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NADH oxidase activity of rat liver xanthine dehydrogenase and xanthine oxidase—contribution for damage mechanisms

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Abstract
The involvement of xanthine oxidase (XO) in some reactive oxygen species (ROS) -mediated diseases has been proposed as a result of the generation of $O_2^{\cdot -}$ and H_2O_2 during hypoxanthine and xanthine oxidation. In this study, it was shown that purified rat liver XO and xanthine dehydrogenase (XD) catalyse the NADH oxidation, generating $O_2^{\cdot -}$ and inducing the peroxidation of liposomes, in a NADH and enzyme concentration-dependent manner. Comparatively to equimolar concentrations of xanthine, a higher peroxidation extent is observed in the presence of NADH. In addition, the peroxidation extent induced by XD is higher than that observed with XO. The *in vivo*-predominant dehydrogenase is, therefore, intrinsically efficient at generating ROS, without requiring the conversion to XO. Our results suggest that, in those pathological conditions where an increase on NADH concentration occurs, the NADH oxidation catalysed by XD may constitute an important pathway for ROS-mediated tissue injuries.

Keywords: *Xanthine dehydrogenase, xanthine oxidase, NADH, superoxide radical*

Abbreviations: *Abs, absorbance; ADP, adenosine diphosphate; AO, aldehyde oxidase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TBArs, 2-thiobarbituric acid reactive substances; XD, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase*

Introduction

Mammalian xanthine oxidoreductase (XOR) exists in two reversibly interconvertible forms, xanthine dehydrogenase (XD, EC 1.1.1.204) and xanthine oxidase (XO, 1.1.3.22). The distinction between XD and XO is based on the electron acceptor used by each form. XO transfers the reducing equivalents only to O_2, whereas XD, which can also use O_2, preferentially transfers them to NAD^+. XD can be readily converted to XO, either reversibly, through oxidation of protein sulphydryl groups or, irreversibly, by proteolysis [1,2].

XD and XO are enzymes of low specificity, catalysing the oxidation, not only of hypoxanthine and xanthine, but also of many other purines, pyrimidines, pterins, aldehydes and NADH [3]. The reaction catalysed by XOR can be separated into a reductive half-reaction in which 2 electrons at a time are transferred from the substrate to the enzyme and an oxidative half-reaction in which electrons are conveyed from the enzyme to O_2 or NAD^+. The reduction of O_2 results in the generation of $O_2^{\cdot -}$ and H_2O_2 [1,2].

Aldehyde oxidoreductase (EC 1.2.3.1) is structurally similar to xanthine oxidoreductase, but exists exclusively as an oxidase, aldehyde oxidase (AO) and shows a broad specificity for reducing substrates [4].

The ability of XOR and AO to catalyse the reduction of molecular oxygen, generating the reactive

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oxygen species (ROS) $O_2^{\cdot -}$ and H_2O_2, led to a considerable and increasing interest in these enzymes. ROS may have useful physiological functions (e.g. phagocytosis and signal transduction) [5,6]. Nevertheless, when generated in excess, these species can damage biomolecules and be implicated in several disease mechanisms and ageing [7–10]. In the mechanism underlying ischaemia-reperfusion injury, the hypoxanthine and xanthine oxidation by XO has been considered to play a main role [11,12]. The acetaldehyde oxidation by AO and xanthine oxidation by XO have also shown to be involved in ethanol-dependent ROS generation and contribute to the hepatotoxicity of ethanol [13–17]. *In vivo*, however, XD, rather than XO, is the predominant form of the enzyme, raising some questions concerning the proposed mechanisms, which involve the conversion of XD in XO.

In the pathological situations of ischaemia-reperfusion injury and ethanol hepatotoxicity, there is an accumulation of NADH [15,18], the oxidation of which catalysed by XD may be an alternative pathway for ROS generation [19–21]. The aim of this work was, therefore, to evaluate the relative physiological relevance of these three enzymes, XD, XO and AO, towards NADH oxidation, in order to find potential new metabolic pathways for the generation of ROS, the formation of which can mediate tissue injury in several human diseases.

Many pathological conditions arise from free radical oxidation of DNA, proteins and lipids. Although lipid oxidation may be a secondary event and not a cause of the primary disease process, lipid peroxidation products and in particular aldehydes, directly or indirectly, affect many functions integral to cellular homeostasis [22].

We decided, therefore, to study the ability of the XO/NADH, XD/NADH and AO/NADH systems to promote the ADP-Fe^{3+}-dependent liposomal lipid oxidation. For this purpose, a simple procedure for the purification of xanthine oxidase and aldehyde oxidase from rat liver was developed [23]. The lipid peroxidation extent was assessed using three different and complementary methods and the total $O_2^{\cdot -}$ generated during the lipoperoxidation assays was evaluated through the reduction of ferricytochrome c. In addition, comparative studies were performed using xanthine as reductive substrate of those enzymes (XO/xanthine and XD/xanthine systems).

Materials and methods

Adult male Sprague-Dawley rats (3–4-months-old) were obtained from the Instituto de Investigação Científica Bento da Rocha Cabral (Lisboa–Portugal). All the reagents were of the highest quality available and were used as supplied. ADP (di(monocyclohexylamonium) salt), horse heart ferricytochrome c, *p*-dimethylaminocinnamaldehyde, dithiothreitol, NADH, NAD^+, xanthine and bovine erythrocytes superoxide dismutase (SOD), were from Sigma Chemical Co. (Madrid, Spain). All the other reagents were from Merck (Darmstadt, Germany). Activity assays and spectra were recorded on a PC-linked UV2-100 Unicam spectrophotometer with a temperature controlled cell unit.

Enzyme assays

AO activity was assayed by following the oxidation of 25 μM *p*-dimethylaminocinnamaldehyde at 398 nm ($\varepsilon = 30500\,M^{-1}cm^{-1}$) in 50 mM phosphate buffer pH 7.8, at 25°C, in air-equilibrated solution. XO activity was measured using 20 μM xanthine in 50 mM phosphate buffer pH 7.8, at 25°C, in air-equilibrated solution, monitoring the production of urate at 295 nm ($\varepsilon = 9500\,M^{-1}\,cm^{-1}$). XD activity was measured using the same assay mixture as described for XO plus 85 μM NAD^+ and monitoring NADH production at 340 nm($\varepsilon = 6220\,M^{-1}cm^{-1}$). One unit (U) of catalytic activity is defined as the amount of enzyme that catalyses the oxidation of 1 μmol/min of substrate, under our experimental conditions.

XO and AO purification

XOR, in its reversible XO form, and AO were purified from rat liver as previously described by Maia et al. [23]. Briefly, the rat liver homogenate was fractionated by heat denaturation and by ammonium sulphate precipitation to give a crude extract containing both enzymes. This extract was chromatographed on a Hydroxyapatite column (BioRad, CA, USA) that completely separated AO from XO. Further purification of XO by anion exchange chromatography on a Q-sepharose fast flow column (Pharmacia Biotech, Uppsala, Sweden) resulted in a highly purified (about 1200-fold) preparation, with a specific activity of 3.5–3.7 U/mg. AO was purified about 1000-fold with a specific activity of 3.4–3.6 U/mg, by affinity chromatography on Bezamidine-Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden).

Preparation of XD

XD was obtained through reversible reduction of oxidised XO sulphydryl groups. Purified XO was incubated with 5 mM dithiothreitol, for 1–2 h, at 30°C, and then passed through a small G-25 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 100 mM Tris–HCl buffer, pH 7.8, to remove dithiothreitol. Dithiothreitol treatment resulted in a 80–85% decrease in the XO activity (xanthine:O_2 oxidoreductase activity) and a dehydrogenase to oxidase ratio of 5–6 was achieved, as previously

described [23]. Considering the intrinsic xanthine:O_2 oxidoreductase activity of the XD form [29–32], the XD batches thus prepared are highly purified, with less than 5% contaminating irreversible XO.

Preparation of microsomal lipid liposomes

Rat liver microsomes were prepared as previously described [24] and the microsomal phospholipids were extracted by the method of Folch [25]. Lipid phosphate was quantified by the method of McClare [26]. Stock solutions of phospholipds in chloroform were evaporated under nitrogen and the resulting thin lipid films were dispersed in 50 mM Tris–HCl buffer, pH 7.4, in a vortex mixer.

Lipid peroxidation assays

Lipid peroxidation experiments were conducted in 50 mM Tris–HCl buffer, pH 7.4, containing 0.5 μmol/cm^3 lipid phosphate and 2–0.1 mM ADP-Fe^{3+}, with the other reactants concentrations (NADH, xanthine, enzymes) as indicated in the figure legends. All lipid peroxidation incubations were performed for 30 min, at 37°C, in a reaction volume of 1 cm^3, and the reactions were initiated by addition of the enzyme. The lipid peroxidation extent was evaluated measuring the formation of 2-thiobarbituric acid reactive substances (TBArs), and of malondialdehyde (MDA) by HPLC, and monitoring the oxygen consumption. The lipid peroxidation of the control assays, performed in the absence of ADP-Fe^{3+}, was found to be negligible.

TBArs determination

After incubation, reactions were stopped by addition of 0.090 cm^3 of 2% (w/V) butylated hydroxytoluene and 1 cm^3 of 20% (w/V) trichloroacetic acid. The TBArs were determined adding 1 cm^3 of 0.67% (w/V) 2-thiobarbituric acid and heating the mixtures at 100°C for 10 min. After cooling, 1-butanol was added for extraction of the chromophore, and the absorbance (Abs) of the butanol layer at 532 nm was measured. The TBArs values (absorbances at 532 nm) were expressed as equivalents of MDA, determined on bases of a calibration curve performed with accurately prepared standard MDA solutions. The stock solution of MDA was prepared by hydrolysis of 1 mmol of 1,1,3,3-tetramethoxypropane in 100 cm^3 of 1% (V/V) sulphuric acid, for 2 h. MDA concentrations were checked measuring the absorbance at 245 nm ($\varepsilon = 13700\,M^{-1}\,cm^{-1}$) [27].

MDA determination by HPLC

After incubation, reactions were stopped by addition of 0.090 cm^3 of 2% butylated hydroxytoluene (w/V) and 1 cm^3 of acetonitrile. A solid-phase extraction was performed on a LiChrosolv RP-18 column (500 mg, Merck, Darmstadt, Germany) using methanol as eluent. HPLC analyses were carried out on an aminophase column (LiChrospher 100 RP-18 (5 μm) in LiChroCART 250-4, Merck, Darmstadt, Germany), using as elluent a mixture of 7:3 (V:V) acetonitrile:54 mM Tris–HCl, pH 7.4, with a flow of 1 cm^3/min. The effluent was monitored at 270 nm, the absorption maximum of free MDA in the enolate anionic form. The MDA peak was completely resolved with a retention time of 6.30 mins.

The MDA concentrations were calculated based on a calibration curve performed with accurately prepared standard MDA solutions as described in the "TBArs determination".

Oxygen consumption

The oxygen consumption during the lipid peroxidation assays was measured with an yellow springs instruments electrode connected to a water bath set at 37°C. An oxygen concentration of 200 μM, in the initial incubations mixture, was assumed.

Total $O_2^{\cdot-}$ formation

Total $O_2^{\cdot-}$ generated during the lipid peroxidation assays (30 min) were estimated through the SOD-inhibitable accumulation of ferrocytochrome c [28]. The reaction mixtures contained 50 mM Tris–HCl, pH 7.4, 100 μM ferricytochrome c, 2500 U/cm^3 SOD and the indicated NADH, xanthine and enzymes concentrations. In the assays with xanthine, 1000 U/cm^3 catalase was also included to prevent the ferrocytochrome c reoxidation. The total $O_2^{\cdot-}$ formed was estimated through the absorbance increase at 550 nm ($\Delta\varepsilon = 21000\,M^{-1}\,cm^{-1}$) in the absence and in the presence of 2500 U/cm^3 SOD.

Direct reaction of NADH + XO + ADP-Fe^{3+}

The direct reduction of ADP-Fe^{3+} by NADH + XO was evaluated spectrophotometrically, following NADH (50 μM) oxidation at 340 nm, in the presence of 60 mU/cm^3 XO and 2–0.1 mM ADP-Fe^{3+} in 50 mM Tris–HCl, pH 7.4, with and without O_2. The assays were carried out in anaerobic cuvettes and all solutions used were bubbled with argon. The absorbance decreases observed, in the absence of O_2, were too low to be measured accurately, showing that the direct reaction of NADH + XO + ADP-Fe^{3+} is negligible.

Percentage of oxygen univalent reduction

Total NADH and xanthine consumed during the lipid peroxidation assays (30 min) were determined

spectrophotometrically, following the NADH oxidation at 340 nm ($\varepsilon = 6220\,M^{-1}\,cm^{-1}$) and the urate formation at 295 nm ($\varepsilon = 9500\,M^{-1}\,cm^{-1}$). The reaction mixtures contained 50 mM Tris–HCl, pH 7.4 and the indicated NADH, xanthine and enzymes concentrations. The percentage of oxygen univalent reduction was, then, calculated as half of the ratio between the total $O_2^{\cdot -}$ formed and the total NADH (or xanthine) oxidised.

Data analysis

Each experiment was performed at least with two different enzyme batches and in duplicate, resulting in at least four independent measurements. The results are presented as means ± standard deviation.

Results

Lipid peroxidation induced during NADH oxidation catalysed by rat liver XO, XD and AO

The ability of the XO/NADH, XD/NADH and AO/NADH systems to promote the ADP-Fe^{3+}-dependent liposomal lipid oxidation was evaluated by the formation of malondialdehyde (MDA), through a specific HPLC method [27], and by measuring the thiobarbituric acid reactive substances (TBARs). The extent of lipid peroxidation was also evaluated monitoring the oxygen consumption. These different approaches were used since individual methodologies have their own limitations. Therefore, it is desirable to measure more than one reactant or product of lipid peroxidation. The TBARs assay is a non-specific assay, whereas the HPLC method used is specific for free MDA. Nevertheless, as shown in Figure 1, a very good correlation existed between the concentration of MDA, determined by HPLC, and the equivalents of MDA, measured as TBARs, when either NADH or XO concentrations are varying ($r = 0.998$ and 0.993, respectively). This suggests that, in this particular system, the TBARs assay measures only free MDA and not significant amounts of "MDA-like" substances. The measurement of oxygen consumption is an index of early stages of lipid peroxidation, whereas MDA is an index of late products of lipid peroxidation. Nevertheless, a good correlation was also observed between those indexes of lipid peroxidation ($r = 0.991$ and 0.958 for NADH and XO variations, respectively). Since the TBARs assay, when applied to our simple systems, provided useful information and is furthermore easy to perform and inexpensive, it was the method chosen for the subsequent comparative studies.

The NADH oxidation catalysed by XO and XD, in the presence of ADP-Fe^{3+}, induces the lipid peroxidation of liposomes in a NADH and enzyme concentration-dependent manner (Figure 2).

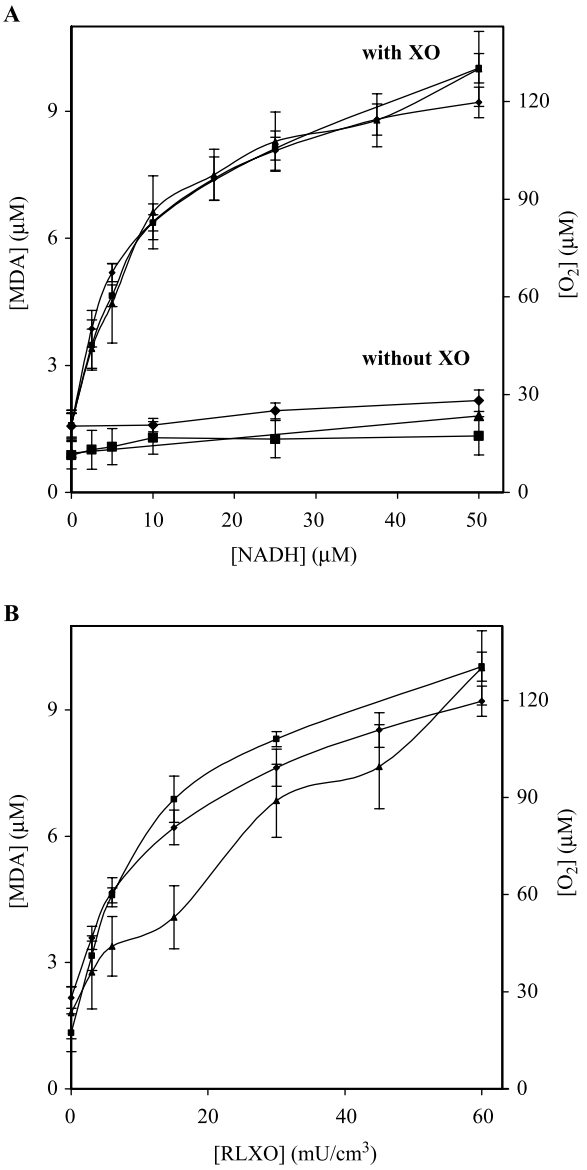

Figure 1. Comparison between the evaluation methods of lipid peroxidation. The lipid peroxidation extent, induced during NADH oxidation catalysed by XO, was evaluated measuring the formation of TBARs (◆), the formation of MDA (■), by HPLC, and monitoring the oxygen consumption (▲), as described under "Materials and methods". (A) Lipid peroxidation extent induced by various NADH concentrations and in the absence and in the presence of 60 mU/cm^3 XO. (B) Lipid peroxidation extent induced by 50 μM NADH and various XO concentrations. Lipid peroxidation assays were conducted in 50 mM Tris–HCl buffer, pH 7.4, containing 0.5 μmol/cm^3 lipid phosphate and 2–0.1 mM ADP-Fe^{3+}, with the indicated NADH and XO concentrations.

The lipid peroxidation extent observed in the presence of XD is, however, higher than that observed with XO. AO, comparatively to XD and XO, is poorly effective at oxidising liposomal lipids and the extent of lipid peroxidation showed almost no dependence on NADH or enzyme concentration (Figure 2).

In the absence of enzymes, the peroxidation extent is very small and not dependent on NADH concentration. These results highlight the importance

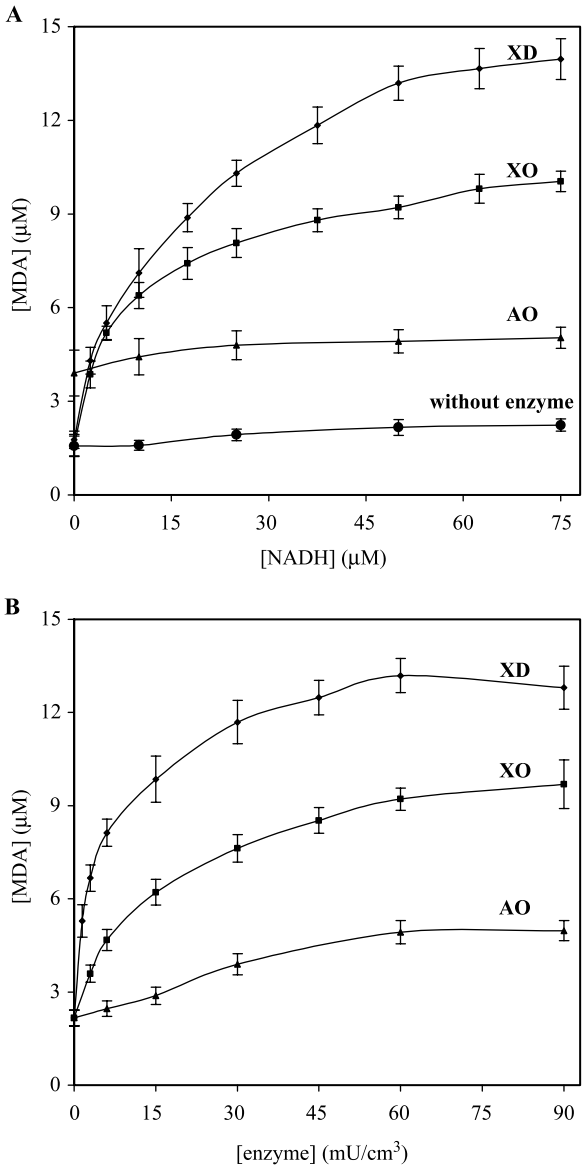

Figure 2. Lipid peroxidation induced during NADH oxidation catalysed by XD (◆), XO (■) and AO (▲). (A) Lipid peroxidation induced by various NADH concentrations in the absence and in the presence of the indicated enzyme (60 mU/cm^3). (B) Lipid peroxidation induced by 50 μM NADH and various enzyme (as indicated) concentrations. Lipid peroxidation assays were conducted in 50 mM Tris–HCl buffer, pH 7.4, containing 0.5 μmol/cm^3 lipid phosphate and 2–0.1mM ADP-Fe^{3+}, with the indicated NADH and enzyme concentrations; the lipid peroxidation extent was evaluated measuring the formation of TBArs, as described under "Materials and methods".

of XO and XD presence in the induction of lipid peroxidation.

Comparison between NADH and xanthine at promoting lipid peroxidation

XO has been hypothesized to be a potential source of ROS during reoxygenation of ischaemic tissues, based on the enzymatic oxidation of purines accumulated from nucleotide catabolism during the ischaemic

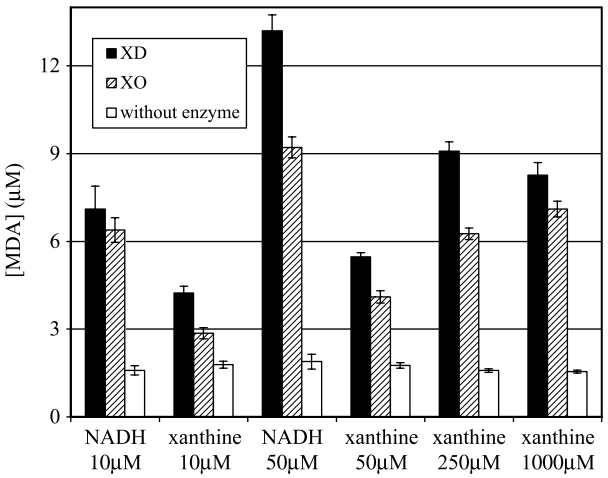

Figure 3. Comparison between the lipid peroxidation induced during NADH or xanthine oxidation catalysed by XO or XD. Lipid peroxidation assays were conducted in 50 mM Tris–HCl buffer, pH 7.4, containing 0.5 μmol/cm^3 lipid phosphate and 2–0.1 mM ADP-Fe^{3+}, with and without 60 mU/cm^3 enzyme and the indicated NADH or xanthine concentrations; the lipid peroxidation extent was evaluated measuring the formation of TBArs, as described under "Materials and methods".

period. Thus, it seemed important to undertake a comparative study between the oxidation of either xanthine or NADH catalysed by XO and XD at promoting lipid peroxidation (Figure 3).

When comparing equimolar concentrations of NADH and xanthine, it was found that those systems containing NADH were the most efficient ones at promoting the lipid peroxidation. Even with an excess of xanthine (1000 μM), the systems with NADH (50 μM) were more efficient (Figure 3).

Total $O_2^{\cdot-}$ generated during NADH or xanthine oxidation

Iron is required for lipid peroxidation induction, and Fe^{3+} reduction precedes the initiation of peroxidation and the decomposition of lipid hydroperoxides [29–31]. The possible direct reaction of NADH + XO + ADP-Fe^{3+} was found to be negligible (data not shown), leaving $O_2^{\cdot-}$ generated, during NADH oxidation by either XO and XD, as the only reducing agent available for iron reduction.

To establish a relationship between the $O_2^{\cdot-}$ generated and peroxidation, induced by either NADH or xanthine, in the presence of either XD or XO, the total $O_2^{\cdot-}$ generated during 30 min of reaction time was evaluated. Consistent with the peroxidation data, it was found that much more $O_2^{\cdot-}$ is generated when NADH oxidation is catalysed by XD than when it is catalysed by XO (Figure 4A). Similarly, more $O_2^{\cdot-}$ is generated when xanthine oxidation is catalysed by XD than when it is catalysed by XO (Figure 4B). The time course of $O_2^{\cdot-}$ generation during either NADH or xanthine (50 μM) oxidation catalysed by XO and XD is also shown (Figure 4 insets).

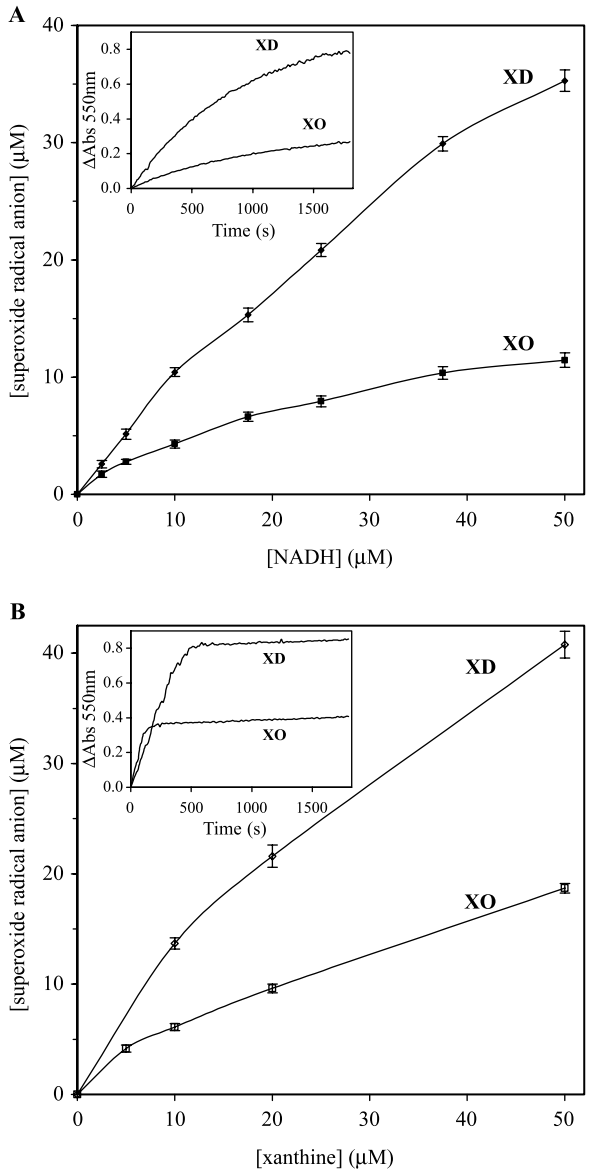

Figure 4. Total $O_2^{\cdot-}$ formed during NADH (A) or xanthine (B) oxidation catalysed by XD (◆) and XO (■). A: $O_2^{\cdot-}$ formed in the presence of various NADH concentrations and the indicated enzyme (60 mU/cm^3). Inset: Time course of $O_2^{\cdot-}$ formation during 50 μM NADH oxidation catalysed the indicated enzyme (60 mU/cm^3). B: $O_2^{\cdot-}$ formed in the presence of various xanthine concentrations and the indicated enzyme (60 mU/cm^3). Inset: Time course of $O_2^{\cdot-}$ formation during 50 μM xanthine oxidation catalysed the indicated enzyme (60 mU/cm^3). $O_2^{\cdot-}$ generation was estimated through the SOD-inhibitable accumulation of ferrocytochrome c, with 50 mM Tris–HCl buffer, pH 7.4, 100 μM ferricytochrome c, 2500 U/cm^3 SOD (when present) and the indicated enzyme, NADH and xanthine concentrations.

Oxygen univalent reduction during NADH or xanthine oxidation

The percentages of oxygen univalent reduction during the oxidation of xanthine catalysed by XD or XO are shown in Figure 5. It is observed that the univalent reduction is higher for the XD/xanthine system than for the XO/xanthine system and decreases when xanthine concentration increases. The percentages of oxygen univalent reduction occurred during NADH oxidation catalysed by XD or XO were similar (45–50%), remaining constant when NADH concentration increases (data not shown).

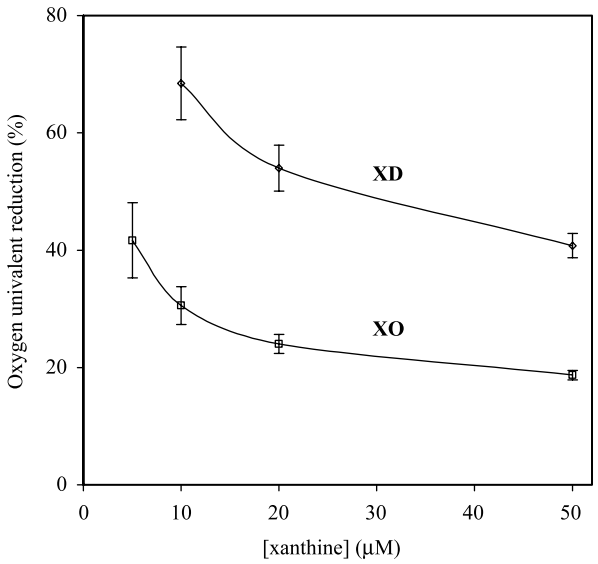

Figure 5. Oxygen univalent reduction during xanthine oxidation catalysed by XD (◆) and XO (■). The oxygen univalent reduction observed in the presence of the XD/xanthine and XO/xanthine systems (with 60 mU/cm^3 enzyme). The percentage of univalent reduction was calculated as described under "Materials and methods".

Discussion

XOR from mammalian sources can exist in two interconvertible forms, XO and XD, which differ in the electron acceptor. While XO transfers the reducing equivalents to O_2 generating $O_2^{\cdot-}$ and H_2O_2, XD preferentially transfers electrons to NAD^+, although it can also use O_2. The xanthine:O_2 oxidoreductase activity of XD has been recognised for enzymes from chicken liver [32], bovine milk [33] and rat liver [34,35].

The most widely accepted physiological role of xanthine oxidoreductase is the oxidation of the purines, hypoxanthine or xanthine, to uric acid. XO is a well documented biologic source of superoxide anion radical and has been the subject of numerous studies due to its possible involvement in some disease mechanisms. Increased purine degradation, associated with the conversion of XD to XO, was proposed as a possible mechanism for ROS generation during ethanol metabolism or in ischaemia-reoxygenation injury [11,36]. These mechanisms depend critically on conversion of the *in vivo*-predominant XD to XO, which extent and rate have been questioned. Therefore, it appears to be consistent and stimulating to search for alternative pathways to the XO/hypoxanthine, xanthine ROS generating systems.

It is noteworthy that XO and XD, from different sources, besides their xanthine:O_2 oxidoreductase activity, have also a NADH:O_2 oxidoreductase activity. This NADH oxidase activity was shown for bovine milk enzymes [33,37–39], for human milk [39], for chicken kidney [40] and liver [32], and for rat liver [35]. Nevertheless, this oxidase activity has been re-evaluated, only recently, when it was realised that it leads to the generation of reactive oxygen species [39]. It is also known that after ethanol administration and during ischaemia-reoxygenation injury, there is an increase in the NADH concentration [15,17,18] and the oxidation of this nucleotide, catalysed by either XO or XD, may play a role in ROS generation implicated in the pathogenesis of those tissue injuries, as it was discussed elsewhere [19–21]. Therefore, it is important to evaluate the relative physiological relevance of these enzymes, towards NADH oxidation, as potential ROS sources and to compare their performance in relation to the commonly used substrate, xanthine. For this purpose, and due to the lipid oxidation relevance to biological systems, the relative ability of these rat liver enzymes to induce the lipid oxidation was evaluated.

The lipid peroxidation extent induced during NADH oxidation, catalysed by XD, was found to be higher than that observed with XO, which is in agreement with the total $O_2^{\cdot-}$ generated by both enzymes during the same period of time. These results are consistent with the catalytic and Michaelis-Menten constants for $O_2^{\cdot-}$ generation during NADH oxidation, determined in a previous work (k_{cat}^{app} of 9.38 and 27.3 min^{-1} and a K_m^{app} of 8.35 and 3.30 μM, for XO and XD, respectively) [35].

For comparative purposes, the ability of rat liver XO and XD to promote the oxidation of lipids during xanthine:O_2 turnover was also evaluated. Likewise during NADH oxidation, it was found that XD induced the lipid peroxidation in a higher extent than XO. Moreover, there was a good correlation between the peroxidation extent and the amount of $O_2^{\cdot-}$ generated by either XD or XO. According to the specificity constants (k_{cat}^{app}/K_m^{app}) towards xanthine oxidation (280 $\mu M^{-1}min^{-1}$ for XO and 238 $\mu M^{-1}min^{-1}$ for XD) [35], it would be expected a greater production of $O_2^{\cdot-}$ with the XO/xanthine than with the XD/xanthine system. The oxygen univalent reduction during xanthine oxidation is, however, higher when catalysed by XD than when catalysed by XO. Taking into a count the general idea, which associates free radical-mediated oxidative damage to the enzymes of the oxidases class, these results seem in some way unexpected. The oxidation of xanthine catalysed by XO is by far the system most frequently used as a ROS source and, to the best of our knowledge, this is the first study showing that xanthine oxidation by XD can induce lipid peroxidation.

When the two reductive substrates, NADH and xanthine, were compared for their ability to induce the oxidation of lipids, higher peroxidation extents were observed during NADH oxidation (by either XD or XO). In addition, it was observed that the systems with NADH generate $O_2^{\cdot-}$ in a slower and continued manner, during 30 min, while the systems with xanthine generate a burst of $O_2^{\cdot-}$ which declines after a few minutes. Therefore, it seems that $O_2^{\cdot-}$ generated at a slow and continued level would be more efficient at inducing ROS-mediated damages on biological molecules.

Iron is a known catalyst of lipid peroxidation [29–31]. The negligible peroxidation extent observed in the absence of ADP-Fe^{3+} showed that peroxidation of liposomes was due to a potent oxidant, generated by the interaction of H_2O_2 and $O_2^{\cdot-}$ in the presence of Fe^{3+}, rather than to either $O_2^{\cdot-}$ or H_2O_2 *per se*. The exact role played by iron in accelerating lipid peroxidation is, however, controversial. Common to all proposals for the role of iron is the requirement of "redox active" iron for iron-dependent lipid peroxidation. In our systems, the rates of $O_2^{\cdot-}/H_2O_2$ generation, catalysed by XD and XO, seem to control the rates of ADP-iron reduction/oxidation, which, in turn, would greatly influence the rates of lipid peroxidation. A relationship between the kinetics of $O_2^{\cdot-}/H_2O_2$ production and the lipid peroxidation extent, as proposed by Miller et al. [41] is, thus supported by our data. In all cases, the primary event must be the Fe^{3+} reduction, which, in our assays, is carried out by the $O_2^{\cdot-}$ generated during the NADH or xanthine oxidation catalysed by XO or XD.

With respect to rat liver AO, the formation of $O_2^{\cdot-}$ (data not shown) and the extent of lipid peroxidation showed almost no dependence on NADH or enzyme concentration. Comparatively to XD and XO, AO seems to play a minor role in the NADH-dependent liposomal lipids damages, which is in agreement with the comparatively low kinetic parameters values found (k_{cat}^{app} value of 2.59 min^{-1} and a K_m^{app} value of 5.04 μM [35]).

In conclusion, our results stress several important points. First, NADH oxidation catalysed by either rat liver XD or XO, generates $O_2^{\cdot-}$ and induces lipid peroxidation. The $O_2^{\cdot-}$ generated and the lipid peroxidation induced during NADH or xanthine oxidation, catalysed by rat liver XD, are higher than those observed with XO. Second, our results with the mammalian liver enzymes confirm that the *in vivo*-predominant dehydrogenase is, thus intrinsically more efficient at generating ROS, with no need of conversion to XO. Third, $O_2^{\cdot-}$ generated in a slow and continued manner seems to be more efficient at inducing ROS-mediated damages on biological molecules.

In the light of these conclusions, we can suggest that, in those pathological conditions where an

increase on NADH concentration exists, the oxidation of this nucleotide catalysed by XD may constitute an alternative pathway for ROS-mediated tissue injuries.

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