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Superoxide-dependent and -independent mechanisms of iron mobilization from ferritin by xanthine oxidase

Implications for oxygen-free-radical-induced tissue destruction during ischaemia and inflammation

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Xanthine oxidase is able to mobilize iron from ferritin. This mobilization can be blocked by 70% by superoxide dismutase, indicating that part of its action is mediated by superoxide (O_2^-). Uric acid induced the release of ferritin iron at concentrations normally found in serum. The O_2^--independent mobilization of ferritin iron by xanthine oxidase cannot be attributed to uric acid, because (1) uricase did not influence the O_2^--independent part and (2) acetaldehyde, a substrate for xanthine oxidase, also revealed an O_2^--independent part, although no uric acid was produced. Presumably the amount of uric acid produced by xanthine oxidase and xanthine is insufficient to release a measurable amount of iron from ferritin. The liberation of iron from ferritin by xanthine oxidase has important consequences in ischaemia and inflammation. In these circumstances xanthine oxidase, formed from xanthine dehydrogenase, will stimulate the formation of a non-protein-bound iron pool, and the O_2^--produced by xanthine oxidase, or granulocytes, will be converted by 'free' iron into much more highly toxic oxygen species such as hydroxyl radicals (OH·), exacerbating the tissue damage.

INTRODUCTION

It has been known for a long time that xanthine oxidase and ferritin interact with each other (Mazur *et al.*, 1955, 1958; Green & Mazur, 1957). Xanthine oxidase is able to mobilize iron from ferritin in both the presence and in the absence of O_2 (Duggan & Streeter, 1973). Mazur *et al.* (1958) suggested that xanthine oxidase is important in the mobilization of iron from liver ferritin *in vivo*. Various studies have provided data both supporting (Mazur & Carleton, 1965; Powell & Emmerson, 1966) and opposing (Kinney *et al.*, 1961; Kozma *et al.*, 1968) this hypothesis. Thopham *et al.* (1982) concluded that the proposed hypothesis is true, because they found an accumulation of iron in the liver after inhibition of xanthine oxidase. They suggested that previous negative findings were due to an incomplete inhibition of xanthine oxidase.

Ferritin is the main iron-storage protein in the body. The physiological mechanism of the mobilization of iron from ferritin is unclear. A reducing substance is needed to form ferrous iron, and subsequently the ferrous iron leaves the ferritin core and must be complexed by a suitable chelator (Crichton, 1973; Harrison, 1977; Crichton *et al.*, 1980).

The mechanism by which xanthine oxidase is able to release iron from ferritin remained unknown for many years. McCord & Fridovich (1968) found that xanthine oxidase was able to produce superoxide (O_2^-). Xanthine oxidase is now the most commonly used source of O_2^- in experiments performed *in vitro*. It is extraordinary that until recently no-one suggested that O_2^- might be responsible for the mobilization of ferritin iron, since it was known that a one-electron reduction is necessary. However, investigations in our laboratory demonstrated that ferritin iron could be mobilized by O_2^- derived from granulocytes or solid KO_2 (Biemond *et al.*, 1984).

Subsequently Thomas *et al.* (1985) showed that O_2^- produced by xanthine oxidase is also able to release iron from ferritin. In the present study additional information about the mobilization of iron from ferritin by xanthine oxidase has been obtained. The consequence of the mobilizaton of iron from ferritin in ischaemia and inflammation are discussed.

MATERIALS AND METHODS

Materials

4,7-Diphenylphenanthroline-1,10-disulphonic acid sodium salt (bathophenanthroline), uric acid and xanthine were obtained from E. Merck, Darmstadt, Germany. Acetaldehyde was from BDH Chemicals, Poole, Dorset, U.K. Cytochrome *c*, horse spleen ferritin (22% iron, 50% saturated), transferrin (70% saturated with iron; removal of 'free' iron accomplished by gel filtration), catalase (65000 units/mg), SOD (5000 units/mg) and uricase (6 units/mg) came from C. F. Boehringer und Soehne, Mannheim, Germany. All other reagents are of highest analytical grade. Milk xanthine oxidase was purified from fresh cow's milk by the method of Massey *et al.* (1969).

O_2^- production

Xanthine oxidase was tested for its capacity to produce O_2^-. The reaction mixture (total volume 1 ml)

Abbreviation used: SOD, superoxide dismutase.

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contained xanthine oxidase (0.02 g/l), xanthine (0.2 mM) and cytochrome *c* (30 μM) in 0.1 M-Tris/HCl buffer, pH 7.4. Incubation at 20 °C was started by addition of xanthine oxidase. The O_2^- production was calculated from the increase in the absorption at 550 nm, by using a molar absorption coefficient of 21 100 $M^{-1} \cdot cm^{-1}$ for cytochrome *c*. Cytochrome *c* reduction slowed down with time, because accumulation of H_2O_2 caused reoxidation of cytochrome *c*, which could be inhibited by catalase. So for the proper evaluation of O_2^- production the initial cytochrome *c* reduction has to be used.

Mobilization of ferritin iron

Mobilization of iron from ferritin by xanthine oxidase was measured by incubation of a mixture containing xanthine oxidase (0.02 g/l), xanthine (0.2 mM), ferritin (0.5 g/l) and bathophenanthroline (1 mM) in 0.1 M-Tris/HCl buffer, pH 7.4. A series of experiments was performed with acetaldehyde (10 mM) as substrate instead of xanthine. Additions were performed as indicated in the Figure legends. Incubation at 20 °C was started by the addition of xanthine oxidase. Mobilization of iron was calculated from the absorption at 530 nm by using a molar absorption coefficient of 22 140 $M^{-1} \cdot cm^{-1}$ for bathophenanthroline. Release of iron from ferritin by uric acid was tested in the same system but with the replacement of xanthine oxidase and xanthine by uric acid.

RESULTS

Mobilization of ferritin iron by xanthine oxidase

Incubation of horse spleen ferritin with xanthine oxidase and xanthine resulted in the release of iron detected by bathophenanthroline (Fig. 1*b*). In control experiments without xanthine oxidase, xanthine or ferritin hardly any iron could be detected. Addition of SOD, at a concentration that is able to destroy all O_2^- produced by xanthine oxidase (Fig. 1*a*), inhibited the release of ferritin iron by 70%, indicating that 70% of the iron mobilization is caused by O_2^-. Catalase had little effect on iron release. Mannitol and dimethyl sulphoxide were also without significant effect (results not shown). It is concluded that H_2O_2 and $OH^\cdot$ are not involved in the mobilization of ferritin iron by xanthine oxidase. Xanthine oxidase was unable to release iron from transferrin (70% iron saturation) (3 g/l) in our system.

Mobilization of ferritin iron by uric acid

Although O_2^- seemed to be responsible for 70% of the mobilization of ferritin iron by xanthine oxidase, we looked for an additional mechanism for the release of ferritin iron. FAD or FMN on their own did not reveal mobilization of iron from ferritin unless the incubation mixture was illuminated. However, uric acid, the product of the reaction between xanthine oxidase and xanthine, was able to release iron from ferritin, as shown in Fig. 2. The amounts depended on the uric acid concentration, which were within the range normally found in serum. Addition of uricase caused a decrease in the mobilization of iron. Control experiments excluded a direct interaction between uric acid and bathophenanthroline. The contribution of uric acid in the mobilization of ferritin iron by xanthine oxidase was investigated by using uricase (Fig. 3). In the presence of SOD, uricase did not have an additional inhibitory effect on the release of ferritin iron by xanthine oxidase and xanthine, suggesting that the O_2^--independent part was not due to an effect of uric acid. Uricase in the absence of SOD, however, did show a small decrease in iron mobilization. O_2^- production by xanthine oxidase did not change in the

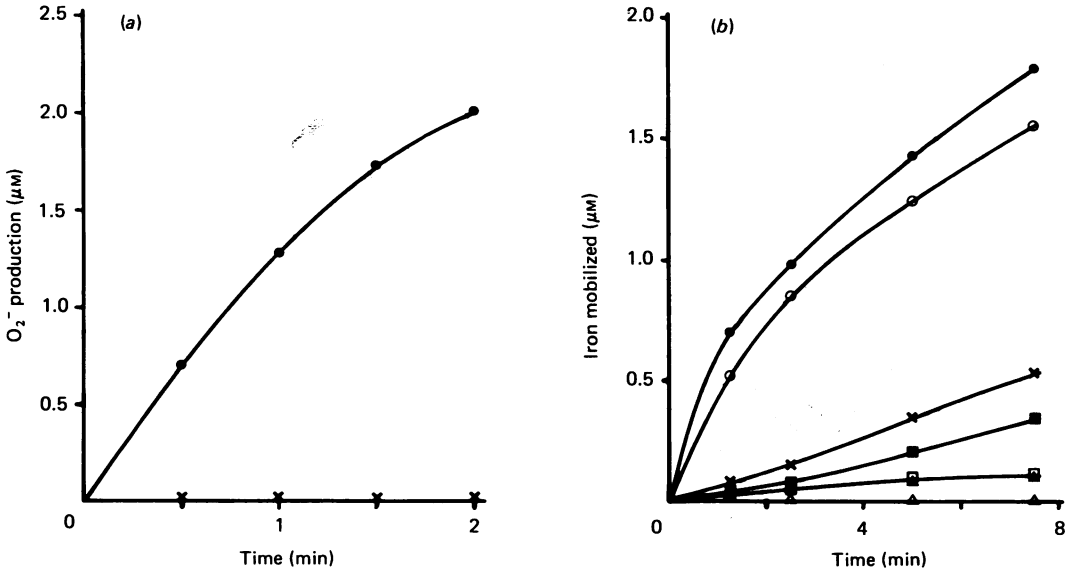


Fig. 1. Production of O_2^- (*a*) and mobilization of iron from ferritin (*b*) by xanthine oxidase and xanthine

(*a*) The reaction mixture (1 ml) contained xanthine oxidase (0.02 g/l), xanthine (0.2 mM) and cytochrome *c* (30 μM) in 0.1 M-Tris/HCl buffer, pH 7.4. Absorbance was measured continuously at 550 nm. (*b*) The reaction mixture (1 ml) contained xanthine oxidase (0.02 g/l), xanthine (0.2 mM), ferritin (0.5 g/l) and bathophenanthroline (1 mM) in 0.1 M-Tris/HCl buffer, pH 7.4. Absorbance was measured continuously at 530 nm. ●, No additions; ×, plus SOD (0.1 g/l); ○, plus catalase (40 mg/l); ■, plus SOD (0.1 g/l) and catalase (40 mg/l); ▲, without xanthine oxidase; □, without xanthine; △, without ferritin.

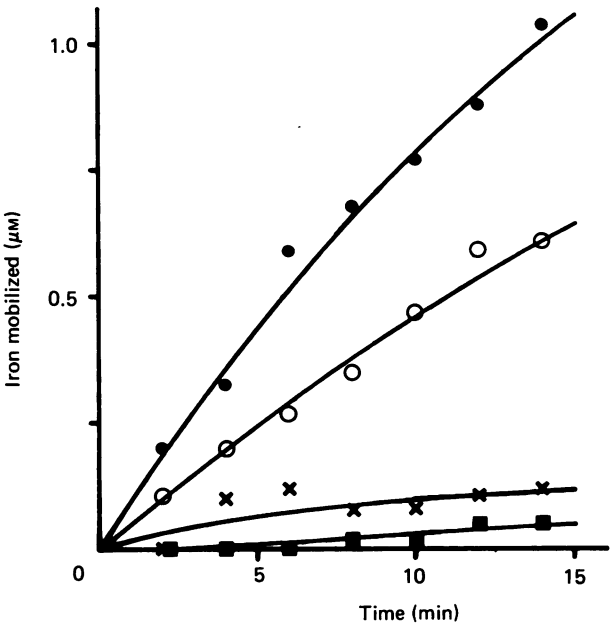


Fig. 2. Mobilization of iron from ferritin by uric acid

The incubation mixture (1 ml) contained ferritin (0.5 g/l), bathophenanthroline (1 mM) and uric acid in 0.1 M-Tris/HCl buffer, pH 7.4. Absorbance was measured at 530 nm. ● 200 μM-Uric acid; ○, 50 μM-uric acid; ×, no uric acid; ■, 50 μM-uric acid plus uricase (10 mg/l).

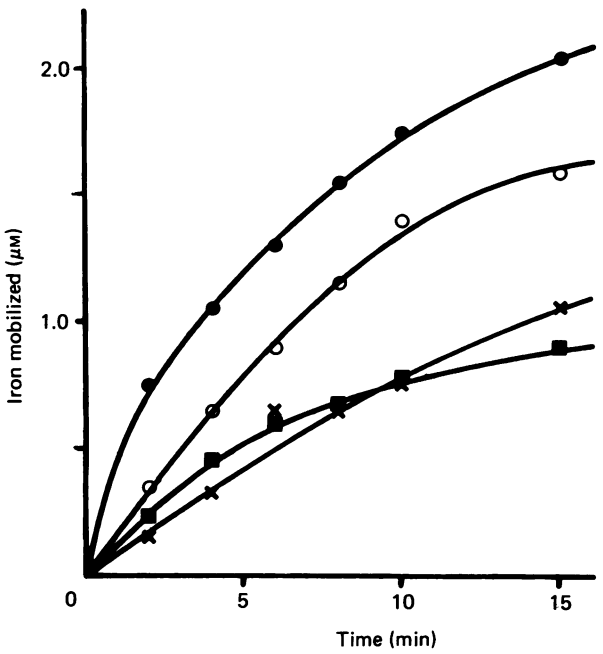


Fig. 3. Mobilization of iron from ferritin by xanthine oxidase and xanthine: inhibition by SOD and uricase

The reaction mixture (1 ml) contained xanthine oxidase (0.02 g/l), xanthine (0.2 mM), ferritin (0.5 g/l) and bathophenanthroline (1 mM) in 0.1 M-Tris/HCl buffer, pH 7.4. Absorbance was measured at 530 nm. ●, No additions; ○, plus uricase (10 mg/l); ×, plus SOD (0.1 g/l); ■, plus uricase (10 mg/l) and SOD (0.1 g/l).

presence of uricase. Liberation of iron from ferritin also occurred after the replacement of xanthine by acetaldehyde. SOD inhibited the release of iron by 80%, which suggested that about 20% of the iron mobilization was O_2^--independent. These various results indicate that, although uric acid is able to release iron from ferritin, its action is insufficient to explain the complete O_2^--independent part of the release of ferritin iron by xanthine oxidase.

DISCUSSION

In the present study xanthine oxidase was found to be able to effect the mobilization of iron from horse spleen ferritin. This mobilization could be inhibited by 70% by SOD at a concentration that is able to inhibit all O_2^- production by xanthine oxidase (Fig. 1). In contrast, catalase, dimethyl sulphoxide and mannitol had no effect. On the basis of the inhibition by SOD, it can be concluded that the greater part of the mobilization of ferritin iron was O_2^--dependent, but that additionally an O_2^--independent part was present. Thomas *et al.* (1985) have previously reported a mobilization of iron by xanthine oxidase and xanthine that could be completely blocked by SOD.

Xanthine oxidase was unable to release iron from human transferrin 70% saturated with iron.

Further investigations were performed to elucidate the mechanism of the O_2^--independent mobilization of iron. At concentrations that are normally found in serum uric acid, the product of the reaction between xanthine oxidase and xanthine, was found to mobilize iron from ferritin (Fig. 2). This mechanism has already been suggested by Green & Mazur (1957). Liberation of iron from ferritin by uric acid may increase damage by oxygen free radicals, which is in sharp contrast with its function as protector against these radicals as suggested by Ames *et al.* (1981). However, their findings could not be reproduced in our laboratory (Koster & Slee, 1983). To measure the part of ferritin iron mobilization by xanthine oxidase and xanthine that is caused by uric acid, uricase was used (Fig. 3). After inhibition by SOD, uricase had no additional inhibiting effect on the release of iron, indicating that the contribution of uric acid was small or absent. A second argument for uric acid not being responsible for the O_2^--independent part was found by using acetaldehyde as a substrate for xanthine oxidase. Iron mobilization from ferritin was then inhibited by SOD by 80%, indicating that 20% of the mobilization of iron was independent of both O_2^- and uric acid. Presumably the amount of uric acid produced by xanthine oxidase is insufficient to cause a measurable amount of mobilization of ferritin iron. FAD and FMN are unable to release iron from ferritin without illumination of the incubation mixture. The O_2^--independent part can possibly be explained by a direct electron transfer from xanthine oxidase to ferritin. However, in view of the fact that these proteins are quite large, steric problems are to be expected. The presumed interaction between xanthine and ferritin is not strong, because after the incubation the proteins can easily be separated by isoelectric focusing.

From consideration of the data we conclude that the mobilization of iron from ferritin by xanthine oxidase depends on more than one mechanism: (1) an O_2^--dependent mechanism, responsible for about 70% of the

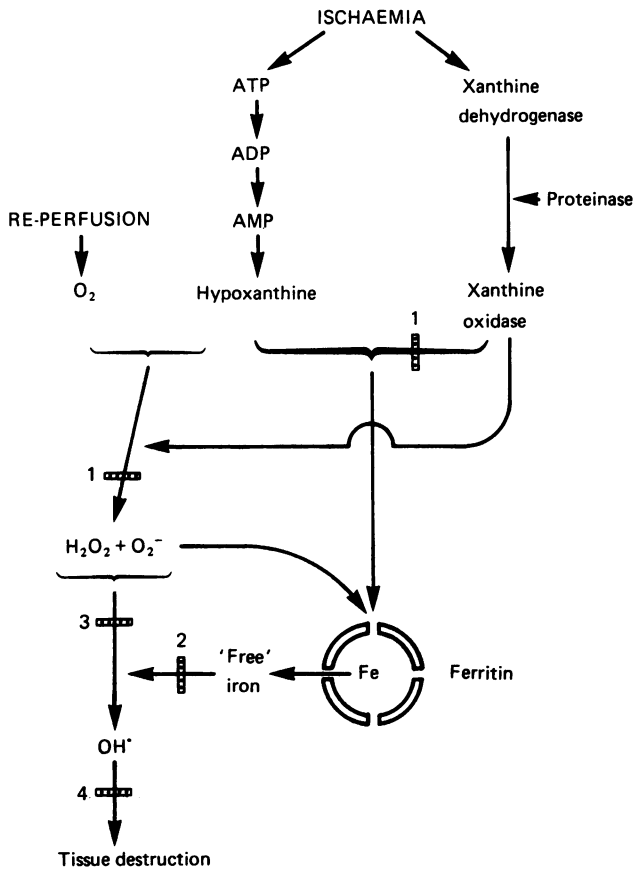
total; (2) an O_2^--independent mechanism, possibly using an electron carrier different from O_2^- that could not be detected, or by direct electron transfer from the enzyme to ferritin, for example by transfer of electrons via the protein shell to the iron core. An O_2^--independent mechanism is substantiated by the release of ferritin iron caused by xanthine oxidase under anaerobic conditions (Green & Mazur, 1957; Duggan & Streeter, 1973). Although the mobilization of ferritin iron by uric acid is possible, it is negligible in comparison with the mobilization of ferritin iron by xanthine oxidase.

These results have serious implications in tissue damage caused by oxygen free radicals. O_2^- is comparatively innocuous on its own, but its toxicity increases enormously in the presence of 'free' iron, owing to formation of hydroxyl radicals (OH·), ferryl radicals or perferryl radicals (Aust & Svingen, 1982; Graf *et al.*, 1984; Anonymous, 1985). Iron present in enzymes, haem-containing proteins or specific iron-binding proteins such as transferrin or lactoferrin is not available for the catalysis of OH· formation (Gutteridge *et al.*, 1981; Winterbourn, 1983; Halliwell & Gutteridge, 1984; Baldwin *et al.*, 1984). Ferritin has been shown to stimulate OH· formation by some workers (Wills, 1966; Gutteridge *et al.*, 1983; Carlin & Djursäter, 1984), although others found no effect (Gutteridge *et al.*, 1981). In view of the present results it is very likely that the stimulation of OH· formation by ferritin can be explained by the release of iron from ferritin by O_2^-, followed by the catalysis by 'free' iron of the formation of OH·.

Evidence is accumulating that oxygen free radicals are involved in the pathogenesis of ischaemia. McCord (1985) suggested that during ischaemia xanthine dehydrogenase, the naturally occurring enzyme, which uses NAD^+ as electron acceptor, is converted into xanthine oxidase, which uses O_2 as electron acceptor, resulting in the formation of O_2^- (Battelli *et al.*, 1972; McCord & Roy, 1982). Xanthine dehydrogenase is present in considerable amounts in many tissues (Krenitsky *et al.*, 1974). Simultaneously, during ischaemia, ATP is broken down via AMP to hypoxanthine, which, together with O_2, present as a consequence of re-perfusion or of partial ischaemia, are the substrates necessary for O_2^- production. This hypothesis was supported by the beneficial effects of SOD during intestinal and mycocardial ischaemia. In addition, post-ischaemic tissue destruction is inhibited by allopurinol, an inhibitor of xanthine oxidase (Crowell *et al.*, 1969; De Wall *et al.*, 1971; Granger *et al.*, 1981; McCord, 1985; Chambers *et al.*, 1985).

The potential consequences of the mobilization of ferritin iron by xanthine oxidase are shown in Scheme 1. During ischaemia xanthine oxidase will release iron from ferritin. Possibly the mobilization of iron already starts before the re-introduction of O_2, by the O_2^--independent mechanism. Non-protein-bound iron accumulates, able to catalyse the formation of the highly toxic OH· radical from O_2^- produced by xanthine oxidase. By this series of events tissue damage is increased dramatically. In ischaemic conditions O_2^- can also be produced by granulocytes (Romson *et al.*, 1983).

The protective effect of allopurinol in ischaemia can be explained by the prevention of O_2^- production by xanthine oxidase. We suggest that the inhibition of the release of iron from ferritin is also important in the effect of allopurinol. The conclusion by Chambers *et al.* (1985) that O_2^- production by granulocytes is not of importance in the ischaemic myocardium because it can be prevented by allopurinol is not correct. Allopurinol will block the release of iron from ferritin by xanthine oxidase, 'free' iron will be depressed and OH· formation from O_2^- produced by granulocytes will diminish. Desferrioxamine is also a potential therapeutic agent in the prevention of ischaemic tissue injury on the basis of our theory, as well as SOD, catalase, antioxidants and radical scavengers. During inflammation xanthine oxidase could possibly be formed from xanthine dehydrogenase by proteinases released from granulocytes, macrophages or dying cells. If this is true, then the mobilization of iron from ferritin by xanthine oxidase could also play a role in a wide spectrum of inflammatory disease.



Scheme 1. Role of xanthine oxidase and iron mobilized from ferritin in tissue destruction in ischaemia

Inhibitors: 1, allopurinol; 2, desferrioxamine; 3, SOD, catalase; 4, radical scavengers.

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