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INHIBITION OF TRANSITION METAL ION-CATALYSED ASCORBATE OXIDATION AND LIPID PEROXIDATION BY ALLOPURINOL AND OXYPURINOL

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Abstract—Allopurinol and its metabolite oxypurinol inhibited basal oxidation of ascorbate and exerted comparable concentration-dependent inhibitory effects on the oxidation of ascorbate catalysed by cupric ion, but the stimulation produced by ferric ion was affected minimally. UV spectral analysis suggested the formation of an allopurinol–ascorbate–copper ion complex. The oxidation of erythrocyte membrane lipids by ferric ion and cupric ion-*t*-butylhydroperoxide was also inhibited by allopurinol and oxypurinol, by the metal chelators EDTA and uric acid, and by the antioxidant butylated hydroxytoluene. The metal chelating actions of allopurinol and oxypurinol may be relevant to their protective actions against ischemia/reperfusion injury.

The important role of reactive oxygen-derived radicals in myocardial ischemia/reperfusion (I/R) injury has been implicated in various experimental and clinical settings [1–3]. This has led to intensive investigation of possible therapeutic interventions aimed at reducing the extent of such oxidative injury, which may have important clinical implications in the areas of post-infarction myocardial salvage, cardioplegia, organ transplantation, and thrombolytic or revascularization procedures [2].

Among those actively investigated agents, allopurinol has been shown to reduce manifestations of I/R injury in a number of systems [4–6]. Studies in our laboratory have demonstrated that chronic allopurinol pretreatment affords significant protection against alterations in ultrastructure [7], subcellular organelle integrity [8] and antioxidant capacity [9] in rabbit hearts subjected to ischemia and reperfusion. Because xanthine activity is virtually undetectable in rabbit myocardium [10], the protective effects of allopurinol cannot be attributed solely to its well-known xanthine oxidase inhibiting properties. A number of other possible mechanisms of allopurinol protection have been proposed, including an increased efficiency of adenosine triphosphate salvage [3], facilitation of mitochondrial electron transfer [11], inactivation of endogenously formed reactive species (such as hydroxyl radicals or myeloperoxidase-derived hypochlorous acid [12]), and direct scavenging of free radicals generated in the ischemic myocardium [13].

The catalytic role of transition metals in mediating oxy-radical-induced tissue damage is well established [14, 15]. Both the iron chelator desferrioxamine and allopurinol have been shown to reduce cellular damage in isolated rabbit hearts subjected to a period of hypoxia followed by reoxygenation [6]. This suggests that another possible mechanism for the protective effect of allopurinol against I/R injury may involve transition metal chelation. In the present study, this idea has been explored by investigating the actions of allopurinol and its metabolite, oxypurinol, on cupric and ferric ion-catalysed oxidation of ascorbate and oxidation of erythrocyte membrane lipids.

MATERIALS AND METHODS

Allopurinol and oxypurinol were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade. Solutions were prepared in double-distilled water to minimize metal contamination.

Ascorbate oxidation. Ascorbate oxidation was monitored spectrophotometrically by the decrease in absorbance at 280 nm. This wavelength was chosen rather than 265 nm, the absorption maximum for ascorbate, in order to minimize the contribution by allopurinol to the absorption. The cupric or ferric ion-catalysed oxidation of ascorbate was assayed in 10 mM phosphate buffer, pH 7.4, either in the absence or presence of 0.1 mM EDTA used to reduce the basal oxidation of ascorbate catalysed by transition metal contaminants present in the reaction medium. The reaction mixture, in a final volume of 3 mL, contained 100 μM ascorbate in the absence or presence of the test compounds. The reaction was initiated by adding 100 μL of cupric or ferric chloride solution at final concentrations ranging from 10 to 100 μM. The change in absorbance at 280 nm of the reaction mixture was monitored continuously when the assay was performed in the absence of EDTA. When 0.1 mM EDTA was present in the reaction mixture, the absorbance at 280 nm was measured 1 min after the addition of cupric or ferric chloride. After a period of incubation at room temperature (15

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or 40 min for cupric or ferric ion-catalysed reactions respectively), the absorbance was again measured. The rate of ascorbate oxidation, expressed in nanomoles per minute, was calculated using a standard calibration curve and was constant over the incubation period. The rate of cupric or ferric ion-catalysed ascorbate oxidation was corrected for the basal oxidation rate in the absence of exogenously added metal ions.

UV absorption spectroscopy. UV absorption spectra of reaction mixtures were recorded with a Perkin–Elmer model Lambda 6B spectrophotometer. Various combinations of allopurinol/ascorbic acid/cupric chloride or EDTA were mixed in the following manner: 1.0 mL of 250 μM allopurinol, 1.0 mL of 125 μM ascorbic acid, 0.5 mL of 500 μM EDTA, 0.5 mL of 500 μM cupric chloride, and sufficient double-distilled water to make up a final volume of 3 mL. Spectra of the reaction mixtures were recorded 2 min after the constituents had been mixed.

Oxidation of erythrocyte membrane lipids. Erythrocyte membranes were prepared from outdated human blood by stepwise hypotonic lysis as previously described [16]. Membrane preparations were stored at −20° and used within 1 week. Ferric ion or cupric ion-*t*-butylhydroperoxide (tBHP)-induced formation of thiobarbituric acid-reactive substances (TBARS) in erythrocyte membranes was measured in isotonic saline adjusted to pH 7.4 with tris[hydroxymethyl]aminomethane. Reactions were performed in either the absence or presence of different concentrations of metal chelators or butylated hydroxytoluene (BHT). All chemicals were dissolved in buffered saline, except BHT, which was dissolved in isopropanol; 10 μL of this isopropanol solution was used in the reaction mixture. The ferric ion-induced formation of TBARS was initiated by adding 250 μL of ferric chloride solution to a mixture containing 0.3 mg membrane protein, in a final volume of 1.0 mL, and the reaction mixtures were incubated at 37° for 30 min. The reaction was stopped by the addition of 0.5 mL of cold 28% trichloroacetic acid, containing 0.1 M sodium arsenite. After centrifugation, the supernatant fraction was assayed for TBARS by mixing 1.0 mL supernatant with 0.5 mL thiobarbituric acid (TBA) reagent [0.5% TBA (w/v) in 0.025 M NaOH], boiling for 15 min, and measuring the absorbance at 532 nm. The cupric ion–tBHP-induced peroxidation reaction was initiated by adding 250 μL cupric ion–tBHP to a mixture containing 0.75 mg membrane protein, in a final volume of 1.0 mL, followed by a 30-min incubation at 37°. Reactions were terminated by adding 3 mL of cold 1% phosphoric acid. Following the addition of 1 mL TBA reagent [0.6% TBA (w/v) in 0.05 M NaOH], the mixture was boiled for 45 min. After cooling, the samples were extracted with 5 mL of *n*-butanol/pyridine (15/2, v/v) and centrifuged to achieve phase separation. The TBARS content of the butanol layer was determined by measuring the absorbance at 532 nm.

RESULTS

Ascorbate oxidation. Ascorbate oxidized spontaneously in phosphate buffer in the absence of added transition metal ions (Fig. 1a). This basal ascorbate oxidation was decreased by allopurinol (100 μM final concentration). Cupric chloride, at a final concentration of 10 μM, dramatically increased the rate of ascorbate oxidation (Fig. 1b). Allopurinol also inhibited this cupric ion-catalysed oxidation, in a concentration-dependent manner, with almost complete suppression at a final concentration of 100 μM. On the other hand, the addition of ferric chloride, up to a final concentration of 100 μM, did not alter significantly the rate of ascorbate oxidation (data not shown).

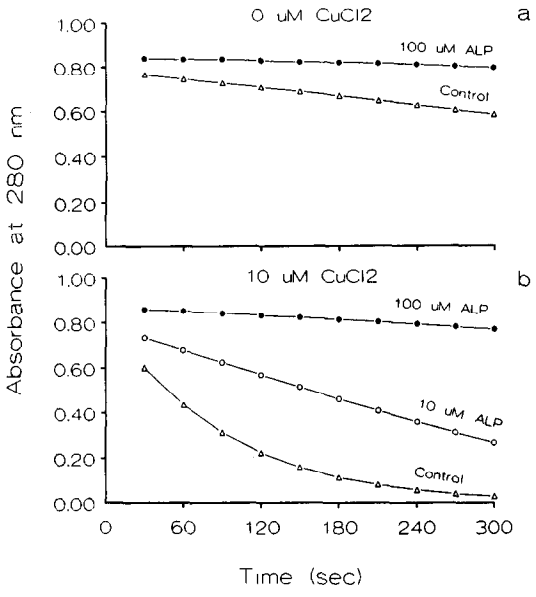

Fig. 1. Cupric ion-catalysed oxidation of ascorbate: effects of allopurinol. Ascorbate oxidation, as indicated by the decrease in absorbance at 280 nm, was monitored for 5 min in reaction mixtures containing 100 μM ascorbate in 10 mM phosphate buffer (pH 7.4), in either the absence or presence of allopurinol. The basal level of ascorbate oxidation was measured in the absence of added cupric chloride, in which the reaction was initiated by the addition of 100 μL of ascorbate solution at time 0. The cupric chloride-stimulated reaction was initiated by adding 100 μL of cupric chloride solution at a final concentration of 10 μM. Each point is the mean value of three experiments, with the SE < 5% of the mean.

When the oxidation of ascorbate was measured in the presence of 100 μM EDTA, the oxidation rate was decreased from the basal value of 3.40 ± 0.11 (SE) nmol/min (Fig. 1a) to 1.01 ± 0.12 nmol/min (Fig. 2). The addition of cupric chloride, at final concentrations of 10 and 30 μM, did not stimulate ascorbate oxidation (data not shown), but when present at a final concentration of 100 μM, cupric chloride significantly increased the rate of ascorbate oxidation to 17.2 ± 1.05 nmol/min (Fig. 2). Both allopurinol and oxypurinol (500 μM final concentrations) significantly inhibited this cupric ion-catalysed oxidation of ascorbate, reducing the oxidation rate to 22 and 16% of control respectively. However, when allopurinol and oxypurinol were added at a

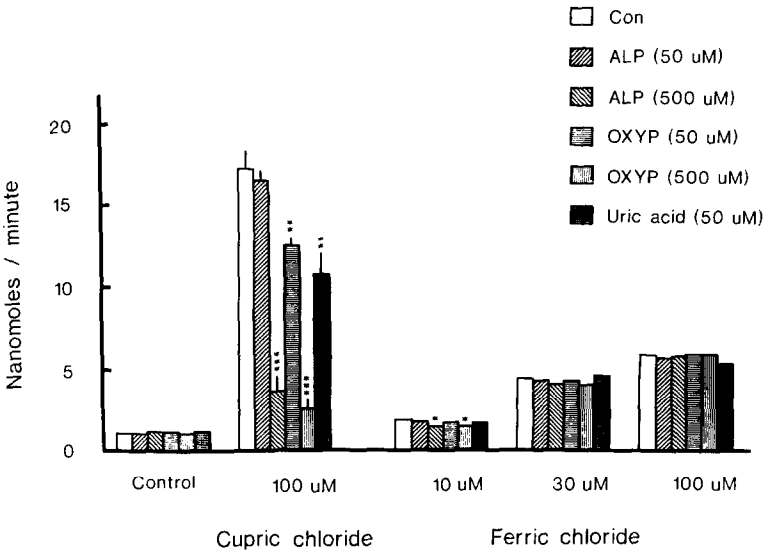

Fig. 2. Cupric and ferric ion-catalysed oxidation of ascorbate. Assays were performed as described in Materials and Methods. Reaction mixtures contained a 100 μM concentration of EDTA and of ascorbate in 10 mM phosphate buffer, pH 7.4. The reaction was initiated by adding 100 μL of cupric chloride or ferric chloride solution, at final concentrations ranging from 10 to 100 μM, in either the absence or presence of the tested compounds in the reaction mixture. Rates of ascorbate oxidation, expressed in nanomoles/minutes, are given as means of three experiments. Vertical bars represent the SE. Key: (*), (**) and (***) denote $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, when compared with the control, using Student's *t*-test.

final concentration of 50 μM, only oxypurinol produced a significant degree of inhibition. Ferric chloride, when added in the presence of 100 μM EDTA, stimulated the oxidation of ascorbate in a concentration-dependent manner to a maximum of 5.88 ± 0.49 nmol/min (Fig. 2). Both allopurinol and oxypurinol at final concentrations of 500 μM slightly inhibited the oxidation of ascorbate catalysed by 10 μM ferric chloride, but at increased concentrations of ferric chloride, this inhibitory effect of allopurinol and oxypurinol was abolished. Uric acid, at a final concentration of 50 μM, inhibited the cupric ion-catalysed oxidation of ascorbate, reducing the rate of oxidation to 63% of control, but had no significant inhibitory effect on the ferric ion-catalysed oxidation.

UV absorption spectroscopy. The spectrum generated from a mixture of allopurinol and ascorbate (spectrum B in Fig. 3a) was very similar to the sum of the individual spectra of allopurinol and ascorbic acid (data not shown). Cupric chloride, although it did not alter appreciably the spectrum of allopurinol (data not shown), generated a distinctive spectrum in the presence of allopurinol and ascorbate (spectrum A in Fig. 3a). This spectrum showed a decrease in absorption between 250 and 280 nm, presumably resulting from the oxidation of ascorbate. Moreover, there was a slight decrease and a moderate increase in absorbances at 206 and 231 nm, respectively, and a slight increase in absorption between 280 and 340 nm, which became apparent when absorbances of the allopurinol–ascorbate and cupric chloride spectra were subtracted from those of the allopurinol–ascorbate–cupric chloride spectrum (see Table 1). The addition of EDTA to the allopurinol–ascorbate–cupric chloride mixture abolished these spectral changes (Table 1), with the production of a spectrum (labelled A′ in Fig. 3b) comparable to that expected for the simple addition of individual absorbances for allopurinol and an ascorbate–cupric chloride–EDTA mixture (spectra B′ and C′ in Fig. 3b). Similar changes in spectral characteristics were observed following the addition of cupric chloride to an oxypurinol–ascorbate mixture (data not shown). In contrast, the addition of ferric chloride to an allopurinol–ascorbate mixture merely resulted in a spectrum similar to that produced by the allopurinol–ferric chloride mixture (data not shown).

Lipid oxidation in erythrocyte membranes. Incubation of erythrocyte membranes with ferric chloride increased the production of TBARS in a concentration-dependent manner (Fig. 4). BHT reduced the extent of ferric ion-induced lipid oxidation, with almost complete inhibition at a final concentration of 4 μM at all ferric chloride concentrations tested. Isopropanol, the solvent in which the BHT was dissolved, had no effect in this system (data not shown). EDTA at a final concentration of 0.4 mM strongly suppressed lipid oxidation induced by 0.2 mM ferric chloride, but the inhibitory action was overcome almost completely by increasing the concentration of ferric chloride to 0.4 mM (Fig. 4). Uric acid, when added at a final concentration of 2 mM, had a comparable inhibitory effect to EDTA, but the higher concentration of ferric chloride only partly overcame the inhibition (Fig. 4). Allopurinol (4 mM) or oxypurinol (3 mM) significantly inhibited lipid peroxidation induced by 0.2 mM ferric chloride;

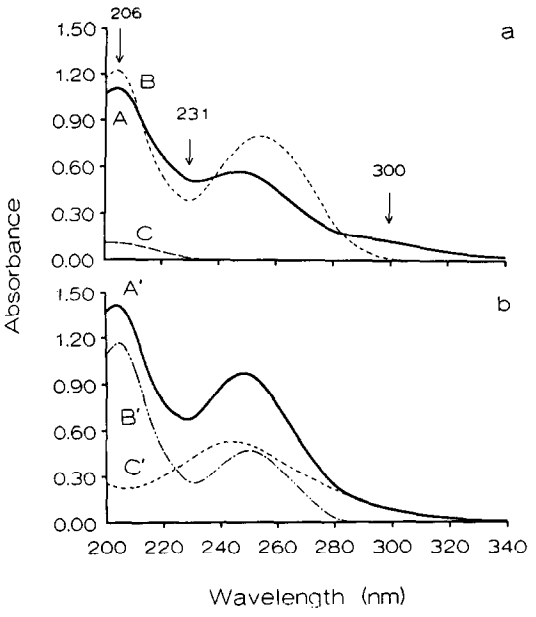

Fig. 3. UV absorption spectrum of the allopurinol–ascorbate–cupric chloride mixture: Effects of EDTA. Reaction mixtures were prepared as described in Materials and Methods. Spectra in (a) were recorded from the allopurinol–ascorbate–cupric chloride (A, ———), allopurinol–ascorbate (B, – – – –) and cupric chloride (C, — — —) mixtures respectively. Spectra in (b) represent the allopurinol–ascorbate–cupric chloride–EDTA (A', ———), allopurinol (B', — · · —) and ascorbate–cupric chloride–EDTA (C', – – – –) mixtures respectively. Cupric chloride, if present in the mixture, was added to start the reaction. Spectra of the reaction mixtures were recorded 2 min after the constituents had been mixed.

however, at the higher concentration of ferric chloride, the inhibitory effects of both these compounds were no longer significant (Fig. 4). None of these compounds interfered with the TBA color reaction (data not shown).

The peroxidation of erythrocyte membrane lipids was stimulated by tBHP, as indicated by the production of TBARS (Fig. 5). This peroxidation was not affected by either allopurinol or oxypurinol. When tBHP was combined with cupric chloride, there was a marked increase in the extent of lipid peroxidation, even though cupric chloride alone produced no significant effect on peroxidation (Fig. 5). Allopurinol and oxypurinol at final concentrations of 500 μM reduced this enhanced peroxidation to 87 and 88% of control, respectively (Fig. 5), but no further decreases in the TBARS level were observed at concentrations of allopurinol up to 2 mM. BHT strongly inhibited lipid peroxidation induced by the cupric chloride–tBHP mixture, in a concentration-dependent manner, reducing the TBARS level in the presence of 100 μM BHT to below that produced by tBHP alone (Fig. 5). Uric acid also significantly inhibited the cupric chloride–tBHP-induced peroxidation in a concentration-dependent manner, with the production of TBARS reduced to 72% of control at a final concentration of 500 μM (Fig. 5).

DISCUSSION

Transition metal ions catalyse the non-enzymatic oxidation of ascorbate through the intermediacy of redox reactions in which ascorbate oxidation occurs in one-electron steps [17]. Since the mechanism of ascorbate oxidation likely involves formation of an ascorbate–transition metal ion complex preceding intramolecular electron transfer [17], transition metal chelators would be expected to interfere with this interaction, thereby decreasing the reaction rate. Allopurinol and oxypurinol, as well as the metal chelator EDTA, are capable of suppressing the basal oxidation of ascorbate, presumably caused by transition metal ion contaminants present in the phosphates and double-distilled water (Fig. 1a). The stimulatory effects of exogenously added cupric ions on the oxidation of ascorbate were also inhibited by allopurinol in a concentration-dependent manner (Fig. 1b). The use of EDTA to minimize basal oxidation of ascorbate permitted measurement of the relatively modest increase in the rate of ascorbate oxidation induced by ferric ions at micromolar concentrations. Under the assay conditions employed, cupric ions were found to be 2-fold more effective than ferric ions in catalysing ascorbate oxidation (Fig. 2). The cupric ion-catalysed reaction was also more susceptible to the inhibitory actions of allopurinol and oxypurinol than that catalysed by ferric ions. A similar trend was observed for uric acid, a compound closely related structurally to allopurinol and oxypurinol.

Table 1. Absorbance changes of the allopurinol–ascorbate–cupric chloride (ALP–ASC–Cu) mixture: Effects of EDTA

	Δ Absorbance*		
	206 nm	231 nm	300 nm
ALP–ASC–Cu	−0.1339 ± 0.0370	0.0947 ± 0.0091	0.0494 ± 0.0126
ALP–ASC–Cu–EDTA	0.0189 ± 0.061†	−0.0060 ± 0.0032†	0.0011 ± 0.0012†

* Changes in absorbances were calculated by subtracting the absorbances both of the B or B' and C or C' spectra from those of the A or A' spectrum respectively (refer to Fig. 3).

† Denotes $P < 0.005$ ($N = 3$) when compared with the ALP–ASC–Cu mixture, using Student's *t*-test.

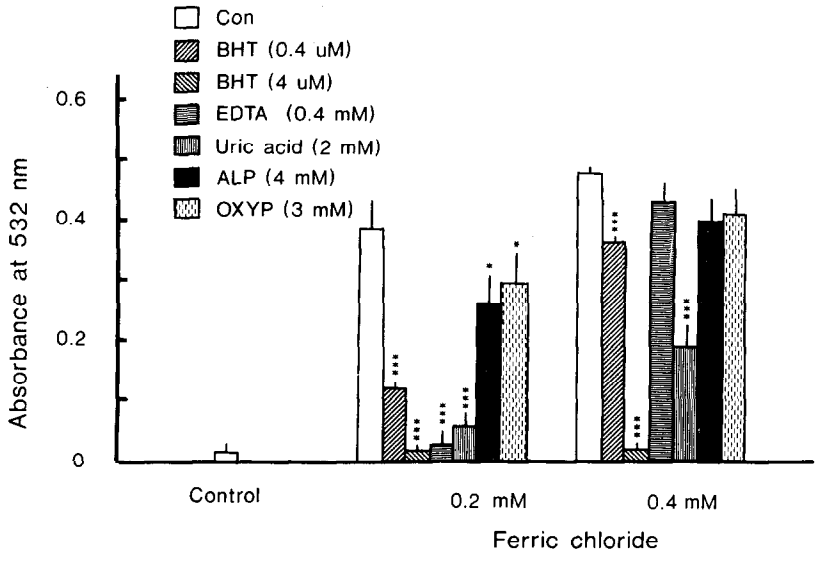

Fig. 4. Ferric ion-induced formation of TBARS in erythrocyte membranes. Assays were performed in the absence or presence of the test compounds in the reaction mixtures, as described in Materials and Methods. Reaction mixtures contained 0.3 mg membrane protein in buffered saline, pH 7.4. The reaction was initiated by adding 250 μL of ferric chloride solution. TBARS values, expressed in absorbance at 532 nm, are given as means of at least three experiments. Vertical bars represent the SE. Key: (*) and (***) denote $P < 0.05$ and $P < 0.001$, respectively, when compared with the control, using Student's *t*-test.

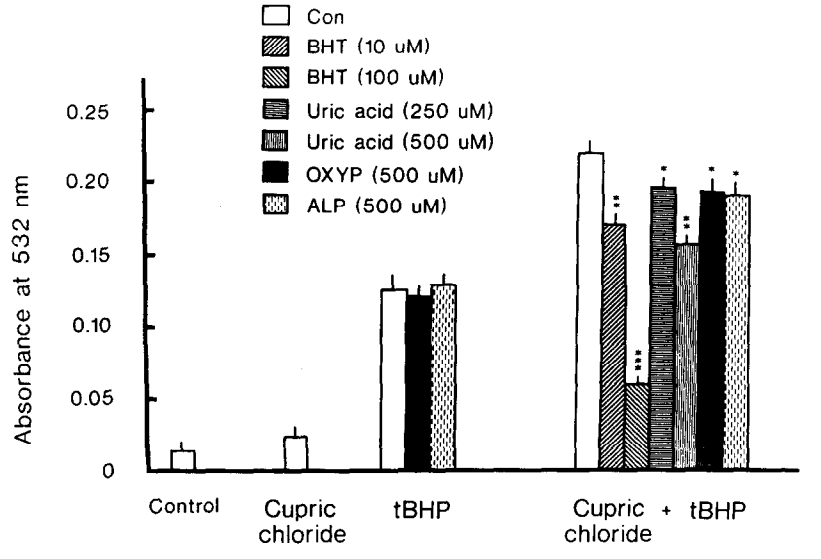

Fig. 5. Cupric ion–*t*-butylhydroperoxide-induced formation of TBARS in erythrocyte membranes. Assays were performed in the absence or presence of the tested compounds in the reaction mixtures, as described in Materials and Methods. Reaction mixtures contained 0.75 mg membrane protein in buffered saline, pH 7.4. The reaction was initiated by adding 250 μL of a cupric chloride (0.2 mM)–tBHP (10 mM) mixture. The basal level of TBARS production in the reaction mixture was measured in the absence of both cupric chloride and tBHP. TBARS values, expressed in absorbance at 532 nm, are given as means of three experiments. Vertical bars represent the SE. Key: (*), (**) and (***) denote $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, when compared with the control, using Student's *t*-test.

UV spectral analysis suggested the formation of an allopurinol–ascorbate–copper complex which is unstable in the presence of EDTA (Fig. 3, Table 1). A similar complexation process has been implicated previously in the inhibition by uric acid of the cupric ion-catalysed oxidation of ascorbate, as reported by Lam *et al.* [18]. The absence of similar spectral changes in the allopurinol–ascorbate–ferric chloride mixture presumably indicates that complex formation in this system is much less highly favored. This is consistent with our observation that ferric chloride is less potent as a catalyst of ascorbate oxidation and its pro-oxidant action is less susceptible to allopurinol inhibition than that of cupric chloride. Although antioxidant enzymes, such as catalase and superoxide dismutase, have been shown previously to inhibit the cupric ion-catalysed oxidation of ascorbate, their protective effects have been attributed to protein binding of cupric ions rather than to scavenging of reactive oxygen-derived radicals [19]. In addition, neither the transition metal ion-catalysed oxidation of ascorbate nor the formation of the allopurinol–ascorbate–copper complex was affected by BHT, a lipophilic antioxidant whose radical scavenging activity was also detectable in some aqueous systems, such as the hydroxyl radical-mediated degradation of deoxyribose (data not shown). Moreover, allopurinol and oxypurinol also inhibited the cupric ion–tBHP-catalysed peroxidation of membrane lipids (Fig. 5), presumably by chelating cupric ions, thereby preventing their reaction with hydroperoxides which can generate reactive oxy-radicals [20]. The lack of inhibitory effect on lipid peroxidation induced by tBHP alone (i.e. in the absence of exogenously added transition metal ions) suggests that the actions of allopurinol or oxypurinol are not mediated by direct free radical scavenging. This is further strengthened by the observation that increasing concentrations of allopurinol or oxypurinol did not cause a greater degree of inhibition of cupric ion–tBHP-induced lipid peroxidation. All this evidence seems to suggest that the inhibition by allopurinol of the transition metal ion-mediated processes is more likely attributable to its metal chelating properties rather than to an oxy-radical scavenging action.

Allopurinol and oxypurinol, as well as other iron-chelators such as EDTA and uric acid, inhibited the ferric ion-induced oxidation of erythrocyte membrane lipids. Based on the hypothesis that an optimal ratio of ferric to ferrous ion is important for the initiation of iron-dependent lipid peroxidation [21–23], iron chelators may act by changing the redox potential of iron. A higher affinity of ferric ions for lipid regions of membranes than for allopurinol or oxypurinol may explain the weak inhibitory effects of allopurinol and oxypurinol on the ferric ion-induced lipid peroxidation in erythrocyte membranes. However, the lipid-soluble antioxidant BHT strongly suppressed this lipid peroxidation. The finding that the inhibitory actions of allopurinol and oxypurinol in the ferric ion-induced oxidation of erythrocyte membrane lipids were surmountable by increasing the concentration of ferric ions, similar to the results obtained with EDTA (Fig. 4), further suggests the involvement of metal chelation in the actions of allopurinol. Although the inhibitory effect of a low concentration of BHT can be overcome, in part, by increasing the concentration of ferric ions, this is probably due to the complete depletion of exogenously added BHT as a result of the increasing production of oxidants [24], which eventually leads to a greater extent of lipid peroxidation. Moreover, the ability of BHT to reduce the extent of peroxidation induced by the cupric ion–tBHP mixture to a level lower than that produced by tBHP alone further suggests a free radical scavenging mode of action.

During the course of tissue ischemia and reperfusion, the availability of catalytically active transition metal ions, such as ferric and cupric ions, might be increased by a process of decompartmentalization, i.e. mobilization from cellular sites [25, 26]. This, in turn, could lead to an enhancement of reactive oxygen radical generation and subsequent amplification of tissue damage. The effective concentrations of allopurinol or oxypurinol shown here to inhibit the transition metal ion-catalysed reactions are much higher than the plasma concentrations attained following allopurinol treatment in animals subjected to I/R studies [27]. However, the fact that optimal protection by allopurinol against I/R injury is usually observed only following multiple drug dosing [7–9, 28] may reflect the progressive attainment of tissue allopurinol or oxypurinol levels sufficient to inhibit the transition metal ion-catalysed generation of reactive oxidant species. Our data, therefore, support the hypothesis that the transition metal chelating actions of allopurinol and oxypurinol are relevant to their protective action against I/R injury.

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