Antioxidant activity of allopurinol on copper-catalysed human lipoprotein oxidation

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Abstract We found that allopurinol, at therapeutically relevant concentrations (9–58 μM), significantly counteracted copper-catalysed human non-HDL lipoprotein oxidation, as assessed by thiobarbituric acid reactant content and kinetics of conjugated diene formation. Oxypurinol was ineffectual. Both drugs had no activity on metal-independent, peroxyl radical-induced lipoprotein oxidation. Specific fluorescence-quenching experiments revealed that only allopurinol could interact with copper antagonizing metal binding to lipoproteins. Thus, therapeutic allopurinol concentrations can inhibit copper-catalysed lipoprotein oxidation through metal complexation, suggesting some antioxidant-antiatherogenic activity of the drug in vivo.

Key words: Allopurinol; Oxypurinol; Copper; Lipoprotein oxidation; Antioxidant

1. Introduction

Allopurinol has been reported to possess antioxidant properties, which may partly be attributable to inhibition of tissue xanthine oxidase, an enzyme capable of generating reactive oxygen species especially under ischemic conditions [1,2]. However, a direct scavenging capacity of allopurinol against highly reactive prooxidants, such as hydroxyl radical and hypochlorous acid, has been shown [3,4]. Some scavenging-type antioxidant effects have also been reported for oxypurinol [3,4], which represents the main metabolite of allopurinol in vivo [5,6]. Moreover, allopurinol has been shown to exert an inhibitory activity against copper-mediated ascorbate and DNA oxidation [7].

In spite of the aforementioned experimental evidences concerning some antioxidant properties of allopurinol and oxypurinol, so far little is known about the effects of these drugs on human lipoprotein oxidation, which is usually driven by catalytic transition metals, especially copper, and represents a focal aspect of atherogenesis [2,8]. The present study, therefore, was designed to investigate potential antioxidant effects of allopurinol and oxypurinol on human lipoprotein oxidation in specific model systems.

2. Materials and methods

2.1. Lipoprotein isolation and oxidation

Reagents were from Sigma Aldrich s.r.l., Milano, Italy, except for 2,2′-azobis(amidinopropane) dihydrochloride (AAPH), which was from Polisciences, Warrington, USA.

The non-high-density lipoprotein (non-HDL) fraction was obtained from EDTA plasma of male healthy adults (age 32–60 years) essentially as reported by Phelps and Harris [9] and Zhang et al. [10], using dextran sulfate (mol. wt. 500 000) plus $MgCl_2$ to precipitate the fraction itself and remove EDTA. Since prolonged ultracentrifugation and dialysis are avoided with this method, which allows the separation of the non-HDL fraction and the removal of EDTA within only 90 min [9,10], artifactual lipoprotein oxidation and loss of endogenous antioxidants resulting from these procedures [11,12] are minimized [9,10]. It is also noteworthy that the non-HDL fraction contains both LDL and VLDL, which are apo-B-containing lipoproteins with a recognized oxidisability and atherogenic potential [8,13–16].

Allopurinol and oxypurinol, pre-dissolved in 5 mM NaOH [6] to give initial drug concentrations of 1–4 mM, were generally used at therapeutically relevant final concentrations of 14, 28 and 58 μM. In this regard, plasma allopurinol and oxypurinol concentrations of about 30 and 60 μM have been reported in humans after oral administration of 300 mg allopurinol [5,6].

In a first set of experiments, non-HDL lipoproteins (0.15 mg non-HDL protein/ml) were oxidized by 13 μM $CuCl_2$ in phosphate buffered saline (PBS), pH 7.4, allowing 5 h incubation at 37°C with and without the drugs. Lipoprotein oxidation was evaluated through a thiobarbituric acid (TBA)-dependent test, which can detect mono- and bi-functional aldehydic products of lipid peroxidation implicated in the atherogenic modifications of the lipoprotein particle [8]. TBA reactive substances (TBARS) were measured basically according to Ohkawa et al. [17], with some modifications. A suitable aliquot of the non-HDL sample was added to a reaction mixture containing 0.25 mM EDTA, 20 μM butylated hydroxytoluene in absolute ethanol, 0.1 ml of 8.1% sodium dodecyl sulfate, 1.3 ml of 20% acetic acid, pH 3.5, and 1.3 ml aqueous solution of 0.6% TBA, followed by 30 min heating at 95°C. After cooling, the chromogen was extracted with n-butanol and read spectrophotometrically at 532 against an appropriate blank. Results were calculated as nmol TBARS/mg non-HDL protein, using a molar extinction coefficient of 154 000. The drugs gave no interference in the TBA-test.

In other specific experiments, the effects of allopurinol and oxypurinol on the kinetics of copper-catalysed human non-HDL lipoprotein oxidation were investigated through continuous spectrophotometric monitoring of absorbance increase at 234 nm, reflecting conjugated dienes (CD) formation during lipid peroxidation [8,11,18]. Molar extinction coefficient of CD was considered to be 29 500 at 234 nm [11]. Since the VLDL-containing non-HDL lipoprotein fraction may be characterized by a slight turbidity which is favored by higher copper concentrations, in these UV experiments the content of lipoprotein and $CuCl_2$ was lowered to 0.1 mg non-HDL protein/ml and 8 μM, respectively. Drug concentrations were also decreased to 9, 17 and 37 μM, so that drug:copper molar ratios were similar to those of the first set of experiments. Incubation was at 37°C in quartz cuvettes containing PBS, pH 7.4; reference cuvettes contained lipoprotein or lipoprotein plus various drug concentrations, where appropriate, in PBS, pH 7.4.

Finally, non-HDL lipoproteins (0.15 mg non-HDL protein/ml) were oxidized using a metal ion-independent prooxidant, namely the peroxyl radical-generating azo-initiator AAPH at 4 mM final concentration in the presence of 0.1 mM diethylenetriaminepentaacetic acid [19]; incubation was for 5 h at 37°C in PBS, pH 7.4, with and without 14, 28 and 58 μM allopurinol or oxypurinol. Lipoprotein oxidation was evaluated through the TBA-test, as reported above.

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Non-HDL-protein was measured by the method of Lowry et al. [20].

2.2. Drug-copper interaction

Copper complexation is a key aspect of the pharmacological antagonism of lipoprotein oxidation [2,8,21]; such a complexation by a drug may prevent metal binding to the particle apo B 100 moiety, which is an essential requisite for copper-mediated lipoprotein oxidation [8,21]. To investigate possible copper-binding properties of allopurinol and oxypurinol, drug fluorescence-quenching experiments were specifically carried out for oxypurinol, which shows an intrinsic fluorescence. Indeed, the relative fluorescence of a substance can be quenched by catalytic transition metals, as a result of formation of a complex between such a substance and metals themselves [22]. Excitation and emission wavelengths of oxypurinol were determined to be 390 and 450 nm, respectively; the drug was used at final concentrations of 100 and 200 μM, while the concentration of $CuCl_2$ (which gave no interference at the aforementioned wavelengths) ranged from 5 to 120 μM.

Another fluorescence-quenching methodological approach was specifically used for allopurinol, which does not apparently show an intrinsic fluorescence. Such an approach, which was however applied also to oxypurinol, is based on the quenching of the endogenous tryptophan-related lipoprotein fluorescence by copper [8,23,24], a phenomenon which may be counteracted by substances able to bind the metal. Optimal operative conditions were found using non-HDL lipoproteins at a final protein content of 5.5 μg/ml, 5 μM $CuCl_2$ and drug concentrations ranging from 5 to 100 μM. Fluorescence emission spectra were recorded after excitation at 280 nm, the emission values around 335 nm being related to lipoprotein tryptophan residues [23,24].

2.3. Statistics

Data were calculated as means ± SD of 7 different experiments, unless otherwise indicated. Specific drug effects were evaluated by the one-way analysis of variance (ANOVA) plus Student-Newman-Keuls test [25]. $P < 0.05$ was regarded as statistically significant [25].

3. Results

3.1. Drug effects on lipoprotein oxidation

In the tests where the drugs were ineffective, we will report only the results observed with the highest therapeutic drug concentrations.

Table 1
Effect of allopurinol on copper-mediated human non-HDL lipoprotein oxidation

	nmol TBARS/mg non-HDL protein
Control	54.8 ± 15.3
Control plus:	
14 μM allopurinol	37.5 ± 9.5 [a]
28 μM allopurinol	24.7 ± 7.5 [a,b]
58 μM allopurinol	18.5 ± 7.0 [a]

Lipoprotein oxidation was induced by 5 h incubation at 37°C with 13 μM $CuCl_2$ in PBS, pH 7.4, and evaluated through the TBA-test (see Section 2 for further explanations).
Means ± SD of 7 different experiments.
[a] $P < 0.05$ vs control; [b] $P < 0.05$ vs the value that precedes (one-way analysis of variance plus Student-Newman-Keuls test).

As shown in Table 1, allopurinol, beginning from 14 μM, was inhibitory on copper-catalysed non-HDL lipoprotein oxidation and TBARS formation. The drug at 58 μM was not significantly more effective than at 28 μM; thus, a virtually maximal antioxidant capacity of allopurinol may be considered to be operative at a drug:copper molar ratio of 2:1. On the other hand, oxypurinol had no specific antioxidant activity; in fact, 53.7 ± 14.5 nmol TBARS/mg non-HDL protein were detected in the presence of 58 μM oxypurinol, not different from the values observed in control experiments (54.8 ± 15.3 nmol TBARS/mg non-HDL protein, P = NS).

Allopurinol also showed antioxidant effects on the kinetics of copper-mediated non-HDL lipoprotein oxidation (Fig. 1). Lag time of oxidation was 52 ± 6 min in control experiments, and it was markedly prolonged by the drug (92.5 ± 13.5, 154.5 ± 24 and 186 ± 38 min with 9, 17 and 37 μM allopurinol, respectively, all $P < 0.05$ vs control; 17 and 37 μM vs 9 μM, $P < 0.05$; $n = 5$). Moreover, oxidation rate was significantly

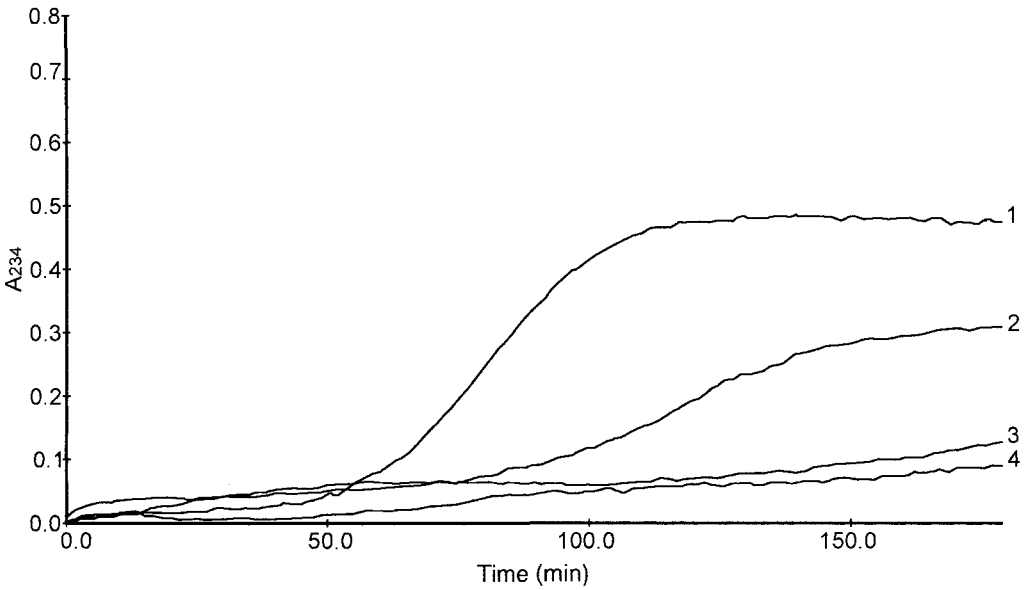

Fig. 1. Antioxidant activity of allopurinol on copper-catalysed human non-HDL lipoprotein oxidation. Lipoproteins were incubated at 37°C in PBS, pH 7.4, with 8 μM $CuCl_2$ in the absence (trace 1, control) or presence of 9, 17 and 37 μM allopurinol (traces 2, 3 and 4, respectively). Conjugated diene formation during oxidation was followed spectrophotometrically as an increase of absorbance at 234 nm (A_2). The figure represents results from a typical kinetic experiment (see Sections 2 and 3 for further explanations).

decreased by allopurinol (from 2.45 ± 0.55 nmol CD/min/mg non-HDL protein of control experiments to 1.33 ± 0.45, 0.83 ± 0.4 and 0.55 ± 0.3 nmol CD/min/mg non-HDL protein with 9, 17 and 37 μM allopurinol, respectively, all $P < 0.05$ vs control; 37 vs 9 μM, $P < 0.05$; $n = 5$). Thus, the kinetics of pharmacological inhibition of lipoprotein oxidation characterized also by a decreased propagation rate suggests that allopurinol may have copper binding and not radical scavenging

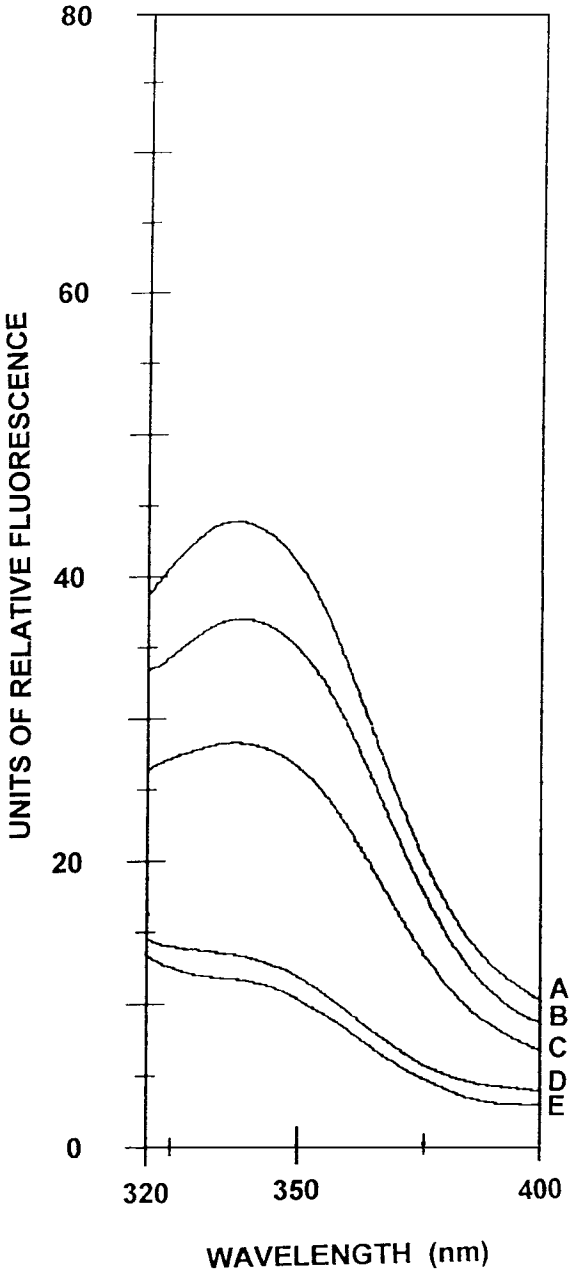

Fig. 2. Inhibition of copper-induced lipoprotein fluorescence quenching by allopurinol. Traces: A, non-HDL lipoprotein alone (5.5 μg non-HDL protein/ml); B, non-HDL lipoprotein (5.5 μg non-HDL protein/ml) plus 5 μM $CuCl_2$ and 10 μM allopurinol; C, non-HDL lipoprotein (5.5 μg non-HDL protein/ml) plus 5 μM $CuCl_2$ and 5 μM allopurinol; D, non-HDL lipoprotein (5.5 μg non-HDL protein/ml) plus 5 μM $CuCl_2$ and 50 μM oxypurinol; E, non-HDL lipoprotein (5.5 μg non-HDL protein/ml) plus 5 μM $CuCl_2$. The figure represents a fluorescence emission spectrum of non-HDL lipoprotein after excitation at 280 nm (see Sections 2 and 3 for further explanations).

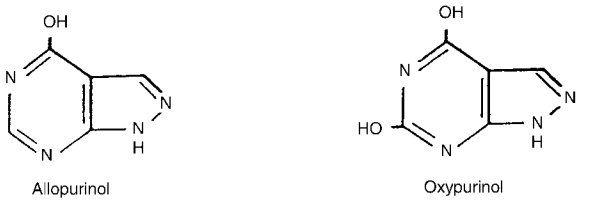

Fig. 3. Chemical structure of allopurinol and oxypurinol.

properties [18]. Oxypurinol, however, showed no specific antioxidant activity (lag time and oxidation rate of 53 ± 6.7 min and 2.6 ± 0.7 nmol CD/min/mg non-HDL protein with 37 μM oxypurinol, as compared to 52 ± 7.3 min and 2.45 ± 0.55 nmol CD/min/mg non-HDL protein of controls, P = NS; $n = 5$).

When non-HDL lipoproteins were oxidized in a metal-independent fashion through peroxyl radicals generated thermally by the azo-initiator AAPH, both allopurinol and oxypurinol were ineffective. In fact, 21.6 ± 7.3 and 21.8 ± 6.9 nmol TBARS/mg non-HDL protein were detected with 58 μM allopurinol and oxypurinol, respectively, as compared to 22.05 ± 7.5 nmol TBARS/mg non-HDL protein of controls (P = NS; $n = 5$). These results further suggest that the antioxidant effects of allopurinol against copper-mediated lipoprotein oxidation are not due to scavenging of peroxyl (or alkoxyl) radicals, which represent the main lipoperoxidation propagating species [2,8].

3.2. Drug-copper interaction

Fluorescence of oxypurinol was not quenched by copper, indicating no metal binding capacity of the drug. Indeed, under the experimental conditions used, fluorescence values of 100 and 200 μM oxypurinol alone were 2.9 ± 0.3 and 5.7 ± 0.6 units of relative fluorescence, not significantly different from those detected, for example, with 100 and 200 μM oxypurinol plus 100 μM $CuCl_2$ (2.75 ± 0.2 and 5.8 ± 0.55 units of relative fluorescence, respectively, P = NS; $n = 5$).

As depicted in Fig. 2, the intrinsic lipoprotein fluorescence (trace A) underwent a marked quenching by 5 μM $CuCl_2$ with decreased emission values around 335 nm (trace E), suggesting metal binding to and degradation of the tryptophan residues of the particle protein moiety [8,23,24]. In line with the lipoprotein oxidation experiments, 5 and 10 μM allopurinol (Fig. 2, traces C and B, respectively), but not 50 μM oxypurinol (Fig. 2, trace D), counteracted copper-mediated lipoprotein fluorescence quenching in a dose-related fashion, pointing to specific metal binding by allopurinol with protection of critical tryptophan residues against copper oxidative attack. Inhibition of copper-induced lipoprotein fluorescence quenching by allopurinol was apparently maximal at a drug:copper molar ratio of 2:1; indeed, increasing allopurinol concentrations up to 100 μM was not further inhibitory, and oxypurinol, even at 100 μM, was ineffective (not shown).

4. Discussion

The present study shows that allopurinol significantly counteracts copper-driven human lipoprotein oxidation, pointing to copper complexation as the mechanism involved in specific drug antioxidant activity. Interestingly oxypurinol, although structurally similar to allopurinol (Fig. 3), has apparently no antioxidant effect. It is evident that the insertion of a further

hydroxyl group in the pyrimidine-type heteroaromatic ring of allopurinol to form oxypurinol (Fig. 3) results in a loss of the endogenous antioxidant capacity. In such a context, it is possible that copper complexation by allopurinol is related to the unused metal electrons which interact with molecular orbitals in the drug ligand. These electrons made available by the metal may then flow in the direction of the aromatic ring, reinforcing ligand-metal bond [26]. The further hydroxyl group of oxypurinol could dissociate to form O^-, the unused electron of which might migrate in direction of the heteroaromatic ring, resulting in higher molecular stability with incapability of the ring itself to accept electrons from catalytic transition metals.

Allopurinol may reach concentrations of about 30 μM in human plasma after oral drug administration of 300 mg [5,6]. It is worth emphasizing, therefore, that the antioxidant effects of allopurinol herewith reported are evident at therapeutically relevant concentrations. On the other hand, allopurinol has a short half-life in human plasma, since it is rapidly metabolized to oxypurinol [5,6]. Hence, a specific antioxidant activity of allopurinol in vivo might be somehow questioned. However, it has been reported than the patients subjected to long-term therapy with allopurinol show a slower drug metabolism than those receiving single doses of the drug [27]; moreover, allopurinol is present not only in the plasma environment, but also at tissue level [27]. This latter aspect appears relevant, considering that lipoprotein oxidation may not occur in the bloodstream due to the high antioxidant capacity of plasma, but, rather, directly in the vascular tissue [28]. It is noteworthy that copper is particularly active at promoting lipoprotein oxidation as a result of its peculiar ability to bind to the particle apo B 100 moiety even in the presence of phosphates [29], and that redox-active copper has been detected in human atherosclerotic lesions [30], pointing to copper as a mediator of prooxidant-atherogenic responses in vivo [2,8,29,30]. Thus, allopurinol, once present especially in the vascular tissue during chronic therapy, could directly counteract copper-mediated lipoprotein oxidation and atherogenic processes. Such an issue deserves further clinical and epidemiological studies.

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