When and why a water-soluble antioxidant becomes pro-oxidant during copper-induced low-density lipoprotein oxidation: a study using uric acid

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The inclusion of uric acid in the incubation medium during copper-induced low-density lipoprotein (LDL) oxidation exerted either an antioxidant or pro-oxidant effect. The pro-oxidant effect, as mirrored by an enhanced formation of conjugated dienes, lipid peroxides, thiobarbituric acid-reactive substances and increase in negative charge, occurred when uric acid was added late during the inhibitory or lag phase and during the subsequent extensive propagation phase of copper-stimulated LDL oxidation. The pro-oxidant effect of uric acid was specific for copper-induced LDL oxidation and required the presence of copper as either Cu(I) or Cu(II). In addition, it became much more evident when the copper to LDL molar ratio was below a threshold value of approx. 50. In native LDL, the shift between the antioxidant and the pro-oxidant activities was related to the availability of lipid hydroperoxides formed during the early

phases of copper-promoted LDL oxidation. The artificial enrichment of isolated LDL with α -tocopherol delayed the onset of the pro-oxidant activity of uric acid and also decreased the rate of stimulated lipid peroxidation. However, previous depletion of α -tocopherol was not a prerequisite for unmasking the pro-oxidant activity of uric acid, since this became apparent even when α -tocopherol was still present in significant amounts (more than 50 % of the original values) in LDL. These results suggest, irrespective of the levels of endogenous α -tocopherol, that uric acid may enhance LDL oxidation by reducing Cu(II) to Cu(I), thus making more Cu(I) available for subsequent radical decomposition of lipid peroxides and propagation reactions.

Key words: antioxidants, bathocuproine, hydroperoxides, oxidation.

INTRODUCTION

The concerted activity of several enzymic and non-enzymic antioxidant systems represents, under physiological conditions, a rather efficient defence barrier against the potential damage exerted by free radicals and reactive species to biologically relevant molecules such as lipids, proteins, carbohydrates and nucleic acids [1]. This is especially true in the water-soluble fraction of plasma where this kind of protection is typical of a number of low-molecular-mass compounds including ascorbic acid, bilirubin, thiols and uric acid [2]. Uric acid (and its monoanion urate) has received much attention, especially because of its relatively high concentration (300 μ M) in plasma as compared with that of ascorbic acid (30–50 μ M), bilirubin (5–20 μ M) and glutathione (< 2 μ M) [2]. Moreover, in a recent study, Nyyssonen et al. [3] have found that urate is the strongest determinant of plasma antioxidant capacity and of the resistance of plasma lipids to oxidation.

Among the various substrates which can be oxidized by free-radical-mediated reactions are low-density lipoproteins (LDL) and the formation of oxidatively modified LDL has been critically associated with the development and progression of atherosclerotic lesions both in laboratory animals and in man [4,5]. Little is known about the molecular mechanisms underlying LDL oxidation *in vivo*, but reactions involving transition metals, such as copper and iron [6], free radicals [6], hypochlorous acid [7], peroxynitrite [8] and the activity of selected enzymes, such as myeloperoxidase [9] and lipoxygenase [10], have been alternatively claimed to play a role. Experimental, clinical and epidemiological data indicate, in addition, that these processes can be efficiently halted by either LDL-associated (lipid-soluble) and non-associated (water-soluble) antioxidants [11]. Urate may be a key compound among the latter, since a major site of uric acid

production is the microvascular endothelium [12], the place where LDL oxidation is thought to take place [5].

Several *in vitro* systems have been developed to mimic the reactions occurring *in vivo* and to investigate the susceptibility of isolated LDL to oxidation. Among them, the experimental set-up involving the pro-oxidant activity of copper ions has become more and more popular [13]. This system has also been successfully employed to investigate the inhibitory effect exerted by a variety of different lipid-soluble as well as water-soluble antioxidants, including α -tocopherol, carotenoids, ascorbic acid and urate [11]. All these compounds have been demonstrated to significantly increase the resistance of isolated LDL to oxidation and to decrease the maximal rate of oxidation itself.

In a number of very elegant studies Stocker and co-workers [14–16] have demonstrated that the antioxidant α -tocopherol may behave, under appropriate conditions, as pro-oxidant, thus paradoxically stimulating LDL oxidation promoted by a constant radical flow originating from the thermal decomposition of the azo-compound 2,2'-azobis-(2-amidino propane) hydrochloride (AAPH) or by low concentrations of copper. The pro-oxidant activity of α -tocopherol is mediated through the one-electron oxidation catalysed by peroxy radicals or by the reductive activation of Cu(II) to Cu(I) with the concomitant formation of the α -tocopheroxyl radical which, in turn, may abstract a hydrogen atom from a polyunsaturated fatty acid in LDL, thus initiating a radical chain ultimately leading to extensive lipid peroxidation. It is not known whether this mechanism can occur with other classical antioxidants, such as uric acid, although evidence has been obtained indicating that free radical metabolites originate from the oxidation of uric acid [17] and that the combination of Cu(II) and uric acid may promote oxidative DNA breakage [18]. Moreover, studies performed with other water-soluble antioxidants, such as Trolox C [19], ascorbic

Abbreviations used: AAPH, 2,2'-azobis-(2-amidino propane) hydrochloride; BC, bathocuproine disulphonate.

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acid [20], caffeic acid [21] and green tea catechins [22], have revealed that these compound may act either as antioxidants or pro-oxidants during copper-mediated LDL oxidation.

The present study was performed in order to investigate in detail the effects exerted by uric acid on the oxidative LDL modifications promoted by copper ions. The results obtained indicate the possibility that uric acid may act as an additional pro-oxidant in LDL particles challenged with Cu(II) when lipid peroxides are formed, independently of the presence of endogenous α -tocopherol.

MATERIALS AND METHODS

Materials

EDTA, ebselen (2-phenyl-1,2-benzisoxselenazol-3[2H]-one), glutathione (reduced form), type V soybean lipoxidase (linoleate: oxygen 13-oxidoreductase, EC 1.13.11.12), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, uric acid, α -tocopherol, cholesteryl linoleate and bathocuproine disulphonate (BC) were obtained from Sigma. Cholesteryl [9-hydroxy]linoleate was from Cayman (Ann Arbor, MI, U.S.A.). Cholesteryl linoleate hydroperoxide was prepared by oxidation of a microsuspension of cholesteryl linoleate with the peroxy radical generator AAPH, obtained from Polysciences (Warrington, PA, U.S.A.). All other reagents were of the highest purity available and were obtained from local suppliers.

Lipoprotein donors

Unselected healthy normocholesterolaemic volunteers were used as lipoprotein donors. Venous blood (20 ml) was taken after an overnight fast and drawn in polypropylene tubes containing EDTA (1 mg/ml blood). Plasma was separated by centrifugation and immediately employed for lipoprotein isolation.

α -Tocopherol supplementation

EDTA-plasma (3 ml) was incubated at 37 °C in a thermostated water bath with continuous gentle stirring, without or with 0.25 mmol/l α -tocopherol dissolved in absolute ethanol [final ethanol concentration less than 1% (v/v)] for 3 h. Ethanol (at the same final concentration) was included in control, non-supplemented samples. α -Tocopherol in LDL was determined as described [23]. Briefly, LDL was precipitated using ethanol (50% final concentration) and subsequently extracted with hexane. The hexane phase was then evaporated and the residue was dissolved in acetonitrile/dichloromethane/methanol (67:19:14, by vol.) and separated by HPLC.

Lipoprotein isolation

The LDL fraction was isolated from the whole plasma by ultracentrifugation through a KBr discontinuous gradient and collected as the fraction floating at a density of 1.019–1.063 g/ml. EDTA was then removed by rapid filtration through disposable desalting columns (Econo-Pac 10 DG; Bio-Rad) and LDL was resuspended in PBS (10 mM phosphate, pH 7.2) at the concentrations indicated in the different sets of experiments {assuming that 1 mg of LDL (mass) = 200 μ g of LDL protein/ml = 0.4 μ M [11]}. Filtered LDL was immediately employed for oxidation experiments.

Lipoprotein oxidation

LDL oxidation was performed at 37 °C in disposable polypropylene tubes with continuous gentle shaking. As a pro-oxidant stimulus, CuSO_4 was employed at the concentrations indicated in the Figure legends. The occurrence of oxidative

modifications in LDL was measured as generation of either conjugated dienes in polyunsaturated fatty acids or lipid hydroperoxides. The formation of conjugated dienes was measured spectrophotometrically in quartz cuvettes in a Beckman DV 650 spectrophotometer, equipped with a thermostatic control (37 °C) and an automatically exchangeable six-positions cuvette holder, operating at 234 nm. The formation of lipid hydroperoxides was measured at various time-points using the ferrous oxidation–Xylenol Orange assay [24] or an HPLC procedure for specific quantification of cholesteryl linoleate hydroperoxide and hydroxide [25]. For the first assay, samples (100 μ l) were taken and immediately mixed with 1 ml of FOX₂ reagent, incubated at room temperature for 30 min and the absorbance read at 560 nm [24]. Lipid peroxide concentrations were determined, after subtracting the PBS blank at each time point, using a standard curve of known concentrations of t-butyl-hydroperoxide. For the second assay, samples (0.25 ml) were taken and immediately mixed with an equal volume of ethanol and 1 ml of hexane and vigorously vortexed for 1 min. The hexane phase was then evaporated to dryness and the residue was dissolved in 100 μ l of methanol/t-butanol (50:50, v/v). Separation and analysis of cholesteryl linoleate hydroperoxide and cholesteryl linoleate hydroxide were performed by reverse-phase HPLC on a 15 $\times$ 0.46 cm C₁₈ column, eluted with acetonitrile/isopropanol/water (44:54:2, by vol.) at 1 ml/min. Detection was via UV at 234 nm. Results were quantified by area comparison with appropriate standards.

Increases in the electrophoretic mobility of modified lipoproteins were measured on CelloGel strips (Labometrics, Milano, Italy) following the manufacturer's directions for electrophoretic conditions, gel staining and destaining.

Reduction of hydroperoxides in LDL

Isolated human LDL (1 mg/ml) was incubated for 30 min at 37 °C in PBS, pH 7.4, with 65 μ M ebselen and 750 μ M glutathione (reduced form). At the end of the incubation procedure the LDL samples were rapidly filtered through Econo-Pac 10 DG disposable desalting columns (Bio-Rad) and subsequently resuspended in PBS (10 mM phosphate, pH 7.2) at the concentrations indicated in the different sets of experiments.

RESULTS

Uric acid may exert either an antioxidant or pro-oxidant effect during copper-stimulated LDL oxidation

The incubation of isolated human LDL with 2.5 μ M CuSO_4 resulted in the oxidation of polyunsaturated fatty acids with the concomitant formation of conjugated dienes (Figure 1A). The oxidation profile was characterized by an initial inhibition (or lag) phase followed by a propagation phase where the rate of conjugated diene formation was maximal. The addition of uric acid (at a final concentration of 10 μ M) in the incubation medium exerted either an antioxidant or a pro-oxidant effect, depending on the time-point of addition. When uric acid was included in the incubation mixture at time 0, before the addition of copper, the subsequent formation of conjugated dienes was significantly inhibited, as revealed by the marked prolongation of the lag phase. However, when uric acid was included in the incubation medium well after copper, during either the lag phase or the propagation phase, an almost immediate stimulation of both the rate and the extent of LDL oxidation was detectable (Figure 1A). The oxidation process involved both the lipid and the protein moiety of the lipoprotein particles, as suggested by

Figure 1 Effects of uric acid on copper-, AAPH- and lipoxidase-induced oxidation of isolated human LDL

LDL was isolated from human plasma as described in the Materials and methods section and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with either 2.5 μ M CuSO₄ (A) or 1 mM AAPH (B) or 20 units/ml soybean lipoxidase (C) at 37 °C in the absence (○) or presence (●) of uric acid. At the indicated time points uric acid (10 μ M, final concentration) was added and LDL oxidation was monitored at 234 nm to follow the formation of conjugated dienes.

Figure 2 Dose-dependency of uric acid-stimulation of copper-induced LDL oxidation

The experimental conditions are similar to those reported in the legend to Figure 1, except that different concentrations of uric acid were included in the incubation medium 30 min after the addition of copper. In (A) the maximal rates of LDL oxidation are reported, whereas in (B) the decrease in the remaining lag phase (calculated as percentage of the lag phase measured in the absence of added uric acid) is reported.

their increased electrophoretic mobility on nitrocellulose strips (results not shown).

The pro-oxidant effect exerted by uric acid was apparently specific for copper-induced LDL oxidation. In fact, in another model of LDL oxidation promoted by the azo-initiator AAPH, the addition of 10 μ M uric acid at any time-point during the lag or propagation phase always resulted in a marked inhibition of lipid peroxidation, as demonstrated by the immediate inhibition of the subsequent formation of conjugated dienes (Figure 1B). As an additional model of LDL oxidation we have employed soybean lipoxidase which does not necessarily involve aqueous radical intermediates. The addition of 10 μ M uric acid at any time-point only slightly decreased the rate of conjugated diene

Figure 3 Effects of EDTA on uric acid-stimulation of copper-promoted LDL oxidation

LDL was isolated from human plasma as described in the Materials and methods section and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with 2.5 μM CuSO_4 (○). At the indicated time points uric acid (10 μM , final concentration) was added without (●) or with (▲) 200 μM EDTA. As a control, at the same time-points 200 μM EDTA (△) was added. LDL oxidation was then monitored at 234 nm to follow the formation of conjugated dienes.

formation (Figure 1C), but never stimulated an extensive oxidation comparable with that detected in the copper model.

Dose-dependency experiments using concentrations of uric acid ranging from 0.1 to 30 μM revealed that concentrations as low as 1 μM were effective in both decreasing the remaining lag phase by approx. 80 % and stimulating the maximal rate of conjugated diene formation by about 100 %. The half-maximal effects on the duration of the lag phase and the maximal oxidation rate were obtained with 0.25 and 2.5 μM uric acid respectively (Figure 2).

The pro-oxidant activity of uric acid is strictly dependent on the availability of either Cu(II) and/or Cu(I)

In order to characterize further the dependence of the pro-oxidant effect of uric acid on the availability of copper, experiments were performed using the copper chelators EDTA and BC in order to remove efficiently either Cu(II) and Cu(I) or exclusively Cu(I) [23,26]. As reported in Figure 3, the inclusion of 200 μM EDTA immediately halted the formation of conjugated dienes during both the lag phase and the propagation phase of the LDL oxidation kinetics. Furthermore, EDTA was also able to inhibit completely the stimulation of conjugated diene formation promoted by the addition of uric acid. These findings indicated that the availability of copper was a prerequisite for both the propagation of lipid peroxidation and the stimulation of lipid peroxidation by uric acid.

We have recently reported that, in the presence of relatively high concentrations of copper, the Cu(I) chelator BC significantly stimulated the peroxidation of polyunsaturated fatty acids of isolated human LDL [27]. As reported in Figure 4(A), the inclusion of 360 μM BC in the incubation mixture containing isolated human LDL and 10 μM CuSO_4 promoted accelerated formation of peroxides. The presence of 10 μM uric acid from the beginning of the incubation procedure, however, completely prevented LDL oxidation, as mirrored by the production of peroxides, irrespective of the absence or the presence of BC

Figure 4 Effect of BC on uric acid-stimulation of copper-promoted LDL oxidation

(A) LDL were isolated from human plasma as described in the Materials and methods section and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with 10 μM CuSO_4 in the absence (○, △) or presence (●, ▲) of 360 μM BC and in the absence (○, ●) or presence (△, ▲) of 10 μM uric acid. (B) LDL was isolated from human plasma as described in the Materials and methods section and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with 10 μM CuSO_4 in the absence of added BC or uric acid. After 15 and 30 min of incubation, samples were taken and incubated with 10 μM uric acid in the absence (closed bars) or presence (cross-hatched bars) of 360 μM BC. LDL oxidation was monitored by following the generation of lipid peroxides, as described in the Materials and methods section.

(Figure 4A). Moreover, in the presence of BC, the stimulation of peroxide production triggered by the addition of 10 μM uric acid during the lag phase was completely abolished, thus indicating that the availability of Cu(I) was a prerequisite for the stimulation of lipid peroxidation by uric acid (Figure 4B).

Uric acid stimulates the generation of cholesteryl linoleate hydroperoxides during copper-induced LDL oxidation

The possibility that uric acid could enhance copper-mediated LDL oxidation through activation of a sequence of chemical events different from those operating in the simple copper model was investigated by measuring the amount of lipid peroxides (namely cholesteryl linoleate hydroperoxide) and their corresponding hydroxides during the time-course of oxidation. It has in fact been reported that the incubation of isolated human LDL by different copper concentrations triggered the oxidative process

Figure 5 Generation of cholesteryl linoleate hydroperoxide and cholesteryl linoleate hydroxide during uric acid-stimulated, copper-dependent LDL oxidation

LDL was isolated from human plasma as described in the Materials and methods section and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with 2.5 μ M CuSO_4 without (○) or with (●) the subsequent addition of 10 μ M uric acid after 30 min, a condition that rapidly stimulates LDL oxidation. At different time points samples were taken from the two incubation mixtures for the contemporary measurement of conjugated dienes (absorbance at 234 nm) and cholesteryl linoleate hydroperoxide (CL-OOH) and cholesteryl linoleate hydroxide (CL-OH).

by different mechanisms, leading to the generation of different amounts of lipid hydroperoxides and lipid hydroxides [28]. As reported in Figure 5, during both copper-promoted LDL oxidation and uric acid stimulated- and copper-dependent LDL oxidation, the amounts of cholesteryl linoleate hydroperoxide and cholesteryl linoleate hydroxide formed at comparable values of conjugated dienes were practically identical. This finding suggests that uric acid did not recruit additional mechanisms of lipoprotein oxidation, but rather probably accelerated the same oxidation mechanisms involved in LDL oxidation by copper alone.

Figure 6 Effects of uric acid added at different time-points during LDL oxidation promoted by different copper concentrations

LDL was isolated from human plasma as described in the Materials and methods section and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with 1.25 (●), 2.5 (○), 5 (△), 10 (▲), 25 (□) or 50 (■) μ M CuSO_4 . Uric acid (10 μ M final concentration) was included in the incubation medium at time zero (A), 30 min (B) or 60 min (C) after the addition of copper. LDL oxidation was then monitored at 234 nm to follow the formation of conjugated dienes.

The pro-oxidant activity of uric acid is related to the copper to LDL molar ratio

Experiments were then performed in order to investigate further some quantitative aspects of uric acid-induced enhancement of copper-promoted LDL oxidation. Isolated human LDL (at a

Figure 7 Effect of uric acid added at different time-points during LDL oxidation promoted by copper at different LDL concentrations

LDL was isolated from human plasma as described in the Materials and methods section and subsequently incubated (in phosphate buffer, pH 7.4) with 1.25 (●), 2.5 (△), 5 (▲) or 10 (■) μM CuSO_4 . Uric acid (10 μM final concentration) was included in the incubation medium 30 min after the addition of copper. The LDL concentrations employed were 0.125 mg/ml (A), 0.250 mg/ml (B) and 0.375 mg/ml (C). LDL oxidation was then monitored at 234 nm to follow the formation of conjugated dienes.

concentration of 0.1 μM) were incubated with different concentrations of CuSO_4 in the presence of 10 μM uric acid added at different time-points during the lag-phase of the oxidation profile. In this set of experiments the duration of the lag phase was 82.7 ± 2.4 min when 1.25 μM CuSO_4 was employed and decreased to 76.8 ± 3.2 min with 2.5 μM CuSO_4 . Higher copper concentrations did not further decrease the duration of the lag phase as previously reported [29]. As illustrated in Figure 6(A), the

inclusion of uric acid together with copper exerted a marked inhibition of diene formation promoted by all the concentrations of copper employed. However, when uric acid was included in the incubation medium 30 min after the addition of copper, but still during the lag phase, a stimulation of diene formation was detectable exclusively when the oxidation process was triggered with 1.25 and 2.5 μM CuSO_4 at copper to LDL ratios of 12.5 and 25 respectively (Figure 6B). On the other hand, at higher copper concentrations (and at a copper to LDL ratio equal to or higher than 50), an antioxidant effect of uric acid was still evident. Finally, the inclusion of uric acid at 60 min of incubation, just at the end of the lag phase, immediately stimulated the formation of conjugated dienes at all the concentrations of copper employed to trigger the oxidation process (Figure 6C).

In order to validate the hypothesis that at a selected time-point during the lag phase the pro-oxidant effect of uric acid appeared at specific copper to LDL ratios, experiments were performed using different LDL concentrations and fixed copper concentrations. As reported in Figure 7, the stimulation of diene formation promoted by uric acid appeared at copper to LDL ratios of 8.3, 12.5, 16.6, 25 and 33 but was not evident when they exceeded 50 (50, 66.6, 100 and 200).

The pro-oxidant activity of uric acid is related to the availability of lipid hydroperoxides

In a recent study, Ziouzenkova et al. [28] have demonstrated that, at a low copper to LDL ratio, substantial amounts of lipid hydroperoxides are generated during the early phases of LDL oxidation, even in the presence of substantial amounts of α -tocopherol and other endogenous lipid-soluble antioxidants. The possibility that the increased susceptibility to uric acid-stimulated oxidation of LDL incubated with low concentrations of copper could be linked to an increased content of lipid hydroperoxides was therefore investigated. As reported in Figure 8, cholesteryl linoleate hydroperoxides were detectable already after 10 min of incubation and their concentration progressively increased at later time-points. On the other hand, in LDL incubated with high copper concentrations, cholesteryl linoleate hydroperoxides became measurable exclusively after 45 min (Figure 8A). Interestingly, the presence of lipid hydroperoxides coincided with the appraisal of the pro-oxidant activity of uric acid in both models (Figures 8B and 8C).

The necessary involvement of lipid hydroperoxides in uric acid-stimulated, copper-dependent LDL oxidation was further investigated using partially peroxidized LDL treated with the combination of ebselen and glutathione, a condition which has been reported previously to reduce lipid hydroperoxides [30]. As reported in Figure 9, this treatment led to the complete disappearance of cholesteryl linoleate hydroperoxides with the concomitant formation of the corresponding hydroxides. The complete reduction of lipid peroxides was also associated with a dramatic inhibition of the subsequent stimulation of LDL oxidation induced by the contemporary presence of copper and uric acid.

The depletion of endogenous α -tocopherol is not a prerequisite for the pro-oxidant activity of uric acid

Although the relative contribution of endogenous α -tocopherol in protecting LDL from copper-induced oxidation is still a matter of active debate, all the available evidence indicates that artificial enrichment of isolated lipoproteins with exogenous α -tocopherol significantly increases the resistance to oxidation and prolongs the lag phase [31]. Albertini and Abuja [19] have

Figure 8 Relationship between the pro-oxidant activity of uric acid and the presence of lipid hydroperoxides in copper-dependent LDL oxidation

Two different experimental conditions were employed. LDL isolated from human plasma as described in the Materials and methods section were incubated at a concentration of 0.50 mg/ml in phosphate buffer, pH 7.4, with either 0.625 μ M CuSO_4 (low copper to LDL ratio) or 10 μ M CuSO_4 (high copper to LDL ratio). (A) Samples were taken at the indicated time-points from both incubation mixtures (●, low copper to LDL ratio; ○, high copper to LDL ratio) for quantification of cholesteryl linoleate hydroperoxide as described in the Materials and methods section. *Statistically significant ($P < 0.01$) difference. (B) Formation of conjugated dienes was measured using LDL incubated with a high copper concentration (10 μ M) without (○) or with (●) the subsequent addition of 10 μ M uric acid at the indicated time-points. (C) Formation of conjugated dienes was measured using LDL incubated with low copper concentration (0.625 μ M) without (○) or with (●) the subsequent addition of 10 μ M uric acid at the indicated time-points.

Figure 9 The reduction of lipid hydroperoxides by ebselen and glutathione abolishes uric acid-stimulated, copper-dependent LDL oxidation

Isolated human LDL (1 mg/ml) was treated with 1.25 μ M CuSO_4 for 60 min (a condition similar to that reported in Figure 8, and defined as low copper to LDL ratio, in order to promote a partial peroxidation of LDL) and subsequently incubated without (A) or with (B) ebselen and reduced glutathione to reduce lipid hydroperoxides as described in the Materials and methods section. (C) At the end of the incubation procedure samples were taken for the measurement of cholesteryl linoleate hydroperoxides (open bar) and hydroxides (hatched bars). The remaining LDL suspension was immediately gel-filtered and resuspended at a final concentration of 0.25 mg/ml in the absence (○) or in the presence of 2.5 μ M CuSO_4 (▲) or 2.5 μ M CuSO_4 and 10 μ M uric acid (●). The formation of conjugated dienes was measured as described in the Materials and methods section.

recently found that the water-soluble analogue of vitamin E, Trolox C, may exert a pro-oxidant effect during copper-catalysed oxidation of LDL only when the endogenous α -tocopherol has been consumed. This finding is consistent with results obtained in the present study employing 0.1 μ M LDL, 2.5 μ M copper and 10 μ M uric acid (results not shown). α -Tocopherol-enriched LDL was additionally employed in this study to investigate the occurrence of uric acid-stimulated lipid peroxidation in these less susceptible lipoproteins. As reported in Figure 10, the pro-oxidant activity of uric acid was still detectable, but it took place at later time-points compared with unsupplemented lipoproteins, and the rate of peroxide formation was lower.

In order to get more insights about the relationship between α -tocopherol content and the susceptibility of LDL to uric acid-stimulated and copper-induced LDL oxidation two experimental models were employed, with respectively low and high copper to LDL ratios. As reported in Figures 8(B) and 8(C) these two conditions were associated with a high and low susceptibility to the pro-oxidant effect of uric acid during early time-points of the lag phase. When high concentrations of copper were employed, the endogenous α -tocopherol was rapidly consumed and became undetectable at 45 min, a time at which uric acid stimulated lipid peroxidation. On the other hand, when low concentrations of copper were employed, α -tocopherol levels declined much more

Figure 10 Effect of α -tocopherol enrichment on uric acid-stimulation of copper-induced LDL oxidation

LDL was isolated from either native, non-supplemented human plasma or plasma supplemented with 250 μ M α -tocopherol, as described in the Materials and methods section, and subsequently incubated (at a concentration of 0.250 mg/ml in phosphate buffer, pH 7.4) with 2.5 μ M CuSO_4 . At the indicated time-points samples were taken for the measurement of both the actual peroxide content ($\circ$, native LDL; $\bullet$, supplemented LDL) and uric acid-stimulated peroxide formation (measured as the amount of peroxides formed in 10 min after the addition of 10 μ M uric acid, hatched bars, native LDL; filled bars, supplemented LDL). The α -tocopherol content in non-supplemented LDL was 3.2 ± 0.6 nmol/mg LDL and increased to 9.7 ± 1.8 nmol/mg LDL in supplemented LDL.

Figure 11 Consumption of endogenous α -tocopherol during LDL oxidation promoted by low and high copper concentrations

Two different experimental conditions were employed. LDL isolated from human plasma as described in the Materials and methods section was incubated at a concentration of 0.50 mg/ml in phosphate buffer, pH 7.4, with either 0.625 μ M CuSO_4 ($\bullet$, low copper to LDL ratio) or 10 μ M CuSO_4 ($\circ$, high copper to LDL ratio). Samples were taken at the indicated time-points from both incubation mixtures for quantification of endogenous α -tocopherol, as described in the Materials and methods section. *Statistically significant ($P < 0.01$) difference.

slowly and significant amounts were still present at 10 and 20 min (5 and 2 mol/mol of LDL respectively) when uric acid had already stimulated lipid peroxidation (Figure 11). These findings clearly indicate that the depletion of endogenous α -tocopherol is not an absolute requirement for the appraisal of the pro-oxidant activity of uric acid.

DISCUSSION

The results reported in this paper indicate that, under selected circumstances, uric acid, at physiological pH, is able to stimulate the oxidation of LDL triggered by copper. The restriction of this pro-oxidant activity to the copper model of LDL oxidation and the absolute requirement for either Cu(II) or Cu(I) point to a critical role of copper-uric acid interactions as the biochemical basis of this effect. The precise molecular mechanisms, however, are still largely obscure and just a few hypotheses can be forwarded.

Uric acid can be directly oxidized by free Cu(II) with the generation of Cu(I) and the concomitant formation of a series of oxidized compounds, including allantoin, oxonic/oxaluric acid and parabanic acid, and also, via a one-electron redox process, urate anion free radical [12,32]. Although the precise reactivity of this free radical is not known, it can be tentatively hypothesized that it could react with a polyunsaturated fatty acid leading to the formation of a lipid radical which, in the presence of molecular oxygen, would generate a lipid peroxy radical thus propagating lipid peroxidation within the LDL particle. The exclusive involvement of a urate anion free radical in accelerating the LDL oxidation process, however, is disproved by three pieces of experimental evidence obtained here. First, not only is Cu(II) required in order to observe the pro-oxidant activity of uric acid, but also Cu(I), as demonstrated by the inhibitory effect promoted by the Cu(I) chelator BC. Secondly, even in the presence of available Cu(I), when uric acid is included in the incubation medium together with copper or when the copper to LDL molar ratio is high, the enhancement of the peroxidative processes is not detectable. Finally, uric acid efficiently protects LDL from the oxidation triggered by AAPH, although it can be speculated that the urate anion free radical could also be formed in this system.

Alternative mechanisms rely on the central role played by Cu(I) formed upon reduction of Cu(II) by uric acid. Uric acid is, indeed, a powerful reductant of Cu(II) in aqueous solutions and its availability could explain both the antioxidant and pro-oxidant effects exerted by uric acid. With regard to the antioxidant activity, we have recently observed that the inclusion of equimolar concentrations of Cu(I), when added together with Cu(II) to an incubation mixture containing isolated human LDL, efficiently prolonged the duration of the lag phase and decreased the initial rate of endogenous α -tocopherol consumption [33]. Moreover, Cu(I) reacts with preformed lipid peroxides with the concomitant formation of lipid alkoxy radicals

which can be quenched by α -tocopherol. Furthermore, since α -tocopherol is an efficient Cu(II) reductant [14,30] and during this reaction it is rapidly consumed [34], the shift of the copper redox equilibrium toward Cu(I) would spare endogenous α -tocopherol and would make LDL more resistant to an oxidative attack. Additionally, it can be proposed that uric acid might act in synergism with α -tocopherol by reducing tocopheroxyl radical back to the parent compound. α -Tocopherol is, indeed, positioned at the LDL-water interface [35,36] and is therefore promptly accessible to react with the water-soluble uric acid. This kind of synergism has also been proposed for ascorbic acid and for the water-soluble analogue of α -tocopherol, Trolox C [37].

The pro-oxidant activity of Cu(I) is much more established and characterized and basically relies on its interaction with lipid hydroperoxides, as described in eqn. (1). This activity requires, in addition to Cu(I), the presence of lipid hydroperoxides and the

progressive consumption of endogenous antioxidants whose regeneration would otherwise stop the progression of the propagation reaction.

A very intriguing aspect of the results obtained in this work is the dependency of the pro-oxidant activity of uric acid on the copper to LDL molar ratio, thus suggesting that a more complex uric acid–copper–LDL interaction is involved in this reaction. The threshold value for observing a decreased pro-oxidant effect of uric acid was about a 50:1 copper to LDL ratio, a value identical with that found previously for BC-stimulated LDL oxidation [27]. A possible explanation of these results can be found in a report by Proudfoot et al. [38] who demonstrated that a similar copper to LDL ratio was the threshold for unmasking a peroxidase-like activity associated with uric acid in serum and operative in promoting the breakdown of lipid hydroperoxides formed during LDL oxidation. At a high copper to LDL ratio, the destruction of hydroperoxides would remove a possible substrate for propagating reactions. This peroxidase activity was strictly linked to the formation of a uric acid–copper–albumin complex and the presence of serum albumin was an absolute prerequisite. The experimental conditions employed in the present work, however, do not include albumin in the incubation mixtures and no contamination of the isolated LDL with albumin was detectable using conventional PAGE.

The higher susceptibility of LDL incubated with low copper concentrations to the pro-oxidant activity of uric acid might be related to the greater generation of lipid hydroperoxides already during the lag phase. This phenomenon has been previously reported by Ziouzenkova et al. [28] and it has been confirmed in this study (see Figure 8). Interestingly, under these experimental conditions, both lipid hydroperoxides are produced despite the presence of substantial amounts of endogenous antioxidants and uric acid becomes pro-oxidant. This clearly indicates that previous depletion of LDL-associated α -tocopherol is not a prerequisite for unmasking the pro-oxidant activity of uric acid, and suggests that, at least under these circumstances, α -tocopherol is not spared by uric acid but, rather, is more rapidly consumed.

When the endogenous antioxidants are completely lost and lipid hydroperoxides are formed, it can be predicted that the propagation of lipid peroxidation in the copper model will follow two distinct routes, one involving Cu(I) as in eqn. (1) and the other involving Cu(II) in the reaction:

where LOO $^\bullet$ is the lipid peroxy free radical. Although these reactions have different rate constants [39], their operation is strictly dependent on: (i) the Cu(II) to Cu(I) ratio; (ii) the rate of reduction of Cu(II) to Cu(I) by LDL-associated reductants; and (iii) the concentration of LDL-derived lipid hydroperoxides. In other words reactions (1) and (2) would be strictly dependent on the copper to LDL molar ratio. At lower copper concentrations, the relative abundance of reducing equivalents would make the Cu(II) to Cu(I) ratio low and the propagation would preferentially involve Cu(I)-mediated reactions (such as that shown in eqn. 1). At high copper concentrations Cu(II) will prevail and the propagation would preferentially involve Cu(II)-mediated reactions. In the presence of uric acid, the reactions involving Cu(I) will prevail and it could be predicted that significant amounts of lipid hydroxides will be formed according to eqn. (3):

where LOH is a lipid hydroxide. This is apparently in contrast with the results obtained in this study which show that, at comparable levels of conjugated dienes formed, the amounts of lipid hydroxide were identical, either in the absence or in the

presence of uric acid. Comparable findings were also obtained by Stait and Leake [20] and Retsky and Frei [40]. This apparent discrepancy can be explained by the assumption that the propagation of lipid peroxidation in the copper model is almost exclusively mediated by Cu(I) and that uric acid simply accelerates the reaction making available increasing concentrations of catalytic Cu(I). Alternatively, it must be stressed that the alkoxyl radical, in addition to that described in eqn. (3) has a number of fates, including its conversion into aldehydes by β -scission and that the peroxy radical may not fragment into aldehydes as rapidly as does the alkoxyl radical, as suggested by Stait and Leake [20].

In conclusion, in the presence of pre-formed lipid hydroperoxides, uric acid may accelerate the peroxidation of human LDL triggered by copper, even in the presence of endogenous antioxidants. Although the operation of copper-catalysed LDL oxidation *in vivo* is still a matter of debate, the occurrence of a form of LDL containing lipid hydroperoxides (LDL $^\bullet$) [41] in plasma and in the subendothelial space, which is well equipped with water-soluble antioxidants such as uric acid, could make it potentially susceptible to extensive oxidation.

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