J. Pharm. Pharmacol. 1994, 46: 487–490
Received September 1, 1993
Accepted December 3, 1993

Induction of the Conversion of Xanthine Dehydrogenase to Oxidase in Rabbit Liver by Cu^{2+}, Zn^{2+} and Selenium Ions

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Abstract—Effects of Cu^{2+}, Zn^{2+}, Fe^{2+} and selenium ions on the conversion of xanthine dehydrogenase to oxidase in rabbit liver were examined. Under basal conditions, xanthine oxidase activity represented only 16% of the total xanthine oxidase plus dehydrogenase activity. Cu^{2+} (2–10 μM), Zn^{2+} (5–30 μM) and selenium ions (5–100 μM) brought about the conversion of xanthine dehydrogenase to oxidase in a dose-dependent manner. The concentrations of Cu^{2+}, Zn^{2+} and selenium ions required for increasing xanthine oxidase activity by 50% was approximately 4, 10 and 20 μM, respectively. On the other hand, Fe^{2+} had no effect on the conversion of the enzyme up to 100 μM. These results suggest that Cu^{2+}, Zn^{2+} and selenium ions have the potential to modulate the conversion of xanthine dehydrogenase to oxidase in rabbit liver.

The hypothesis that reactive oxygen species are responsible for hepatic injury (in particular, ischaemic and postischaemic injury) has obtained much support over the past few years (Braunwald & Lonar 1985; McCord 1985; Cross et al 1987). Reactive oxygen species are able to react with polyunsaturated fatty acids in phospholipids of membranes, resulting in lipid peroxidative damage (Halliwell & Gutteridge 1984; Slater 1984). The generator of reactive oxygen species in ischaemic and reperfused liver tissue is still not known, but a xanthine oxidase-mediated process is considered to be the primary source of reactive oxygen species during ischaemia-reperfusion (Cross et al 1987; McCutchan et al 1990; Hasan et al 1991; Brass et al 1991; Sanhueza et al 1992). Xanthine oxidase is involved in the metabolism of xanthine to uric acid with the generation of reactive oxygen species, such as superoxide ($O_2\cdot$) and hydrogen peroxide (H_2O_2), from oxygen (O_2) (Kuppusamy & Zweier 1989). During ischaemia, it has been reported that this enzyme is induced from xanthine dehydrogenase that utilizes NAD^+ in place of O_2 as an electron acceptor, but the mechanism responsible for the induction of xanthine oxidase from xanthine dehydrogenase, to date, has not been determined clearly.

Various ions are known to be modulators of reactive oxygen species-generating enzymes. Few reports are available, however, concerning the influence of ions on the conversion of xanthine dehydrogenase to oxidase. Cu^{2+}, Zn^{2+}, Fe^{2+} and selenium ions are closely related in lipid peroxidation. We have previously reported that Fe^{2+} and Cu^{2+} induce lipid peroxidation in rabbit kidney (Fujimoto et al 1984; Fujita et al 1987). It has also been shown that Zn^{2+} (Fukino et al 1984) and selenium ions (Rotruck & Pole 1973) act as inhibitors of stimulant-induced lipid peroxidation. In the present investigation, our purpose was to study the effects of Cu^{2+}, Zn^{2+}, Fe^{2+} and selenium ions on the conversion of xanthine dehydrogenase to oxidase in rabbit liver.

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Materials and Methods

Xanthine, NAD^+, NADH, uric acid and allopurinol were purchased from Sigma Chemical Co. (St Louis, MO). Trypsin was purchased from Boehringer (Mannheim, Germany). $CuSO_4$, $ZnCl_2$, Na_2SeO_3 and $FeSO_4$ were obtained from Wako Pure Chemical Co. (Osaka, Japan). Other reagents were of analytical grade.

Enzyme preparation

Male rabbits, 2–2·5 kg, with free access to food, were used as liver donors. Each rabbit was anaesthetized by intravenous administration of sodium pentobarbitone (30 mg kg^{-1}), the abdomen opened and the liver perfused in-situ with ice-cold saline. Homogenization, centrifugation and Sephadex G-25 chromatography were performed as previously described (Krenitsky & Tuttle 1978; Cighetti et al 1989). Briefly, the liver was excised and homogenized in 5 vol 30 mM potassium phosphate buffer (pH 8·3). The homogenate was then centrifuged at 1500 *g* for 10 min, and the resulting supernatant fraction was further centrifuged at 105 000 *g* for 60 min. Aliquots of the supernatant (2·5 mL) obtained after the latter centrifugation were transferred to a precooled Sephadex G-25 (PD-10, Pharmacia) column and eluted with 3·5 mL potassium phosphate buffer, to remove endogenous substrates. This eluate was used for the assay of xanthine oxidase and dehydrogenase activities.

Assay of xanthine oxidase and dehydrogenase activities

Xanthine oxidase and dehydrogenase activities were assayed by monitoring uric acid formation by use of a high-pressure liquid chromatographic (HPLC) method that modified a procedure previously described (Cighetti et al 1989, 1990). The enzyme preparation was incubated at 30°C in 0·1 M Tris-HCl buffer (pH 8·1) containing 60 μM xanthine with or without 0·1 mM NAD^+, in a final volume of 1 mL. Xanthine oxidase activity was determined by uric acid formation in the presence of xanthine alone. Xanthine dehydrogenase activity was calculated by subtracting the amounts of uric acid in the presence of xanthine alone from those in the presence of xanthine plus NAD^+.

Determination of uric acid by HPLC

After incubation, uric acid in the reaction mixture was extracted with 10 vol ethanol (Cighetti et al 1989, 1990). The supernatant after centrifugation at 1000 *g* for 5 min was dried under nitrogen and the residue resuspended in 0·8 mL distilled water/0·1 M ammonium phosphate buffer (pH 5·5) (1:1, v/v). The suspension (0·1 mL) was directly injected in the HPLC system. The analysis was carried out with a reverse-phase column (YMC A-303 type, 4·6 mm i.d. × 25 cm). The mobile phase consisted of 0·1 M ammonium phosphate buffer (pH 5·5). The volume of sample injected was 100 μL, the flow rate 1·5 mL min^{-1}, total running time 15 min. Uric acid was detected at 292 nm at 0·005 absorbance units full-scale. Quantitation was based on peak height, and excellent proportionality was obtained between the different height of the standard or extract injected and the concentration of uric acid. The detection limit of the HPLC method was about 1 pmol. The equipment consisted of a Shimadzu model LC-6AD pump, SPD-6AV UV detector and C-R6A chromatopak.

Statistics

Results are means ± s.e.m. Statistical significance was determined by Student's *t*-test.

Results

Fig. 1 shows the effects of protein concentration (A) and incubation time (B) on the xanthine oxidase and dehydrogenase activities in rabbit liver. The supernatant prepared from this tissue was incubated with xanthine in the presence or absence of NAD^+. Xanthine oxidase activity and dehydrogenase activity were proportional to protein concentration up to 1 mg protein, and linear up to 60 min. In the following experiments, protein concentration was fixed at 0·5 mg protein, and the incubation was performed for 30 min. The value of the activity of xanthine oxidase obtained was 68·9 ± 10·8 pmol min^{-1} mg^{-1} and that of xanthine dehydrogenase was 366·7 ± 47·8 pmol min^{-1} mg^{-1}; xanthine oxidase represented only 16% of the total xanthine oxidase plus dehydrogenase activity (n = 22). A similar tendency has been obtained previously, using a rat liver preparation (Engerson et al 1987; McKelvey et al 1988).

We have investigated the effects of allopurinol, a xanthine oxidase and dehydrogenase inhibitor (Massey et al 1970), and trypsin which converts xanthine dehydrogenase to oxidase partially by proteolysis of xanthine dehydrogenase (Stirpe & Della Corte 1969; Della Corte & Stirpe 1972), on the conversion of this enzyme in rabbit liver (Table 1). Allopurinol (1 μM) reduced xanthine oxidase, xanthine dehydrogenase and the total (xanthine oxidase plus xanthine dehydrogenase) activity by 52, 73 and 70%, respectively. On the other hand, trypsin (50 μg mL^{-1}) did not affect the total activity, but it efficiently increased xanthine oxidase activity, implying that trypsin elicits the conversion of xanthine dehydrogenase to oxidase in rabbit liver. These findings show that our present method is useful for assessment of conversion of xanthine dehydrogenase to oxidase.

Fig. 2 shows the effects of Cu^{2+}, Zn^{2+}, Fe^{2+} and selenium ions on the conversion of xanthine dehydrogenase to oxidase in rabbit liver. In each case, the alteration of xanthine oxidase and dehydrogenase activity was judged to have resulted from conversion of the enzyme activities, since the total activity of xanthine oxidase plus xanthine dehydrogenase remained relatively constant at the concentrations of these ions indicated in the figure (data not shown). Cu^{2+} (2–10 μM), Zn^{2+} (5–30 μM) and selenium ions (5–100 μM) induced the conversion of xanthine dehydrogenase to oxidase in a dose-dependent manner. The concentration of Cu^{2+}, Zn^{2+} and selenium ions required to increase xanthine oxidase activity to 50% was approximately 4, 10 and 20 μM, respectively.

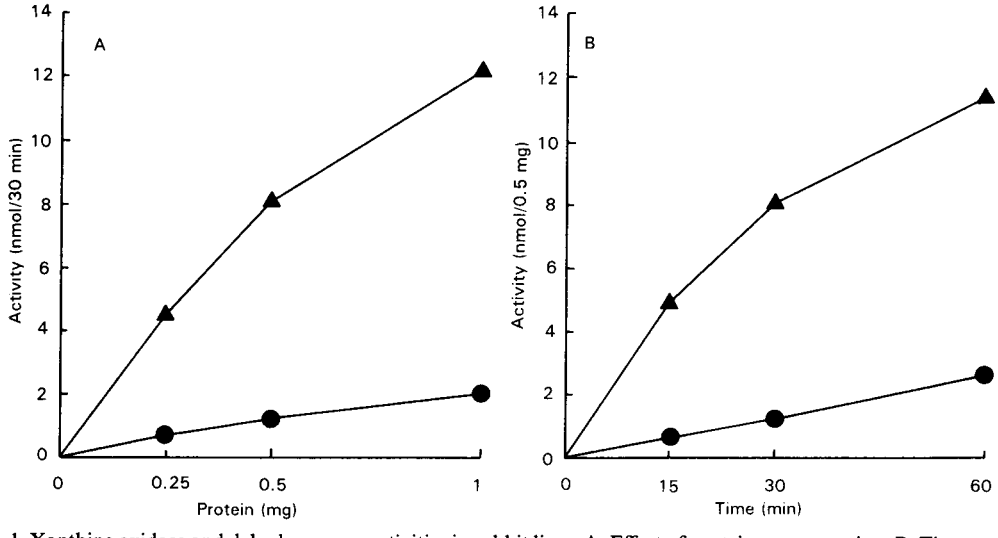

FIG. 1. Xanthine oxidase and dehydrogenase activities in rabbit liver. A. Effect of protein concentration. B. Time course. The enzyme preparation was incubated at 30°C in 1 mL of 0·1 M Tris-HCl buffer (pH 8·1) containing 60 μM xanthine with or without 0·1 mM NAD^+. Xanthine oxidase activity (●) was determined by uric acid formation in the presence of xanthine alone. Xanthine dehydrogenase activity (▲) was calculated by subtracting the amounts of uric acid in the presence of xanthine alone from those in the presence of xanthine plus NAD^+. Each point represents the mean of three experiments.

Table 1. The effects of allopurinol and trypsin on the activities of xanthine oxidase and dehydrogenase in rabbit liver.

Treatment	Xanthine oxidase activity	Xanthine dehydrogenase activity	Total activity
	(pmol min^{-1} (mg protein)$^{-1}$)		
Control	46·5 ± 7·5	267·9 ± 35·9	314·4 ± 42·8
Allopurinol (1 μM)	22·3 ± 3·8	72·9 ± 4·2**	95·2 ± 7·5*
Trypsin (50 μg mL^{-1})	234·3 ± 39·9**	45·2 ± 35·6**	279·5 ± 4·7

The enzyme preparation (0·5 mg protein) was incubated for 30 min at 30°C in 1 mL 0·1 M Tris-HCl buffer (pH 8·1) containing 60 μM xanthine with or without 0·1 mM NAD^+. Xanthine oxidase activity was determined by uric acid formation in the presence of xanthine alone. Total activity (xanthine oxidase activity plus xanthine dehydrogenase activity) was determined by uric acid formation in the presence of xanthine plus NAD^+. Xanthine dehydrogenase activity was calculated by subtracting the amounts of uric acid in the presence of xanthine alone from those in the presence of xanthine plus NAD^+. Values are the mean ± s.e.m. (n = 3), $*P < 0·02$, $**P < 0·01$ vs control.

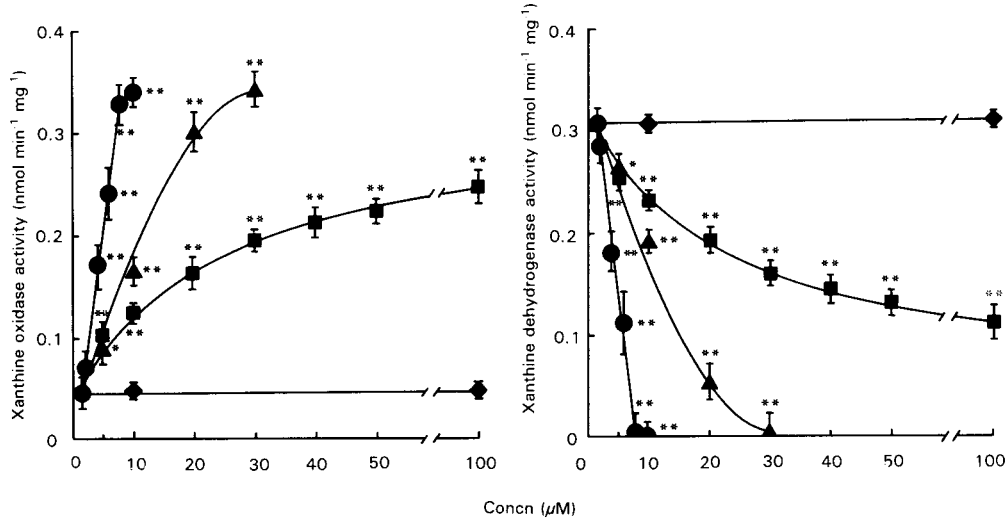

FIG. 2. The effects of various ions on the conversion of xanthine dehydrogenase to oxidase in rabbit liver. The enzyme preparation was incubated at 30°C in 1 mL of 0·1 M Tris-HCl buffer (pH 8·1) containing 60 μM xanthine with or without 0·1 mM NAD^+. Xanthine oxidase activity was determined by uric acid formation in the presence of xanthine alone. Xanthine dehydrogenase activity was calculated by subtracting the amounts of uric acid in the presence of xanthine alone from those in the presence of xanthine plus NAD^+. Each point represents the mean of four experiments; vertical lines show s.e.m. $*P < 0·05$, $**P < 0·01$, compared with the corresponding value in the absence of Cu^2, Zn^{2+}, selenium ions or Fe^{2+}. ● Cu^{2+}, ▲ Zn^{2+}, ■ selenium ions, ◆ Fe^{2+}.

Discussion

In-vitro, xanthine dehydrogenase is converted into oxidase either by addition of proteases (Massey et al 1970) or by oxidation of sulphhydryl groups of the protein into disulphide groups. The former process is irreversible. On the other hand, the latter is easily avoided or reversed by the treatment of the enzyme preparations with thiols, such as mercaptoethanol and dithiothreitol. Linas et al (1990) have concluded in their report that the conversion of xanthine dehydrogenase to oxidase during renal ischaemia is due mainly to its reversible manner.

The present study shows that Cu^{2+}, Zn^{2+} and selenium ions can induce the conversion of xanthine dehydrogenase to oxidase in rabbit liver. Cu^{2+} has been reported to react with sulphhydryl groups of xanthine dehydrogenase, thus inducing the conversion to xanthine oxidase (Della Corte & Stirpe 1972; Kaminski & Jezewska 1979). On the other hand, there are some reports that Zn^{2+} and selenium ions can oxidize sulphhydryl groups and that the action of Zn^{2+} or selenium ions is weaker than that of Cu^{2+} (Albert 1952; Klotz et al 1952). It has also been shown that Fe^{2+} is a much weaker modifier of sulphhydryl groups than Cu^{2+}, Zn^{2+} and selenium ions (Albert 1952). Thus, there is a close relationship between their ability to convert xanthine dehydrogenase to oxidase and to oxidize sulphhydryl groups. Therefore, it seems possible that the conversion of xanthine dehydrogenase to oxidase by Zn^{2+} and selenium ions is due to oxidation of sulphhydryl groups of the xanthine dehydrogenase, as well as Cu^{2+}.

Copper is an essential nutrient, which is widely distributed in the animal, especially the liver. Cu^{2+} has been reported to be a potent initiator of lipid peroxidation (Fujimoto et al 1984; Fujita et al 1987). This metal ion can react with lipid hydroperoxides to form hydroperoxyl, hydroxyl or alkoxyl radicals. The present study shows that Cu^{2+} can convert xanthine dehydrogenase to oxidase in rabbit liver. As Tipton & Cook (1963) and Muller et al (1986) have reported that the total level of copper in human or rat liver is about 140 μM, it is possible that Cu^{2+} may be able to induce the conversion of xanthine dehydrogenase to oxidase, which may relate, in

part, to the peroxidative damage mobilized by Cu^{2+} in-vivo, provided that a significant amount of the metal is free for interaction with the enzyme under ischaemic conditions. Furthermore, we have previously indicated that Fe^{2+} and Cu^{2+} may initiate lipid peroxidation (Fujimoto et al 1984). The fact that Cu^{2+}, not Fe^{2+}, elicits the generation of reactive oxygen species by converting xanthine dehydrogenase to oxidase, in addition to its ability to decompose lipid hydroperoxides to form free radicals, may partially explain such a difference.

Zn^{2+} is thought to be a reducer of lipid peroxidation (Fukino et al 1984). The action of Zn^{2+} has been ascribed to its effect on the hydroperoxide-reducing enzyme activity (Fukino et al 1984). However, the result shown in the present study may indicate that Zn^{2+} inversely induces lipid peroxidation by acting on the conversion of xanthine dehydrogenase to oxidase in the liver. The total level of this metal ion in human or rat liver has been reported to be about 800 μM (Tipton & Cook 1963; Muller et al 1986). Therefore, it seems possible that Zn^{2+} may be involved in modulating the conversion of xanthine dehydrogenase to oxidase in the liver, in addition to Cu^{2+}.

Selenium ions are a main constituent of glutathione peroxidase, which reduces hydroperoxides to the alcohols. Added selenium ions act as inhibitors of lipid peroxidation (Rotruck & Pole 1973), as does Zn^{2+} (Fukino et al 1984). On the other hand, there is evidence that selenium ions give rise to lipid peroxidation under certain circumstances (Batist et al 1986; Kramer & Ames 1988). Therefore, the present results, together with the previous observations, suggest that selenium ions can inhibit or enhance lipid peroxidation, depending on the conditions used.

Further studies are needed to clarify the contribution of Cu^{2+}, Zn^{2+} and selenium ions on the xanthine oxidase induction from dehydrogenase during hepatic ischaemia-reperfusion. However, the present study shows that Cu^{2+}, Zn^{2+} and selenium ions have the potential to increase the generation of reactive oxygen species in the liver by affecting the conversion of xanthine dehydrogenase to oxidase.

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