Ascorbate prevents prooxidant effects of urate in oxidation of human low density lipoprotein

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Abstract Uric acid and ascorbic acid are important low molecular weight antioxidants in plasma. Their interactions and combined effect on Cu^{2+}-catalysed oxidation of human low density lipoprotein were studied in vitro. It was found that uric acid alone becomes strongly prooxidant whenever it is added to low density lipoprotein shortly after the start of oxidation (conditional prooxidant). Ascorbic acid, which is present in human plasma at much lower concentrations (20–60 μM) than urate (300–400 μM), is in itself not a conditional prooxidant. Moreover, ascorbate prevents prooxidant effects of urate, when added to oxidising low density lipoprotein simultaneously with urate, even at a 60-fold molar excess of urate over ascorbate. Ascorbate appears to have the same anti-prooxidant effect with other aqueous reductants, which, besides their antioxidant properties, were reported to be conditionally prooxidant. Such interactions between ascorbate and urate may be important in preventing oxidative modification of lipoproteins in the circulation and in other biological fluids.

Key words: Low density lipoprotein; Uric acid; Ascorbic acid; Antioxidant; Prooxidant; Anti-prooxidant

1. Introduction

Oxidation of low density lipoprotein (LDL) has been suggested to play a key role in the development of atherosclerosis and as a consequence an important role in the prevention of LDL oxidation has been attributed to the antioxidants contained within LDL and in plasma (for a review see [1]). The major low molecular weight antioxidants in plasma are urate (UA, 300 μM, approximately) and ascorbate (AA, 20–60 μM, approximately) [1,2], and they appear to be mainly responsible for the fact that only very small amounts of oxidised LDL are found in the circulation. Recently, many aqueous antioxidants/reductants were found to promote metal-catalysed oxidation of LDL, whenever the major endogenous antioxidant of LDL, α-tocopherol, has been already consumed. The list of substances capable of such redox activation of transition metals includes trolox [3], a water soluble analogue of α-tocopherol, dehydroascorbate (DHA) [4–6], flavonoids [5], anthocyanins [7], catechins [8] and a variety of other aqueous reductants. AA has been reported to act likewise [4,5], and prooxidant effects have also been observed in vivo [9], but other reports [6,10] have left some doubt regarding prooxidant effects of AA in LDL oxidation. This is even more remarkable as in metal-containing solutions, AA is rapidly converted to DHA. However, DHA derived from AA during Cu^{2+}-catalysed oxidation of LDL does not become prooxidant [4,5], suggesting a more general ability of AA to prevent prooxidant effects of antioxidants.

In this paper we report prooxidant properties of UA, in Cu^{2+}- catalysed LDL oxidation and the ability of AA to counteract the prooxidant effects of UA.

2. Materials and methods

Ascorbic acid was from Loba (Fischamend, Austria), dehydroascorbate was from Sigma (Vienna, Austria), all other chemicals used were from Merck or Sigma (Vienna, Austria) and of analytical grade or better.

2.1. LDL preparation

LDL was prepared from pooled EDTA plasma of healthy volunteers of both sexes by ultracentrifugation in a single step discontinuous gradient in a Beckman NVT65 rotor, as described [11]. Prior to use, LDL was stored in an evacuated glass vial under argon at 4°C for a maximum of 2 weeks. The LDL concentration was determined from its cholesterol content, using the CHOD-PAP enzymatic test kit (Boehringer-Mannheim, Germany), assuming a molar mass of LDL of 2.5 MDa and a cholesterol content of 32.2 weight%.

2.2. LDL oxidation

Prior to oxidation of LDL, EDTA and KBr were removed by gelfiltration as described [12]. Unless indicated otherwise, oxidation was performed at 37°C by addition of a 100 μM solution of $CuSO_4$ to a 0.1 μM solution of LDL in PBS (160 mM NaCl, 10 mM Na-phosphate, pH = 7.4) to give a final concentration of 1.6 μM Cu^{2+}. The progress of oxidation was monitored spectrophotometrically (Beckman DU-640, equipped with Peltier-thermostatted six cell holder) by the formation of conjugated dienes at 234 nm (A_{234}) [12]. Lag-times of LDL oxidation were determined as the time coordinate of the intersection of the tangents to the slow and fast phases of conjugated diene formation. Solutions of aqueous antioxidants in PBS were added prior to addition of copper, or at the indicated time points, to give the indicated concentrations.

2.3. Low level chemiluminescence (LL-CL) measurements

LL-CL was measured in a LUCY I luminometer (Anthos Labtech Instruments, Salzburg, Austria) equipped with a single photon counting photomultiplier (sensitivity 300–700 nm) as described [13]. The integration time was set to 90 s, measurements were performed in a white microplate. LL-CL is due to the termination of peroxyl radicals and formation of excited oxygen species (in this case mostly triplet carbonyls are monitored [13]) which emit light upon return to the ground state. The intensity of LL-CL corresponds to the square of the oxidation rate.

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Abbreviations: LDL, low density lipoprotein; AA, ascorbic acid/ascorbate; DHA, dehydroascorbic acid/dehydroascorbate; UA, uric acid/urate; PBS, phosphate buffered saline; EDTA, ethylenediamine tetraacetic acid; A_{234}, absorbance at 234 nm; LL-CL, low level chemiluminescence; LOOH, lipid hydroperoxide; LOO•, lipid peroxyl radical; LO•, lipid alkoxyl radical

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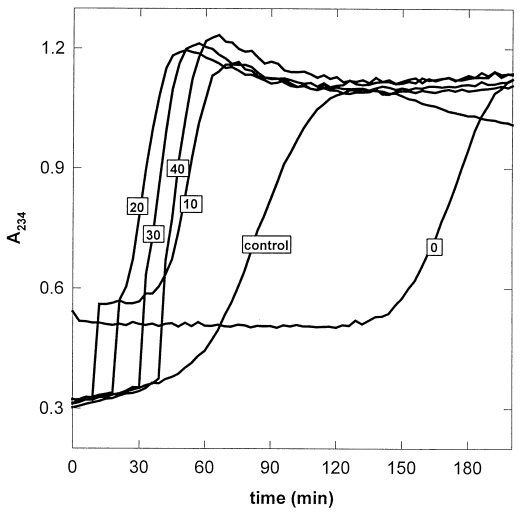

Fig. 1. Antioxidant and prooxidant effect of UA in LDL oxidation. A 0.1 μM solution of LDL was oxidised with 1.6 μM Cu^{2+}, monitoring the A_{234}, in the absence (control) or presence of 20 μM UA. UA was either present from the beginning (0) or added at the indicated time points. The immediate increase of A_{234} (0.3→0.55) after addition of UA is due to UA itself.

3. Results

3.1. Antioxidant and prooxidant effects of urate on the LDL oxidation

Incubation of 0.1 μM LDL with 1.6 μM Cu^{2+} in the presence of 20 μM UA (at t = 0 min) led to a considerable prolongation of the lag-phase (typically from 60 min to 150 min) (Fig. 1 shows one representative experiment of four). Addition of UA at later time points, when oxidation had already started and the α-tocopherol content of LDL was reduced or already exhausted (30 min under these experimental conditions, compare [3]) led to a considerable reduction of the lag-time or even to the immediate start of the propagation phase. After addition of UA, the maximal oxidation rate during propagation was strongly increased compared to the control (the immediate increase in absorbance after addition is due to UA).

3.2. AA, but not DHA prevents prooxidant effects of UA

When 0.1 μM LDL was oxidised by 1.6 μM Cu^{2+} in the presence of 20 μM AA plus 20 μM UA (mixed before addition), no prooxidant effect of UA, as shown in Fig. 1, could be observed, irrespective of the time point of addition (Fig. 2A). Addition of a combination of 20 μM AA and UA at t = 0 min produced a very long lag-phase (450 min), which was about halved when AH and UA were added at t = 30 min, but still much longer than the control. In order to check whether this anti-prooxidant effect of AH extends to other aqueous reductants, we repeated the experiment using 3 μM of trolox, a water soluble analogue of α-tocopherol, which has been shown previously to be a conditional prooxidant [3], and found a similar behaviour (not shown).

Fig. 2B shows that DHA does not exert anti-prooxidant effects. Rather, addition of 20 μM DHA and 20 μM UA at t = 30 min produced an even stronger prooxidant effect on the LDL oxidation. Note also that the antioxidant effect of DHA+UA at t = 0 is about the same as AH+UA at t = 30 min.

3.3. Anti-prooxidant effect of 20 μM AA at 300 μM UA

In human plasma, the concentrations of UA are much higher than those of AA. In order to investigate the anti-prooxidant effect of AA in presence of a large excess of UA, LL-CL was used to monitor the oxidation, due to the strong UV absorbance of UA.

In this experiment, 0.3 μM LDL was incubated with 4.8 μM of Cu^{2+}. Fig. 3 shows that the presence of 20 μM AA and/or 300 μM UA at t = 0 led to a practically complete suppression of the LDL oxidation (longer than 800 min in these experiments). Addition of 300 μM UA at t = 35 min led to an immediate increase of LL-CL, consistent with the prooxidant effect of UA observed already for 20 μM UA, by monitoring the conjugated diene formation (Fig. 1). Note that the maximum rate of oxidation is about four-fold higher (two-fold higher LL-CL, [13]) due to the prooxidant effect of UA. Simultaneous the addition of 20 μM AA and 300 μM UA at 35 min, however, prevented a significant increase in LL-CL. Rather, LDL oxidation was suppressed for more than 800 min, similar to the effect of 300 μM UA added at t = 0. Even at a much higher Cu^{2+} concentration (167 μM), the simultaneous addition of AA and UA led only to a small oxidative burst (data not shown), corresponding to about 10% of the rate produced by adding UA alone at 35 min.

4. Discussion

4.1. Antioxidant and prooxidant properties of UA

Fig. 1 shows that UA is not only a good antioxidant in

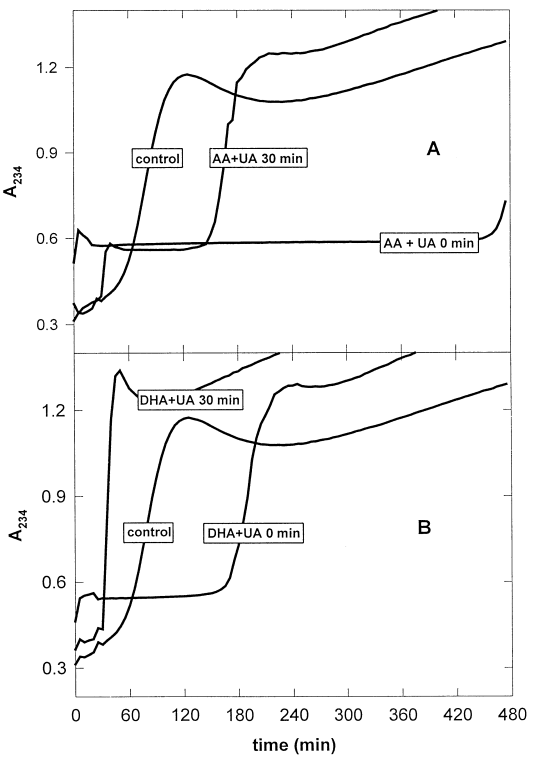

Fig. 2. Effects of AA and DHA on the prooxidant effect of UA. LDL (0.1 μM) was oxidised with 1.6 μM Cu^{2+} (control) and the A_{234} monitored. A: a mixture of 20 μM AA and 20 μM UA was present from the beginning of oxidation (0) or added at 30 min (30). B: the procedure as in A, but 20 μM DHA was used instead of AA.

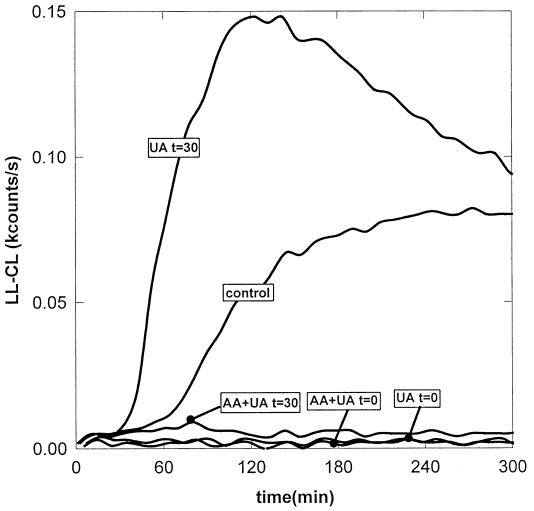

Fig. 3. Anti-prooxidant effect of 20 μM AA at 300 μM UA. LDL (0.3 μM) was oxidised with 4.8 μM Cu^{2+}. UA (300 μM) was either present from the beginning or added 35 min after the start of oxidation, either with or without 20 μM AA and LL-CL was monitored. Note that LL-CL is proportional to the square of the oxidation rate [13].

metal-catalysed LDL oxidation but that it also shares, with many aqueous antioxidants, the property of switching to a prooxidant mode whenever added after (partial) depletion of α-tocopherol (conditional prooxidant). The prooxidant potential of UA appears to be quite high, compared to other conditional prooxidants: only 10 min after oxidation (during which time about one third of the α-tocopherol content is lost under our experimental conditions (compare [3])), UA already acts as a prooxidant, whereas most other water soluble reductants become prooxidant only when most or all of the α-tocopherol in LDL has been consumed (compare [3–5,7–9]).

4.2. Exclusively antioxidant and anti-prooxidant properties of AA but not DHA

AA appears to have very particular antioxidant properties, as it is the only aqueous antioxidant described so far which is not a conditional prooxidant [4,6] in the Cu^{2+}-mediated oxidation of LDL. This is even more surprising, as brief incubation with transition metal ions is sufficient to convert AA to DHA, which itself is a conditional prooxidant.

The antioxidant properties of AA have been attributed largely to its ability to reduce a α-tocopheroxyl radical back to α-tocopherol (reviewed in [14]). However, AA is an extremely effective antioxidant even after consumption of α-tocopherol (and thus of α-tocopheroxyl radical) in LDL, rather than becoming prooxidant: its antioxidant effect is concentration-dependent (unpublished data, [4,5]).

4.3. Possible mechanism of prooxidant effects of aqueous reductants

Consistent with experimental data, the prooxidant properties of water soluble antioxidants can be attributed to an enhancement of redox cycling of transition metal ions at a lipid-aqueous phase boundary: $Cu^{2+}+LOOH \rightarrow Cu^{+}+LOO^{\bullet}+H^{+}$ (lipid phase, slow) (1) $Cu^{+}+LOOH \rightarrow Cu^{2+}+LO^{\bullet}+OH^{-}$ (lipid phase, fast) (2) $Cu^{2+}+RedH \rightarrow Cu^{+}+Red^{\bullet}+H^{+}$ (aqueous phase, fast) (3)In this reaction scheme, the slow, rate limiting reduction of Cu^{2+} by lipid hydroperoxides (LOOH, reaction 1) is enhanced by the reduction of Cu^{2+} through an aqueous reductant (RedH, reaction 3).

As long α-tocopherol is present in LDL, which itself acts as a prooxidant by reducing Cu^{2+} [15,16], such an enhanced reduction has no observable effects because α-tocopherol and α-tocopheroxyl radical can both scavenge the lipid radicals generated.

4.4. Possible mechanisms of the anti-prooxidant action of AA

There are several possible explanations for this peculiar anti-prooxidant effect of AA: (i) It has been suggested that the urate radical, formed by oxidation of UA with Cu^{2+} [17] could re-initiate lipid peroxidation. AA would then scavenge such radicals. However, the anti-prooxidant effect of AA includes not only UA but also AA and Trolox, the radicals of which have different reactivities. Moreover, such a re-initiation by antioxidant radicals is inconsistent with the good antioxidant properties of these substances in the presence of TocOH (compare also (iii)). (ii) We could rule out the possibility that AA is able to regenerate TocOH even after its complete consumption. (iii) It appears reasonable that AA is able to redox inactivate LOOH and keeps the metal in the reduced state (eq. (3)), which would effectively prevent redox cycling. This is corroborated by the observation that higher concentrations of UA in combination with AA lead to a better antioxidant effect even under conditions where UA alone is prooxidant (compare Figs. 2 and 3). This also contradicts (i). (iv) It has been reported that the formation of 2-oxo-histidine residues on apoB-100 might reduce copper binding and hence be in part responsible for the fact that AA does not become prooxidant. However the modifying agent in [6] appears to be DHA and not AA. Moreover, preincubation of LDL with either AA or DHA (data not shown) failed to render LDL insensitive to the prooxidant behaviour of UA. It is conceivable, however, that histidine residues [10,18], copper, LOOH and AA act together in this anti-prooxidant effect.

4.5. Conclusion

AA appears to be an effective anti-prooxidant even in the presence of a large excess of UA, at concentrations of AA which are at the lower boundary of 'normal' AA concentrations in human plasma (Fig. 3). Apparently, AA does not simply compete with the prooxidant, it rather leads to very effective uncoupling of metal reduction from radical formation.

The anti-prooxidant property of AA is particularly important in the light of increasingly frequent reports about conditionally prooxidant properties of many powerful antioxidants in LDL oxidation. Under circumstances, such as moderate depletion of endogenous antioxidants in lipoprotein, the urate-ascorbate system in human plasma appears to be quite 'well-designed' to preserve their integrity.

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