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INTERACTION BETWEEN ALLOPURINOL AND COPPER: POSSIBLE ROLE IN MYOCARDIAL PROTECTION

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Allopurinol, a potent inhibitor of xanthine oxidase, is known to effectively protect the heart against damage in patients undergoing cardiac bypass surgery. There is still an ambiguity concerning the presence of xanthine oxidase in the human heart. Thus, the mechanism underlying the protective effect of allopurinol is unclear. Transition metal ions, such as iron and copper, can participate in single-electron reactions and mediate the formation of oxygen-derived free radicals. In this study the interaction between allopurinol and Cu(II) was investigated. Spectrophotometric investigation shows that allopurinol (0–0.8 mM) form a 1:1 complex with Cu(II) ions (0–0.8 mM) with a specific absorbance peak at 364 nm. Also, the rate constant (k) for the copper-catalyzed aerobic oxidation of ascorbate was markedly decreased in the presence of allopurinol (from 0.068 min^{-1} to 0.014 min^{-1}). Allopurinol substantially reduced the copper-mediated and ascorbate-driven DNA breakage. Spectrophotometric measurements did not indicate a specific interaction between iron ions and allopurinol. It is suggested that the beneficial effects of allopurinol during reperfusion of the heart could stem from its chelation of copper, yielding a complex with low redox activity.

KEY WORDS: Allopurinol, copper, chelation, ascorbate, DNA.

INTRODUCTION

There is growing evidence implicating oxygen free radicals in myocardial injury caused by ischemia and reperfusion.[1–4] However, the sources of these free radicals are not clear. It has been suggested that the transition metals, iron and copper, can mediate the formation of these oxygen active species.[5–11] Xanthine oxidase has been proposed as a major source of oxygen free radicals which are detrimental to the heart.[12,13] Although the levels of xanthine oxidase are very low in the human heart,[14] allopurinol, a specific inhibitor of the enzyme, has been found to be effective in preventing reperfusion damage to cellular structures and aberrations in cardiac metabolism in patients undergoing open heart surgery.[15–17]

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This study was directed toward the elucidation of the mechanism of the beneficial effect of allopurinol in the human heart, by examining the interaction between allopurinol and copper or iron ions.

MATERIALS AND METHODS

Allopurinol, glycine, nitrilotriacetic acid (sodium salt) (NTA), calf thymus DNA, sodium ascorbate and ethylenediamine-tetra acetic acid (EDTA) were purchased from Sigma Chemical Co. Desferrioxamine mesylate (Desferal®) was purchased from Ciba-Geigy (Basle, Switzerland).

Cu(II)-allopurinol interaction

The interaction between allopurinol and copper or iron and the stoichiometry of the corresponding complexes were spectrophotometrically determined according to the procedure of Nagano *et al.*[17] Briefly, increasing concentrations of allopurinol (0–0.8 mM) were mixed with the corresponding decreasing concentrations of $CuSO_4$ (0.8–0 mM) in either sodium acetate buffer (10 mM, pH 5.6) or Tris buffer (10 mM or 100 mM, pH 7.4) and the spectra (300–450 nm) were recorded using a Kontron Uvikon 860 spectrophotometer.

Assessment of the stability constant of the allopurinol-copper complex

Cu(II)-allopurinol complex (0.4 mM of each) in HEPES buffer (10 mM, pH 7) was titrated with aliquots of a ligand (chelator), whose stability constant with copper is known (Figure 2). The following chelators and their respective binding constants with copper have been used: glycine, 1.6×10^8 (ref. 18); NTA, 4.0×10^{13} (ref. 19); Desferal®, 1×10^{14} (ref. 20) and EDTA, 8.5×10^{18} (ref. 21). The spectra (250–450 nm) were recorded and the absorbance peaks at 364 nm were used as an indicator of the Cu(II)-allopurinol complex.

The effect of allopurinol on ascorbate oxidation

Allopurinol (1 mM), either with or without DNA (0.25 mg/ml), was preincubated with sodium ascorbate (5 mM), $CuSO_4$ (0.15 mM), and HEPES buffer (5 mM, pH 7) in a shaking water bath at 37°C (aerobic conditions). The residual (reduced form of) ascorbate was measured spectrophotometrically at 515 nm using the ferriphenanthroline reduction assay.[22] At various time intervals, 10 μl samples were withdrawn and added to optical cells containing 0.1 ml of ferriphenanthroline and 0.9 ml imidazole buffer, prepared as previously described. The apparent first order rate constant for ascorbate oxidation (k) was determined.

DNA preparation

Circular single-stranded DNA of M13 bacteriophage was isolated from the mature free phage according to the method of Shreier and Cortese.[23]

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DNA degradation reactions

A sample of 2–5 μg of single-stranded DNA was preincubated with either Cu(II) or Cu(II)/allopurinol (as indicated in the corresponding figure legends) for 5 minutes in HEPES buffer (5 mM, pH 7.4) at 37°C. The mixture was then incubated in the presence of ascorbate (2.5 mM) for an additional 5 minutes at 37°C. The reaction was terminated by the addition of EDTA (10 mM) and the samples were run on agarose gels as described below.

Agarose gel electrophoresis

Reaction mixtures (25 μl) were run on agarose gel (1%) in Tris-borate/EDTA (TBE) buffer (Tris (100 mM), boric acid (10 mM), EDTA (2.5 mM), pH = 8.3). Electrophoresis was carried out at 20 V for 16 hours, and the gels were subsequently stained with ethidium bromide (0.5 μg/ml) in TBE buffer, for 30 minutes. The gels were photographed with a Polaroid Land Camera using Polaroid 667 film.

RESULTS

Cu(II)-allopurinol complex

Figure 1a demonstrates the absorption spectra in the 300–450 nm range of solutions containing allopurinol and Cu(II) in various molar ratios. A new specific absorbance peak at 364 nm, attributed to the copper/allopurinol complex, was observed. This peak was not observed in the absence of either allopurinol or copper ions. Under anaerobic conditions a peak of similar height at 364 nm was observed. Analysis according to Nagano *et al.*[17] using acetate or Tris buffers, at pH 5.6 or 7.4, respectively, indicated a 1:1.05 (±0.04) copper/allopurinol complex (Figure 1b).

The stability constant of the copper-allopurinol complex was assessed by titrating the copper-allopurinol complex (1:1) with different chelators whose stability constant (Cu(II)-chelator) is known. Figure 2 demonstrates the ability of the chelators: NTA, EDTA and Desferal® to dissociate the Cu(II)-allopurinol complex. It is obvious from Figure 2 that glycine has a minimal effect on the stability of the Cu(II)-allopurinol complex, while EDTA has the maximal effect on the disintegration of the complex. The order of activity of these ligands on the Cu(II)-allopurinol complex (HEPES buffer, 10 mM, pH 7.4) is: glycine ≪ Desferal® < NTA < EDTA.

The effect of allopurinol on ascorbate oxidation

Aerobic oxidation of ascorbate without Cu(II) gave an apparent rate constant (k) of less than 0.003 min^{-1} (Table I). Addition of Cu(II) ions to the reaction mixtures markedly increased the rate of ascorbate oxidation ($k = 0.068\ min^{-1}$), however, with the addition of allopurinol or DNA the rate of ascorbate oxidation was significantly reduced, to 0.014 and 0.012 min^{-1}, respectively. An additional two-fold decrease in the oxidation rate was observed when allopurinol **and** DNA were added together (Table I).

Figure 3 presents degradation of DNA under various conditions. Cu(II) causes rapid and extensive ascorbate-driven DNA degradation. The addition of allopurinol resulted in a marked protection against the degradation. This protection

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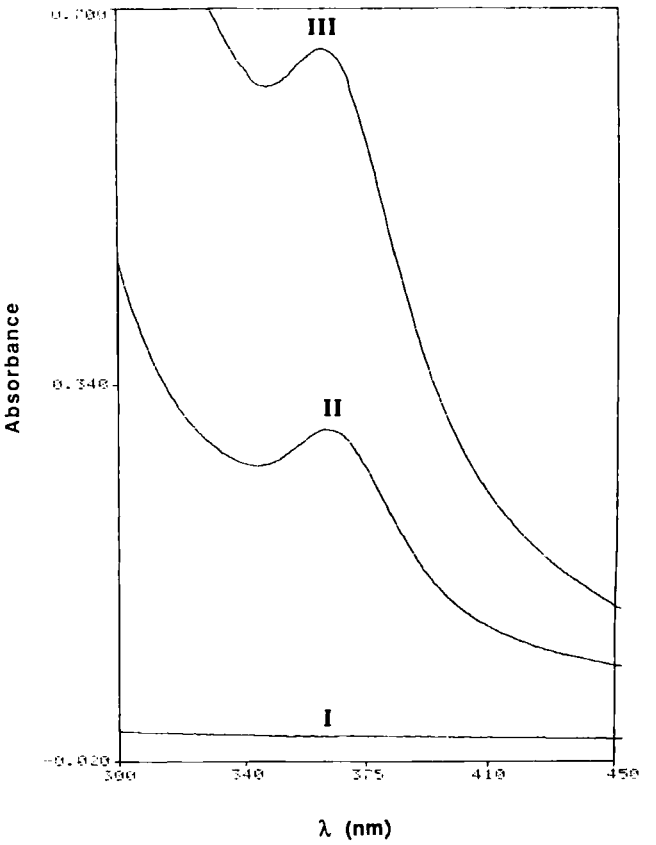

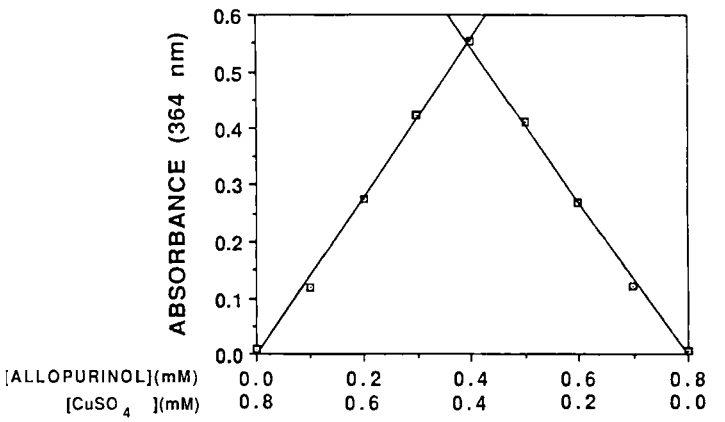

FIGURE 1 Spectral characterization of Cu(II)/Allopurinol complexes. The solutions were prepared in Tris-HCl buffer (0.1 M, pH 7.4). (a) Absorbance spectra of the Cu(II)/allopurinol complex in the 300–450 nm range. Trace I: [Allopurinol] = 0.4 mM, [Cu(II)] = 0.mM; Trace II: [Allopurinol] = 0.4 mM, [Cu(II)] = 0.2 mM; Trace III: [Allopurinol] = 0.4 mM, [Cu(II)] = 0.4 mM. (b) The absorbance at 364 nm of the complex formed at various [Cu]/[allopurinol] ratios: [Allopurinol] + [Cu(II)] = .8 mM.

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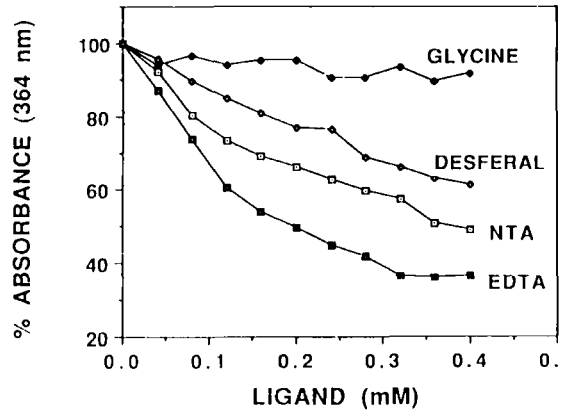

FIGURE 2 Assessment of the Cu(II)-allopurinol stability constant. The complex, prepared by mixing $CuSO_4$ (0.4 mM) and allopurinol (0.4 mM) in a HEPES buffer (10 mM, pH 7.4) was titrated with aliquots of each of the following ligands (chelators): glycine, NTA, Desferal® and EDTA. The absorbance at 364 nm was measured.

increased with [Allopurinol]/[Cu(II)] ratio, reaching about 70% at a [Allopurinol]/[Cu(II)] ≥ 7 (not shown).

Similar results were obtained with the following buffer systems: acetate (5 mM, pH 5), Tris (5 mM, pH 7.4) and phosphate (5 mM, pH 7.4) (results not shown). The optimal protective effect was obtained in HEPES buffer (5 mM, pH 7.4).

DISCUSSION

The present study probed the interaction between allopurinol and Cu(II). A specific absorbance peak at 364 nm was observed in the presence of allopurinol and Cu(II), indicating the production of a complex with a 1:1 stoichiometry. Copper forms

TABLE I
Apparent first order rate constant (k) for the copper-catalyzed ascorbate oxidation in the absence or the presence of allopurinol and DNA

	System					
	Ascorbate (5 mM)	O_2 (ambient air)	Cu(II) (150 μM)	Allopurinol (1 mM)	DNA (250 μg/ml)	k (min^{-1})
1.	+	+	−	−	−	<0.003
2.	+	+	+	−	−	0.068
3.	+	+	+	+	−	0.014
4.	+	+	+	−	+	0.012
5.	+	+	+	+	+	0.007
6.	+	+	−	−	+	<0.006
7.	+	+	−	+	−	<0.0001

For the experimental details see Materials and Methods

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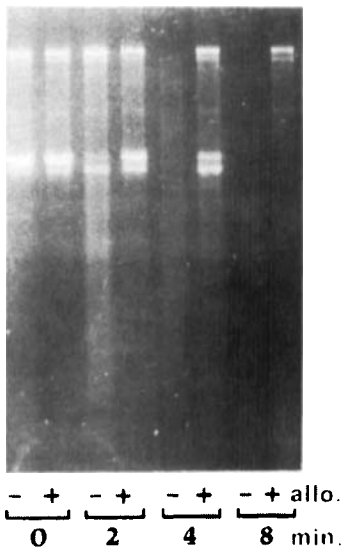

FIGURE 3 Allopurinol provides protection to DNA against degradation. Two μg of M13 bacteriophage single-stranded purified DNA in 10 μl of HEPES buffer (5 mM, pH 7.4) were incubated for 5 min at 37°C with Cu(II) (30 μM) (all lanes) in the presence of allopurinol (200 μM) (marked with "+"). Ascorbic acid (2.5 mM) was then added and samples were incubated further at 37°C for the indicated times. The reactions were terminated by the addition of EDTA (10 mM) and transferred to ice. Gels were run as described in Materials and Methods.

planar, tetrahedral or octahedral complexes with at least four ligands associated with each Cu(II).[24] One allopurinol molecule cannot contribute all the ligands, thus, a ternary complex, Cu(II)-Allopurinol-Tris or Cu(II)-Allopurinol-HEPES or Cu(II)-Allopurinol-acetate, is postulated. Ko and Godin[25] were unable to spectrally detect a Cu(II)-allopurinol complex in an aqueous solution, however, a distinctive complex of Cu(II)-allopurinol-ascorbate was detected. These findings lend support to the suggestion of a ternary complex involving Cu(II)-allopurinol with an additional ligand (Tris, HEPES, acetate or ascorbate). The interaction does not seem to depend on oxidation-reduction reactions. Allopurinol decreased the rate of copper-mediated ascorbate oxidation. DNA caused a similar decrease in the rate of this reaction. The explanation for this observed decrease is that copper ions form stable complexes with either allopurinol or DNA. These are characterized by a smaller turnover number for the cyclic reduction and reoxidation of copper within these complexes. The combination of both allopurinol and DNA caused a more pronounced decrease in the rate of copper-mediated ascorbate-driven oxidation. It is possible that a ternary complex DNA-Cu(II)-allopurinol is being formed and its turnover number is one-half of that of either Cu(II)-DNA-buffer or Cu(II)-allopurinol-buffer complexes. This is in accord with the observation that allopurinol also provided partial protection against copper-mediated ascorbate-driven DNA degradation.

From the titration curve of the Cu(II)-allopurinol complex with different ligands, it is possible to assess the stability constant of the complex. It is obvious that the stability constant of this complex is higher than that of Cu(II)-glycine complex, and is estimated above 10^{10}. Under physiological concentration of allopurinol

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(0.04 mM),[26] and copper concentration of $(0.1-1.0) \times 10^{-6}$ M, this stability constant (10^{10}) is sufficiently high to ensure quantitative binding of all (>99%) the copper by allopurinol. By this, a protection will be afforded against copper-mediated injurious processes.

While the plasma concentration of allopurinol in patients is about 0.04 mM, that of oxypurinol is ten times higher.[26] These concentrations are in the same range as those used in the present study. It has been proposed that allopurinol may also act as a scavenger of hydroxyl radicals,[27] however, at such low concentrations (below 1 mM) the efficiency of scavenging is expected to be quite limited,[28] and the chelation of copper could be the major pathway of protection.[6]

During hypoxic perfusion the efficiency of cellular protective mechanisms against oxygen-derived free radicals is reduced, with significant decreases in the activities of both superoxide dismutase and glutathione peroxidase.[1] Compared to aerobic hearts, cellular levels of acid-soluble thiol groups, insoluble thiol groups and reduced glutathione are decreased by hypoxia, while the oxidized glutathione concentration remains unchanged. Reoxygenation leads to a further reduction in thiol groups and glutathione levels.[1] Physiological systems contain significant concentrations of labile iron and copper ions[6,29,30] which may promote hydroxyl radical production via the Fenton and Haber-Weiss reactions.[5-11] These radicals are known to react effectively with -SH groups. Thus, transition metal chelators are of potential value for the prevention of such biological damage, including reperfusion-induced injury.[5-7]

Urate, a purine analog of allopurinol, also forms stable complexes with copper (and iron) ions.[8,31,32] Urate was found to protect against iron-induced ascorbate oxidation in blood and other biological fluids.[32] In both human serum and in phosphate buffer, urate exerted its protective effect.

Naturally occurring and synthetic pteridines, riboflavins, purines, and amino acids have a high affinity for heavy metal ions.[33,34] Copper ions interact with much greater affinity than iron ions with amino acids.[34] Both Cu(II) and Fe(III) are rather insoluble at physiological pH and can only remain in solution by forming chelates with high- or low-molecular weight cellular components,[6,35,36] which, in turn, can serve as centers for free radical production. During ischemia, increased levels of various cellular reducing agents, including pyridine nucleotides, flavin nucleotides, and cytochromes, which might provide the reducing equivalents involved in the initiation of free radical-induced damage, were observed.

Iron, which is relatively highly abundant in biological systems, can catalyze biological redox reactions. However, in human red cells[37] and bacterial cells,[38] Cu(II) was found to be ten-fold more efficient, on molar basis, then Fe(III) in inducing free radical-mediated cellular damage.

Thus, even minute quantities of mobilized copper, which could be below the level of detection by most available methodologies, might be sufficient to promote tissue injury. The protective effect of allopurinol in patients undergoing open heart surgery is in accord with chelation of copper by allopurinol to yield a stable complex with a low redox activity.

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