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OXYPURINOL AS AN INHIBITOR OF XANTHINE OXIDASE-CATALYZED PRODUCTION OF SUPEROXIDE RADICAL

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Abstract—A recent study of the mechanism by which oxypurinol inhibits uric acid generation [T. Spector, W. W. Hall and T. A. Krenitsky, *Biochem. Pharmac.* **35**, 3109 (1986)] showed that xanthine is ineffective in impeding the binding of oxypurinol to reduced xanthine oxidase. This study prompted the present hypothesis that, at elevated concentrations of substrates, oxypurinol would be superior to allopurinol as an inhibitor of the xanthine oxidase-catalyzed production of superoxide radical. It was found that the potency of allopurinol was attenuated by elevated concentrations of xanthine and hypoxanthine, whereas the potency of oxypurinol was relatively unaffected. Oxypurinol produced immediate inhibition of superoxide radical production as well as progressive inhibition with time. In contrast, allopurinol, which is also a substrate for xanthine oxidase, produced very little immediate inhibition and caused progressive inhibition only after conversion to oxypurinol. The theoretical advantages of treating ischemic tissues with oxypurinol are discussed.

Allopurinol (4-hydroxyprazolo[3,4-*d*]pyrimidine) inhibits the xanthine oxidase-catalyzed production of uric acid by virtue of its own substrate activity with this enzyme. As an alternative substrate, allopurinol competes with the physiological substrates, xanthine and hypoxanthine, for binding and subsequent oxidation. The product of the reaction, oxypurinol (4,6-dihydroxyprazolo[3,4-*d*]pyrimidine; also known as oxipurinol), is a true dead-end inhibitor of xanthine oxidase. Oxypurinol forms an initial complex with electron-rich (reduced) enzyme, which rearranges into a more stable form [1–8 and reviewed in 9 and 10].

The inhibition of xanthine oxidase may serve two purposes. In addition to decreasing the production of uric acid, inhibition can also prevent the formation of superoxide radical, which is generated as a by-product as the reduced enzyme is reoxidized by oxygen. The latter purpose is important for the protection of ischemic tissues from the oxygen toxicity that occurs following reperfusion. During ischemia, the breakdown of ATP leads to a large build-up of hypoxanthine. Reintroduction of oxygen creates a burst of superoxide radical production, which gives rise to the damaging hydroxyl radical [reviewed in 11 and 12].

A recent study [7] of the mechanism by which oxypurinol inhibits uric acid formation has prompted the present hypothesis that oxypurinol may be superior to allopurinol as an inhibitor of the xanthine oxidase-catalyzed production of superoxide radical. Although earlier studies have shown that xanthine and other substrates [13, 14] can bind to reduced xanthine oxidase, this latter report [7] demonstrated that xanthine (and presumably hypoxanthine) is ineffective in impeding the binding of oxypurinol to the reduced enzyme and subsequent inhibition of uric acid formation. In fact, the formation of the stable complex between oxypurinol and reduced human xanthine oxidase occurred faster at 55 μM xanthine than at 25 μM xanthine. Therefore, it is hypothesized that the elevated concentrations of physiological substrates could competitively inhibit the binding of allopurinol but not oxypurinol. Oxypurinol may have an additional advantage because the oxidation of allopurinol, itself, can generate superoxide radical. Thus, although allopurinol can inhibit the production of uric acid, it cannot inhibit the formation of superoxide radical; it must first be converted to oxypurinol to accomplish this.

In the present study, the relative abilities of oxypurinol and allopurinol to inhibit the xanthine oxidase-catalyzed production of superoxide were tested at several concentrations of xanthine and at elevated hypoxanthine. The data clearly support the theory that oxypurinol is superior to allopurinol for protecting ischemic tissues from reperfusion injury.

MATERIALS AND METHODS

Bovine xanthine oxidase was purchased from Boehringer Mannheim; horse heart cytochrome *c*, Type III, from Sigma; and catalase from Worthington Biochemicals.

Reaction mixtures contained 50 mM potassium phosphate buffer at pH 6.8, 0.1 mM EDTA, 200 units/ml catalase, 80 μM cytochrome *c*, 0.03 units/ml xanthine oxidase, and substrates and inhibitors as indicated in the text. The production of superoxide radical was monitored by following the superoxide-dependent reduction of cytochrome *c* at 550 nm [15] with a Gilford recording spectrophotometer. The reaction temperature was electronically regulated to 37°. Catalase was included to prevent the non-enzymic reoxidation of cytochrome *c* by H_2O_2. Reactions were initiated with xanthine oxidase after a 3-min preincubation. Rate constants

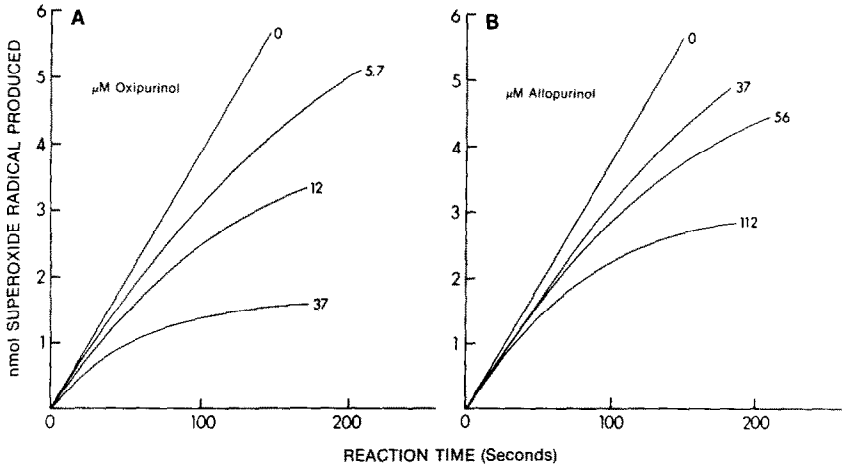

Fig. 1. Abilities of oxypurinol (A) and allopurinol (B) to inhibit the xanthine oxidase-catalyzed production of superoxide radical from 120 μM xanthine.

for the development of progressive inhibition were calculated as described earlier [16].

RESULTS

The abilities of allopurinol and oxypurinol to inhibit the xanthine oxidase-catalyzed production of superoxide radical were tested at initial concentrations of 120, 55 and 12 μM xanthine. The higher concentrations were chosen to represent a conservative estimate of the level of substrate that might build up during tissue ischemia. The concentration of 12 μM xanthine was the lowest that would sustain a linear reaction for a reasonable time. The 12 μM xanthine was completely depleted within 1 min in the absence of inhibitor. Some of the data obtained at 120 μM xanthine are shown in Fig. 1. Although the rate of formation of superoxide radical is linear with respect to time in the absence of inhibitors, it progressively slows down in the presence of either allopurinol or oxypurinol. Two significant differences between the compounds were detected. First, it was calculated by computer analysis [16] that oxypurinol produced significant inhibition of the initial velocity, whereas allopurinol did not (see Figs. 2 and 4 also). Second, the concentrations of allopurinol required to induce rapid, progressive inhibition were significantly higher than those required for oxypurinol. These differences were also noted at 55 and 12 μM xanthine. However, at 12 μM xanthine, high concentrations of allopurinol (> 65 μM) induced more rapid decelerations of superoxide radical production than did comparable concentrations of oxypurinol (Fig. 2).

Apparent first-order rate constants for the development of the progressive inhibition were calculated for reactions inhibited by various concentrations of either allopurinol or oxypurinol. Constants that correspond to inhibitor concentrations of 10 and 25 μM were obtained by extrapolation from graphs of the rate constant versus the concentration of inhibitor. The bar graph of Fig. 3 shows the effect of the concentration of xanthine on these rate constants. It can be seen that the rate of the development

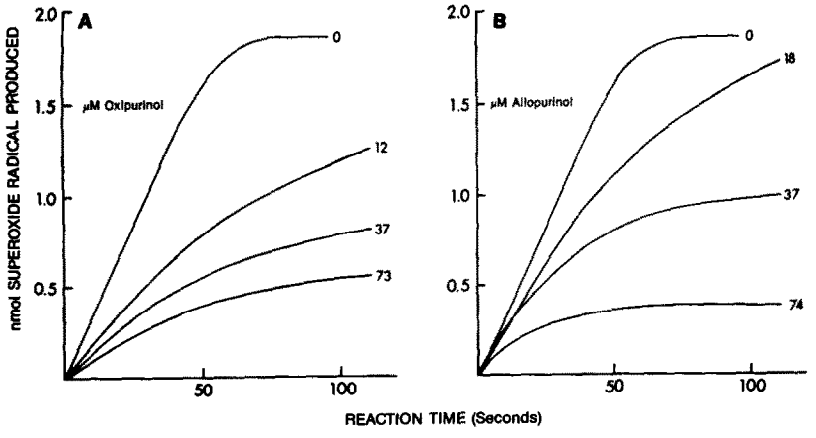

Fig. 2. Abilities of oxypurinol (A) and allopurinol (B) to inhibit the xanthine oxidase-catalyzed production of superoxide radical from 12 μM xanthine.

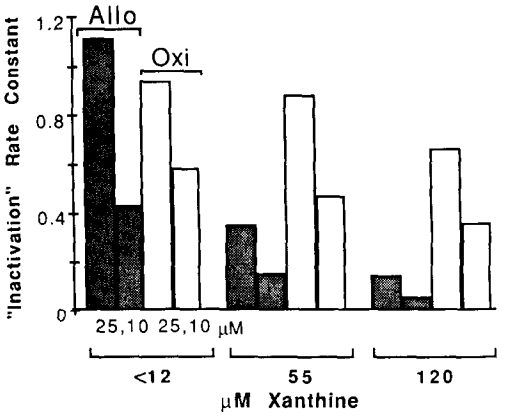

Fig. 3. Rate constants (min^{-1}) for the development of progressive inhibition ("inactivation") of the xanthine oxidase-catalyzed production of superoxide radical from xanthine by oxypurinol and allopurinol. The concentrations of the inhibitors are 25 and 10 μM for the left and right bar, respectively, for each pair: oxypurinol (Oxi; light bars); allopurinol (Allo; shaded bars). The concentration of xanthine is indicated at the bottom of the figure.

of inhibition by allopurinol was retarded significantly as the concentration of xanthine was increased, whereas that for oxypurinol remained much less affected.

The above experiments were repeated with 120 μM hypoxanthine replacing xanthine as the substrate. The results (Fig. 4) show that the patterns of inhibition were very similar to those observed with xanthine. A difference was noted in that the uninhibited reactions increased in velocity during the first 50 sec of the reaction.

DISCUSSION

These studies show that, compared to allopurinol, oxypurinol was a considerably more efficient inhibitor of xanthine oxidase-catalyzed superoxide radical production when the concentrations of the physiological substrates were elevated. An examination of the recent literature reveals that, during ischemia, the breakdown of ATP leads to tissue concentrations of hypoxanthine that are in the 100 to 400 μM range [12, 17–19]. The superiority of oxypurinol lies in its ability to produce immediate inhibition as well as rapidly occurring progressive inhibition at low concentrations. The lack of significant immediate inhibition by allopurinol is probably due to its own production of superoxide radical [2] as it is being oxidized by xanthine oxidase. Allopurinol is an excellent substrate of xanthine oxidase [1, 3, 8, 20, 21] and therefore is not capable of inhibiting superoxide radical production itself. On the other hand, oxypurinol is a true dead-end inhibitor that rapidly forms an initial complex with reduced xanthine oxidase [3, 4], which accounts for its immediate inhibition. The time-dependent rearrangement of this initial complex into a stable complex [3, 5, 7] accounts for the progressive inhibition observed with oxypurinol either added directly or generated from allopurinol. Furthermore, because xanthine and allopurinol compete as substrates for binding, the concentrations of allopurinol required to induce the formation of oxypurinol and subsequent progressive inhibition increased in direct proportion to the concentration of competing substrate. In contrast, the ability of oxypurinol to induce progressive inhibition was relatively independent of the concentration of xanthine because xanthine and hypoxanthine are ineffective in blocking the binding of oxypurinol to reduced xanthine oxidase [7]. Only when the concentration of xanthine was less than 12 μM did high concentrations of allopurinol appear more inhibitory than comparable concentrations of oxypurinol. This is most likely due to the ability of allopurinol to supplement the capacity of xanthine to produce the reduced enzyme species to which oxypurinol binds.

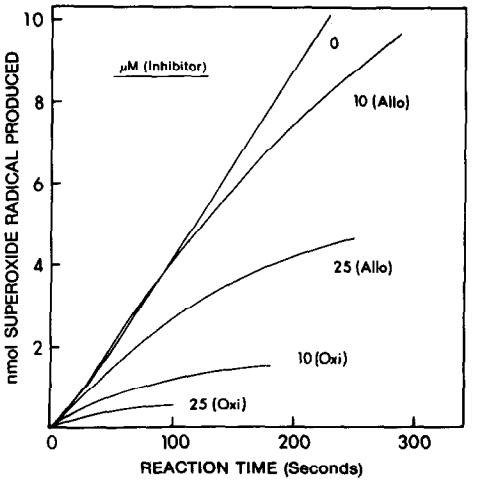

Fig. 4. Abilities of oxypurinol (Oxi) and allopurinol (Allo) to inhibit the xanthine oxidase-catalyzed production of superoxide radical from 120 μM hypoxanthine.

It is important to note that the superiority of oxypurinol to allopurinol at elevated concentrations of physiological substrates should be magnified with human xanthine oxidase. Since the K_m values for allopurinol are similar with bovine and human xanthine oxidases [8], the abilities of substrates to compete with allopurinol should remain the same. However, the two enzymes behaved differently with respect to the ability of elevated concentrations of substrate to affect the rate that oxypurinol induced progressive inhibition. Whereas the progressive inhibition of the bovine enzyme occurred slightly slower at higher concentrations of xanthine, the development of inhibition of the human enzyme was actually faster at elevated concentrations of xanthine [7]. Therefore, one would expect the potency by which allopurinol inhibits human xanthine oxidase to be attenuated by substrate and, in contrast, the potency of oxypurinol should be amplified by elevated substrates.

Although most of the xanthine oxidizing enzyme actually exists as a dehydrogenase (NAD serves as the electron acceptor) *in vivo*, a significant amount is present as an oxidase [22, 23], which is believed to be responsible for the production of superoxide radical that ultimately leads to damage following reperfusion of ischemic tissues [reviewed in 11 and

12]. Therefore, it is the inhibition of xanthine oxidase that appears to be important for the protection from radical damage. However, the inhibition of xanthine dehydrogenase may also be important because the oxidation of substrates by this enzyme produces NADH, which may also result in the generation of superoxide radical as it is reoxidized.

The data of the present report clearly show the theoretical advantages of treatment with oxypurinol once ischemia and subsequent elevation of hypoxanthine and xanthine have occurred. As far as prophylactic treatment is concerned, either allopurinol or oxypurinol should be effective. However, oxypurinol may be more desirable because it does not lead to the production of superoxide radical.

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