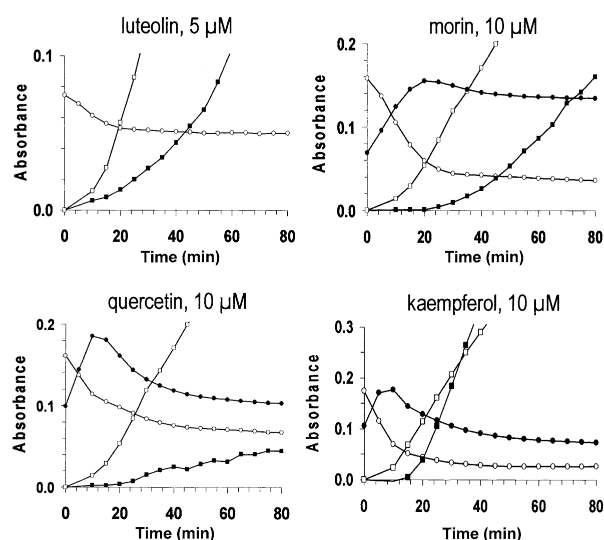

Fig. 4. Time course of lipid peroxidation of LDL (squares) and oxidation of flavonoids (circles) in the MPO/nitrite system for luteolin, morin, quercetin and kaempferol. Open circles refer to bleaching of the parent chromophores with the following maxima: luteolin, 360 nm; morin, 390 nm; quercetin, 375 nm; kaempferol, 372 nm. Filled circles indicate formation of new chromophores with the following maxima: morin, 330 nm; quercetin, 330 nm; kaempferol, 325 nm. Formation of conjugated dienes (A_{234}) is indicated by open squares for control and by filled squares for the reactions with flavonoids, respectively. Representative examples of experiments at least in triplicate are shown.

that the inhibition of lipid peroxidation during the lag phase and the propagation phase may be accounted for by distinct chemical entities and may also explain the opposite effects of kaempferol on the two phases. Generally, there was no clear-cut correlation between the antioxidant effects of flavonoids and their tendency to be oxidized in this complex system.

3.4. *Flavonoids are co-substrates of MPO*

In order to distinguish whether the conversion of flavonoids in the complex system with LDL is due to the peroxidase activity of MPO or a consequence of co-oxidation by intermediate radicals occurring during lipid peroxidation, we also investigated the corresponding reactions in the absence of LDL. As shown in Fig. 5, MPO catalyzed the oxidation of (−)-epicatechin, luteolin and morin up to completeness, the

Table 2
Potencies of selected flavonoids to protect from MPO/nitrite-induced lipid peroxidation of LDL

Flavonoid	IC_{50} (μM)	EC_5 (μM)
Quercetin	2.2	2.0
Rutin	3.0	2.8
Taxifolin	3.8	2.6
Epicatechin	4.4	2.7
Luteolin	4.5	5.8
Morin	11.1	10.0
Kaempferol	> 20[a]	15.6

The reactions were followed in the presence of varying concentrations of flavonoids (1–20 μM) (conditions as in Fig. 2A,B). In each setup the lag time from the kinetic traces and the total amount of conjugated dienes from the lipid extract after 80 min were assessed. From the respective dose–response curves (10–12 single measurements) thus obtained, the concentrations to achieve 50% inhibition of conjugated diene formation (IC_{50}) and those to achieve five-fold prolongation of the lag time (EC_5, $\tau_i/\tau_{contr} = 5$) were estimated.
[a]Stimulatory effects at concentrations between 5 and 10 μM; inhibition at concentrations higher than 20 μM.

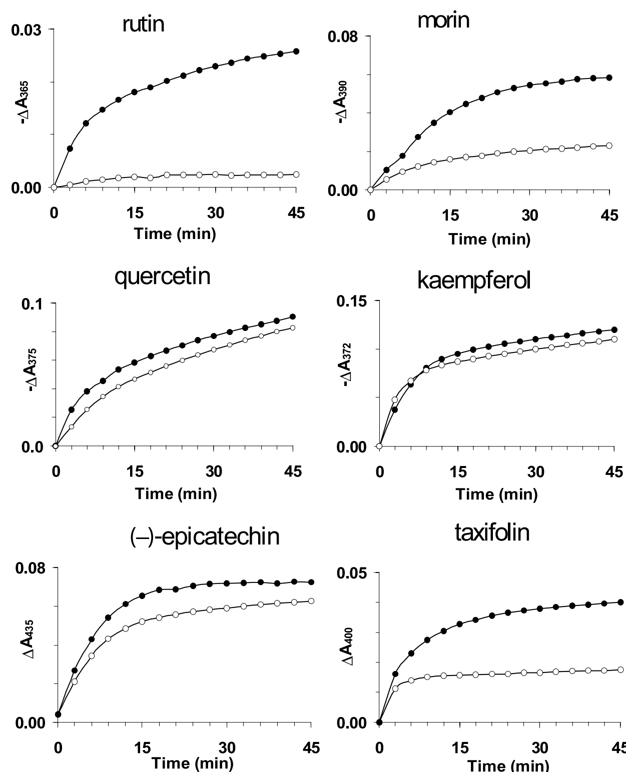

Fig. 5. Time course of oxidation of flavonoids (10 μM) by MPO/GOD/glucose in the presence (filled circles) and absence (open circles) of nitrite. The decrease in absorbance (−Δ*A*) for rutin, morin, quercetin, kaempferol or increase in absorbance (Δ*A*) for (−)-epicatechin and taxifolin, respectively, was followed. Representative examples for three to five independent experiments are shown.

rates being higher in the presence of nitrite than in its absence. In the case of rutin, activity was only observed in the presence of nitrite, which may be due to the fact that the 3-hydroxy group in rutin is blocked and may require unmasking by a MPO/nitrite-derived reactive species. Activity was also observed with quercetin. Here, however, several intermediate products with different absorbance spectra were formed which were consecutively converted to other products, rendering the kinetics fairly complex.

4. Discussion

Using a different experimental approach, our data confirm the work by Buyn et al. [5] reporting that lipid peroxidation of LDL caused by human MPO is strongly stimulated by low concentrations of nitrite but not by physiological concentrations of chloride. The reaction in the presence of nitrite encompasses both a lag phase and a propagation phase. In this respect it is qualitatively similar to the copper-induced lipid peroxidation of LDL. The duration of the lag phase differs, however, by one order of magnitude. Under comparable conditions, we found a τ of 6.6 ± 1.9 min with MPO/GOD/nitrite and 62 ± 20 min with Cu^{2+}.

For the first time, we also demonstrated that MPO/nitrite-induced lipid peroxidation is attenuated by several flavonoids prolonging the lag time and also inhibiting the reaction rate during the propagation phase (Fig. 2, Table 2). The second effect appears to be, however, due to oxidation products of the flavonoids (Fig. 4). By contrast, in the Cu^{2+}-induced reaction the flavonoids only prolonged τ without affecting the rate of the propagation phase (data not shown). The potencies of quercetin and (−)-epicatechin to prolong the lag phase were similar for MPO/nitrite- and Cu^{2+}-induced reactions. Significant effects were seen at concentrations as low as 0.3 μM flavonoid (data not shown), corresponding to circulating plasma flavonoid levels.

For the MPO/nitrite-induced lipid peroxidation, the duration of the lag phase in the presence of flavonoid appears to depend on MPO-catalyzed oxidative conversion of the flavonoid. The oxidation of flavonoids also occurred in the absence of LDL (Fig. 5), showing that flavonoids can serve as immediate substrates for MPO. Consequently, the parent flavonoids, in particular those bearing a catechol arrangement in the B-ring, must be highly potent inhibitors of lipid peroxidation of LDL in the sub-micromolar range, but lose this high inhibitory potency by reaction with the MPO/GOD/nitrite system.

Nitrogen dioxide has been proposed to be the reactive species produced by the MPO/nitrite system [4,5,15] although other species have also been discussed [6]. Recently, direct experimental evidence for NO_2 formation in this system has been presented [9]. We assume that the flavonoids act as potent scavengers of NO_2, thus preventing lipid peroxidation as long as they are not oxidized by MPO. Direct inhibition of MPO activity by reaction with enzyme protein can be ruled out owing to the demonstration of MPO activity with various flavonoids as co-substrates.

MPO-derived nitrogen dioxide has also been proposed to be responsible for a sizeable part of protein tyrosine nitration in vivo [8,9,16]. Interestingly, we could not observe any formation of nitrotyrosine in LDL under the conditions described here (data not shown). This observation implies that the reaction of MPO with LDL at physiologically relevant concentrations of nitrite exhibits high selectivity towards the lipid moiety. A quite opposite behavior has been reported for peroxynitrite [17], which shares with nitrogen dioxide the properties of being both strong oxidant and nitrating agent. We have observed sizeable tyrosine nitration of LDL by peroxynitrite without significant lipid peroxidation (data not shown) which is in line with reported data [17,18].

The products of the reaction of MPO with flavonoids appear to be heterogeneous. Awad et al. [19] separated the reaction products with horseradish peroxidase by high-performance liquid chromatography and observed at least 20 different fractions; this heterogeneity was diminished when the reaction was carried out in the presence of glutathione. The large variation of the oxidation products is presumably due to the presence of several hydroxyl groups (e.g. five in quercetin, taxifolin and (−)-epicatechin), which permits the formation of several types of semiquinones, quinones and quinone methides [20]. Longer reaction periods of MPO/GOD/nitrite with various flavonoids gave rise to a shoulder in the UV spectrum at 295 nm (not visible in Fig. 4), which indicates loss of conjugation between A- and B-rings with the C-ring either by loss of the 2,3-double bond or cleavage of the C-ring (I.M.C.M. Rietjens, Wageningen, The Netherlands, personal communication).

Since nitrite is an oxidation product of nitric oxide under physiological conditions, our data also indicate a favorable modulation of NO metabolism that may contribute to supposed beneficial effects of dietary flavonoids.

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