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Research Article

Synergistic effects of phenolics and carotenoids on human low-density lipoprotein oxidation

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Low-density lipoprotein oxidation is believed to play an important role in the development of atherosclerosis and therefore a high resistance of LDL against oxidation may prevent atherogenesis and accompanying disorders. Several secondary plant metabolites have been tested for their ability to prevent oxidation of LDL and many phenolics as well as carotenoids have been shown to enhance LDL oxidation resistance. We showed that the quercetingylcoside rutin is able to inhibit copper-induced formation of conjugated dienes and loss of tryptophan fluorescence in LDL. However, enrichment of LDL with the carotenoids lutein or lycopene did not result in an alleviation of LDL oxidation. Since there is an agreement that not one antioxidant alone can lead to health benefits but the combination, as found for example in fruits and vegetables, is the active principle, we tested whether the combination of a phenolic compound (*i.e.* rutin) and carotenoids (*i.e.* lutein or lycopene) leads to synergistic effects. Both combinations were shown to exert supra-additive protection of LDL towards oxidation, which is most likely due to different allocation of the antioxidants in the LDL-particle and to different mechanisms of antioxidant action.

Keywords: Antioxidants / Carotenoids / Flavonoids / LDL-oxidation / Synergistic

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1 Introduction

Oxidative stress plays an important role in the development of several chronic diseases. Therefore, a high intake of antioxidants may protect from or delay the onset of those. Supplementation human intervention studies with several antioxidants (α-tocopherol, β-carotene and others), however, led to inconsistent results [1–6]. Therefore, it is supposed that not a single compound is able to prevent diseases but the combination of different antioxidants may lead to beneficial effects and by this means protect from several disorders [7–9]. One important pathological disorder associated with oxidative stress and antioxidants is the oxidation of LDL and the following development of atherosclerosis [11–12]. For this reason a sufficient protection of LDL by antioxidants may provide protection from atherosclerosis [13, 14]. In this context, α-tocopherol and ubiquinol are well known for their ability to protect LDL from oxidation and particularly for their synergistic effects and "pecking order" [15–20]. In recent years, also several phenolics have been investigated for their capability to protect LDL from oxidation and some showed high *in vitro* antioxidative capacity. Another class of antioxidants, *i.e.* the carotenoids, has already been investigated on their antioxidative capacity regarding oxidation of LDL but the obtained data are somewhat confusing. Packer [21] showed that β-carotene may play an important protective role against oxidation of LDL but Gaziano *et al*. [22] could not show an inhibition of LDL-oxidation by β-carotene *in vitro* and *in vivo*. Lycopene alone also did not exert a protective effect on LDL even in quite high concentrations [23]. In several supplementation studies, an enrichment of LDL with diverse carotenoids could be achieved, but this had no effect on oxidation resistance of LDL [24–27], in spite of the fact that a high intake of carotenoids – or rather fruits and vegetables – is associated with a lower risk for cardiovascular diseases. This again supports the assumption that not single substances but the natural combination is the active principle for the pronounced health benefits. It has been shown in several investigations that a combination of antioxidants can result

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in cooperative effects. Especially in case of phenolics, synergistic effects were shown toward LDL oxidation [28, 29]. Ferulic acid together with α-tocopherol, β-carotene and ascorbic acid brought about a synergistic inhibition of lipid peroxidation [30]. Also for carotenoids cooperative effects were shown. Shi *et al*. [31] showed that a carotenoid mixture was more effective in protecting liposomes from oxidation than the individual carotenoid. Likewise, β-carotene combined with α-tocopherol results in a significantly better inhibition of lipid peroxidation than the single antioxidants [32]. We recently proved synergistic effects of the quercetingylcoside, rutin, together with a hydrophilic (ascorbate) and a lipophilic antioxidant (γ-terpinene) in copper-mediated LDL oxidation [33]. In this investigation, we again combined two classes of secondary plant metabolites – the phenolic compound rutin and the carotenoids lutein and lycopene – in order to analyze their single and combined antioxidant activity.

2 Materials and methods

2.1 Materials

Lutein and lycopene were obtained from AquaNova, Germany. All other chemicals were purchased from either Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

2.2 Methods

2.2.1 Preparation of LDL

EDT-Acontaining plasma samples (1 mg/mL) were prepared from blood of ten healthy donors (five females and five males, 23 – 40 years old), pooled and supplemented with sucrose (0.6% final concentration). *In vitro* loading of LDL with carotenoids was performed by addition of 0.1 to 0.4 mL of a carotenoid solution, which contained 0.05% w/w lycopene, or lutein, respectively (SMO80 was used as emulgator) to 3 mL of human blood plasma and ensuing incubation at 37°C for 1.5 h.

The preparation of LDL (d = 1.019 – 1.063 g/mL) by density gradient ultra-centrifugation was conducted as described by Kögl and coworkers [34]. Plasma (3 mL) was adjusted to a density of 1.41 g/mL with KBr in Beckmann Polyallomer Centrifuge tubes (No. 331372) and stratified with approximately 2.5 mL density solution A (1.080 g/mL; 1 g EDTA/L), B (1.050 g/mL; 1 g EDTA/L) and C (1000 g/mL; 1 g EDTA/L) per layer. After 22-h centrifugation at 10°C with 40 000 rpm (285 000 × g) three different layers appear above the plasma: lowest HDL, then LDL, on the surface VLDL and chylomicrones. After gaining the LDL layer, it was filtered through a 0.22-µm filter (Millex1-GS, Millipore Corporation, Bedford, Ireland) and the purity was confirmed by SDS-PAGE. After that, LDL samples were desalted by gel filtration (Econo-Pac 10 DG desalting column, Bio-Rad). To minimize oxidation of the starting material, the samples were utilized immediately after isolation. LDL content was determined by protein quantification using the Bio-Rad protein assay and assuming a formular weight of 540 kDa for ApoB 100.

2.2.2 Oxidation of LDL

Formation of conjugated dienes was followed at 37°C using a Kontron Instruments Uvikon 922 spectrophotometer by monitoring the increase in absorbance at 234 nm every 10 min for 1000 min. The assays contained 25 µg/mL protein of the different LDL samples and 1.68 µM Cu^{2+} ions ($CuSO_4$) to induce oxidation [35]. The lag time, *i.e*. the time until rapid oxidation measurable as a rapid increase in absorption at 234 nm is observed, is a measure for the oxidation resistance of the LDL sample.

Loss of tryptophan fluorescence is a marker for oxidation of ApoB 100 of LDL. Measurements were conducted at 282 nm excitation and 331 nm emission wavelengths using a Hitachi F-4500 fluorescence spectrometer. The assays contained 50 µg protein of the different LDL samples/mL and 3.36 µM Cu^{2+}ions ($CuSO_4$) to induce oxidation [36, 37]. All cuvettes had to be removed from excitation light into darkness between the single measurements to avoid photooxidation of tryptophan residues. Fluorescence was measured every 20 min. The time until rapid loss of fluorescence occurs, is a measure for the oxidation resistance of the LDL sample.

2.2.3 Extraction and quantification of antioxidants

The antioxidant and carotenoid content of the different LDL samples was determined as follows: 250 µL of the LDL sample was mixed thoroughly with 250 µL of ethanol in order to precipitate the proteins. The ethanol contained α-tocopherolacetate as an internal standard. Subsequently, 500 µL of hexane was added and the sample was vortexed for 1 min. After centrifugation (4000 U/min, 3 min, 4°C), 400 µL of hexane was withdrawn. Further 400 µL hexane was added to the sample, which again was centrifuged. Again, 400 µL hexane was removed and the collected hexane phases were evaporated to dryness. The antioxidants were resolved in 50 µL ethanol and analyzed by HPLC on a Nucleosil 300 ODS column (125*4.6 cm; 7 µm) at isocratic conditions (methanol:ACN:tetrahydrofurane 5:4.5:0.5; 1 mL/min), a column temperature of 35°C and detection at 285 nm.

3 Results and discussion

3.1 Content of antioxidants in LDL

After incubation of human blood plasma with lycopene or lutein, both carotenoids were enriched in LDL. Lycopene was increased twofold, *i.e*. content in enriched LDL was 1.8 mol/mol LDL and lutein 5.4-fold (up to 4.9 mol/mol

Table 1. Content of lipophilic antioxidants in LDL

Antioxidant	Content in LDL mol/mol LDL
Lutein	0.9 ± 0.2
α-Tocopherol	11.6 ± 2.1
Lycopene	0.9 ± 0.3
β-Carotene	0.5 ± 0.1
Ubiquinol	0.3 ± 0.1

LDL). Other antioxidants only varied slightly between the samples. Table 1 gives the mean values with SD ($n = 11$).

3.2 Influence of single compounds on LDL-oxidation

Rutin caused a concentration-dependent elongation of the lag phase during copper-induced formation of conjugated dienes: 0.5 μM rutin already led to a slight increase of the lag phase and a concentration of 5 μM delayed the lag phase beyond the measurement period (Fig. 1). Also for protein oxidation a protective effect of rutin towards LDL was observed. When rutin was added 2.5 μM, the loss of tryptophan fluorescence was delayed by nearly 350 min (Fig. 1). The inhibition of LDL-oxidation by flavonoids has been subject of several *in vitro*, *ex vivo* and *in vivo* investigations [38]. The antioxidant capacity of flavonoids is related to their structure [39, 40] and diverse mechanisms are discussed, by which flavonoids can act as antioxidants: they may scavenge free radicals, chelate transition metals, protect or regenerate other antioxidants or inhibit or protect several enzymes [38, 41 – 43].

As shown above, lycopene as well as lutein could be enriched in LDL; however, no increased resistance of LDL to oxidation could be detected. The increase of the lag phase was 9.8 ± 6.8 min for lycopene and 8.1 ± 6.8 min for lutein. This elongation of the lag phase is negligible and within the range of deviation of the test system. The same was observed for protein oxidation of LDL: neither the lycopene-enriched LDL nor the lutein-enriched one showed an increased oxidation resistance. This is in accordance with several trials regarding carotenoid effectiveness to protect LDL from oxidation. A supplementation with tomato juice, carotenoid mixtures or lycopene led indeed to an increase of carotenoids in plasma as well as in LDL, however no effect on the susceptibility of LDL to oxidation could be observed [24, 25]. However, there are also results indicating a protective effect of carotenoids. In a study by Agarwal and Rao [26] lycopene was shown to be increased significantly in serum and this increase was accompanied by a decrease of LDL-cholesterol oxidation. Likewise, for lutein, some results point to a protective effect towards LDL-oxidation and the progression of early atherosclerosis [27]. Thus, regarding LDL-oxidation, several studies came to inconsistent results and therefore the fact that a high

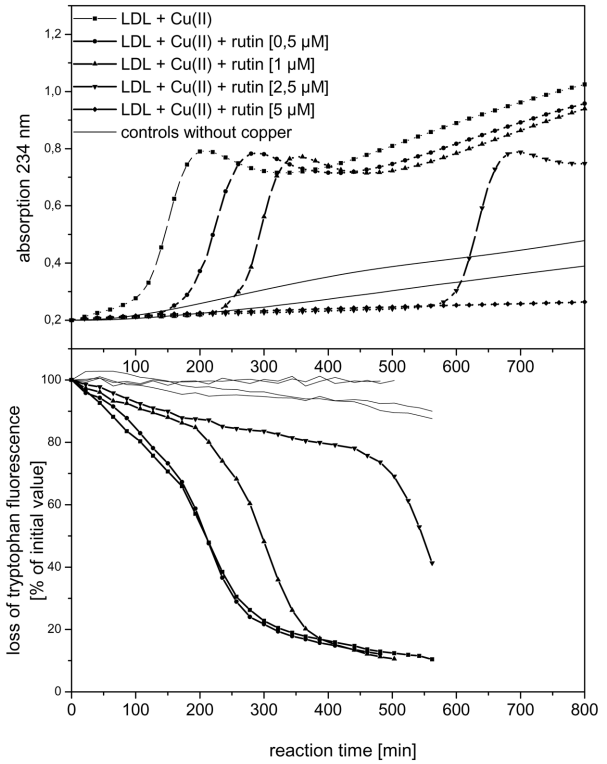

Figure 1. Influence of added rutin on copper-induced formation of conjugated dienes (upper part) and loss of tryptophan fluorescence (lower part) in LDL.

intake of carotenoids is associated with a lower risk for cardiovascular diseases must be due to a more complex mechanism. This led us to the following conclusions: (i) There may be other mechanisms than only the oxidation of LDL by which carotenoids provoke a reduced risk of atherosclerosis, *e. g.* an inhibition of HMG-CoA-reductase as it was shown for lycopene [44] and (ii) not the carotenoids alone bring about an amelioration of oxidative stress, but rather the combination of antioxidants (*e. g.* lipophilic and hydrophilic) is important.

For this reason, in the following experiments we tested whether the combination of rutin, a more hydrophilic antioxidant in combination with lycopene and lutein is able to protect LDL from oxidation.

3.3 Influence of rutin in combination with lycopene or lutein on LDL-oxidation

It is now believed that the combination of phytochemicals from fruits and vegetables is responsible for the antioxidant and disease-preventing activity of these foods. This is explained by synergistic effects of different phytochemicals [7 – 9]. Those effects have been shown for combinations of phenolics [28] as well as for the combination of different carotenoids [31]. But also combining lipophilic antioxidants with hydrophilic ones can lead to synergistic effects

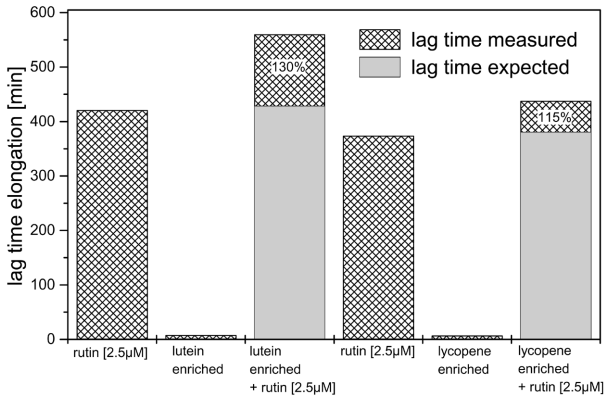

Figure 2. Synergistic effects of lycopene or lutein in combination with rutin on lag time elongation.

[23, 33, 45]. For this reason, we incubated plasma with the lipophilic antioxidants lycopene or lutein to load LDL with these carotenoids. Lycopene was increased 2-fold and lutein 5.4-fold. As shown in Table 1, the contents of the other antioxidants remained unchanged. After LDL isolation, the hydrophilic antioxidant rutin was added and oxidation was initiated by addition of copper. For both carotenoids we found synergistic effects together with rutin regarding formation of conjugated dienes. The expected lag time for the combination of lycopene-enriched LDL and 1 µM rutin is 249 min, the measured value is 303 min. A similar effect is reached when rutin is added in a concentration of 2.5 µM, the expected value is 481 min and the measured one 538 min. This synergistic effect is even more pronounced for a combination of rutin and lutein. For lutein-enriched LDL combined with 1 µM rutin the expected lag time is 305 min, but the measured value is 379 min. For 2.5 µM rutin in combination with lutein-enriched LDL the expected value is 559 min and the measured one 690 min. Figure 2 shows the lag time shift for lutein- or lycopene-enriched LDL, respectively, in combination with 2.5 µM rutin. Percent values show the synergistic effect. In case of protein oxidation the effects were only additive (data not shown).

These results are in accordance with earlier investigations that also proved synergistic effects due to combination of lipophilic and hydrophilic antioxidants (Fuhrman *et al*. [23]).

The antioxidant and possibly synergistic actions of the investigated substances mainly depend on the following factors:

3.3.1 Localization of the antioxidants

Fuhrman *et al*. [23] who showed synergistic effects of lycopene in combination with vitamin E, glabridin, rosmarinic acid, carnosic acid or garlic explained the found effects by different localization of the antioxidants in the LDL-particle. The more hydrophilic compounds are located at the outer surface of the particle whereas the more hydrophobic are stored in the core. Besides an ideal allocation of the antioxidants, this establishes the possibility of α-tocopherol being regenerated at the surface. The localization of carotenoids in the lipophilic core enables them to scavenge lipophilic radicals more effectively than rutin, which is located or even bound at the outer surface [46]. The different localization is also the reason for the fact that the carotenoids are not able to prevent protein oxidation of LDL, because here the action takes place only at the surface, where the carotenoids are not present.

The more pronounced synergistic effect in case of lutein compared to lycopene may also be due to a localization effect. Lutein as a xanthophyll is arranged parallel to the phospholipids at the outer layer of LDL, *i.e*. it adopts a transmembrane orientation [47 – 49]. The more lypophilic lycopene without hydroxyl groups will, however, be placed in the core of the LDL particle. This positioning of the molecules has influence on their reactivity towards, for example, peroxyl radicals. β-Carotene and lycopene are able to react only with the reactive-oxygen species generated in the hydrophobic part of the LDL particle whereas lutein penetrates into the polar zone where it is exposed to the aqueous environment. Therefore, lutein may be able to be regenerated at the surface as it was also reported for α-tocopherol. The "localization effect" was also demonstrated by Stahl *et al*. [50], who showed that among different carotenoid-mixtures lycopene and lutein, *i.e*. a xanthophyll, which spans the membrane, and a carotene, which is located in the core, showed highest synergy regarding the protection of liposomes from oxidation.

3.3.2 Reactivity and reaction mechanism of the antioxidant toward the radical

The variable mechanisms by which the antioxidant action is achieved may account for the synergistic effects. Carotenoids are known to be excellent quenchers of singlet oxygen and scavengers of peroxyl radicals, respectively. Flavonoids may also scavenge diverse radicals but additionally are able to chelate transition metals. Actually, it has been shown by de Souza and de Giovanni [51] that the complexed flavonoids are much more effective free radical scavengers than the free flavonoids, which also may contribute to the synergistic actions.

3.3.3 Fate and features of the antioxidant-derived radical, especially possible prooxidative properties

It has been shown that the α-tocopheroxylradical, which is derived from α-tocopherol after reaction with a free radical, can act as prooxidant. Ascorbic acid reduces α-tocopheroxyl radicals and thus inhibits α-tocopheroxyl radical-mediated oxidation [52]. In our case, rutin may act supplemental to ascorbic acid sparing α-tocopherol and/or regenerating the α-tocopheroxyl radical at the surface, thereby preventing α-tocopheroxyl from promoting oxidation. It has been shown earlier that α-tocopherol, ascorbic acid and

rutin are synergistic antioxidants regarding LDL-oxidation [53]. On the other hand, rutin may act directly as an antioxidant, *i. e*. scavenge free radicals, which results in a rutin-phenoxyl-radical. In case of those polyphenolic compounds it is supposed that the resulting phenoxyl radical is more or less stable, depending on the structure and can be regenerated by ascorbic acid as it was shown for α-tocopheroxyl radical. However, if the phenoxylradical penetrates into the LDL particle, regeneration by ascorbic acid becomes less likely and thus a prooxidant behavior could occur. In case of carotenoid-enriched LDL, however, the phenoxylradicals may be recycled by carotenoids. This reaction of carotenoids and phenoxylradicals has been shown earlier [54, 55] and is probably another component of the synergistic mechanism.

Additionally, the reverse reaction is possible: In contrast to phenolics, carotenoids do not act as hydrogen atom donor but via addition at the double bond. Thereby a carbon-centered radical is formed, which is stabilized by the conjugated polyen chain. Since this carotenoid radical is charged, it will orientate to the more polar interface, where it may react with oxygen and become a peroxylradical, which can promote oxidation. In this case rutin, which is located at the surface, may regenerate the carotenoid and so prevent prooxidative actions of the carotenoid-peroxylradical.

In summary, we can record the fact that the synergism of the tested antioxidants is due to (i) different localization of antioxidants, (ii) the diversity of defense mechanisms provided by carotenoids and flavonoids, and (iii) prohibiting prooxidative actions of the antioxidant-derived radicals.

4 Concluding remarks

It could be shown that combinations of antioxidants are more effective in preventing oxidative stress than single substances. Therefore, the network of phytochemicals is essential for their health-promoting activity, particularly when considering that an antioxidant may become a prooxidant if suitable and sufficient co-antioxidants are missing. This is a basic explanatory approach for the disappointing results of human intervention studies using single supplements, which often failed to prove health benefits. Thus, our results again emphasize the message that not high concentrations of a single antioxidant are “healthy” but that a high intake of fruits and vegetables and in this way a high intake of diverse phytochemicals are beneficial to health.

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