Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions

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Abstract Xanthine oxidoreductase (XOR) catalyses the reduction of the therapeutic organic nitrate, nitroglycerin (glyceryl trinitrate, GTN), as well as inorganic nitrate and nitrite, to nitric oxide (NO) under hypoxic conditions in the presence of NADH. Generation of nitric oxide is not detectable under normoxic conditions and is inhibited by the molybdenum site-specific inhibitors, oxypurinol and (−)BOF 4272. These enzymic reactions provide a mechanism for generation of NO under hypoxic conditions where nitric oxide synthase does not function, suggesting a vasodilatory role in ischaemia.

Key words: Xanthine oxidoreductase; Nitric oxide; Nitrate; Nitrite

1. Introduction

Nitric oxide (NO) is widely recognised as mediating the relaxation of smooth muscle in vasodilation and as initiating many other important biological functions, including inhibition of platelet aggregation and adhesion [1,2]. Its generally accepted physiological source is NO synthase [3], a complex enzyme which is totally dependent on oxygen for its activity and consequently ineffective in a hypoxic environment, where the vasodilatory properties of NO might be seen to be advantageous.

Organic nitrates have been used therapeutically for over 100 years in the treatment and prophylaxis of angina pectoris [4]. These drugs, which include glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN), have a potent vasodilator activity, the most likely mechanism for which is reduction to nitric oxide in the endothelium and/or vascular smooth muscle [5]. The mechanisms by which organic nitrates are reduced in vivo to NO have never been fully explained. Endogenous sulphydryl groups have been implicated in the generation of *S*-nitrosothiols, which subsequently break down to produce NO [6,7]. Enzymic metabolism has also been proposed, involving glutathione-*S*-transferases [8,9] or members of the cytochrome P-450 family of enzymes [10,11]. On the other hand, evidence has been presented showing that glutathione-*S*-transferases are capable of reduction of nitrate to nitrite but not to NO [12].

Xanthine oxidoreductase (XOR) is a complex molybdoflavoprotein, which has been studied as the essentially pure enzyme for almost 60 years [13]. In addition to molybdenum and FAD, it contains two iron-sulphur redox centres and has a wide substrate specificity, typically hydroxylating purines and concomitantly reducing either NAD^+ (dehydrogenase form, EC 1.1.1.204) or molecular oxygen (primarily the oxidase form EC 1.1.3.22) [14]. The latter reaction generates the reactive oxygen species, superoxide anion and hydrogen peroxide, and it is this ability, with its implications for ischaemia-reperfusion injury, that has led to the enzyme's becoming a focus of research activity over the last two decades [15,16]. Much less well known is XOR's capacity to reduce inorganic nitrate to nitrite under conditions of low oxygen tension [17–19]; a property that is perhaps not surprising in view of the enzyme's many similarities with the assimilatory nitrate reductases of fungi, algae and higher plants. Like XOR, the nitrate reductases contain both molybdenum and FAD redox centres and utilise NAD(P)H as reducing substrate [20].

We now report that, under hypoxic conditions and in the presence of NADH, XOR is capable of catalysing the reduction of GTN, as well as inorganic nitrate and nitrite, to NO. We believe that these findings, coupled with the vascular location of the enzyme, suggest a role for XOR not only in the metabolism of GTN but also as a source of NO derived from endogenous nitrate and nitrite, under ischaemic conditions ranging from sub-normoxia to anoxia when NO synthase does not function.

2. Methods and materials

2.1. Materials

Bovine xanthine oxidase (1.4 U/mg) was obtained from Biozyme, Blaenavon, UK. GTN (David Bull Laboratories, Warwick, UK), in injectable form, was diluted in 100 mM potassium phosphate buffer, pH 7.2, containing 0.9% NaCl (PPB) to give a stock concentration of 10 mM. Inorganic nitrate (KNO_3) and nitrite ($NaNO_2$) (Sigma, Poole, UK) were treated in the same manner. NADH (Sigma) was diluted in PPB to a stock concentration of 1 mM. Oxypurinol (Sigma) and (−)BOF-4272 (Otsuka Pharmaceutical Factory, Japan) were made up in PPB also at stock concentrations of 1 mM. All solutions were freshly prepared on the day of use and maintained on ice until required.

2.2. Determination of nitric oxide

Nitric oxide determinations were made by using an ozone chemiluminescence assay in a continuous flow apparatus (Sievers NOA 280) that allows the real time analysis of NO production. The apparatus was modified to allow a constant stream of nitrogen to flow into the reaction chamber. Chemiluminescence data were collected by a data acquisition system; the mean NO produced in parts per billion (ppb) was calculated from readings taken every second and shown as NO ppb/s.

Progress curves, of molar production of NO against time, were

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Abbreviations: XOR, xanthine oxidoreductase; GTN, glyceryl trinitrate; NO, nitric oxide

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calculated by taking into account the gas flow and successively integrating the ppb/s curves. Reactions were carried out in a final volume of 1 ml at 37°C in an atmosphere of $<1\%$ oxygen (Stathkelvin oxygen electrode).

2.3. Determination of inhibition constants

In inhibition experiments, K_i values were determined by fitting to the inhibition function $i = I/I+C$ where I is the concentration of inhibitor, C is a constant and $i = 1\text{-}v_i/v_o$, in which v_o = uninhibited rate, v_i = inhibited rate. Assuming competitive inhibition, $C = K_i(1+S/K_m)$. Here S is the concentration of the competing substrate (GTN) and K_m is its Michaelis constant [21].

3. Results

3.1. XOR-catalysed reduction of GTN to NO

At low oxygen tension, NO is generated when XOR is incubated in the presence of GTN and NADH. A typical rate vs. time profile is shown in Fig. 1, in which it can be seen that, after a lag phase, a steady state rate (represented by the plateau) is achieved. Fig. 1 also illustrates that introduction of air abolished NO generation. Steady-state rates were shown to be directly proportional to enzyme concentration. Generation of NO was found to be dependent on each component of the reaction mixture, without any one of which NO production was not detectable. The reaction followed Michaelis-Menten kinetics (Fig. 2) with apparent K_m and V_{max} values for GTN of 0.33 ± 0.05 mM and $1.83 \pm 0.08 \times 10^{-7}$ mol/min/mg, respectively.

Substitution of xanthine for NADH as reducing substrate under the above conditions gave no detectable NO production. The molybdenum site-directed XOR inhibitors, oxypurinol [22] and (−)BOF-4272 [23] inhibited, in a dose-dependent manner, NO production catalysed by XOR in the presence of GTN and NADH. Xanthine and, to a lesser extent, hypoxanthine similarly inhibited NO generation. K_i values determined as described in the Section 2 are oxypurinol, 2.8×10^{-7} M; (−)BOF-4272, 3.2×10^{-8} M; xanthine, 2.5×10^{-7} M; hypoxanthine, 2.5×10^{-6} M. Representative semilogarithmic plots of relative rates vs. inhibitor concentration for (−)BOF-4272, xanthine and oxypurinol are displayed in Fig. 3.

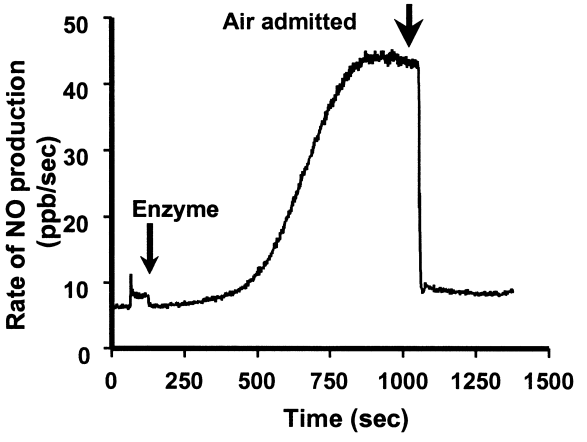

Fig. 1. Chemiluminescence data showing the time dependence of rates of NO production (ppb/s) catalysed by XOR in the presence of 3 mM GTN and 300 μM NADH in an atmosphere containing $<1\%$ oxygen. Solutions of GTN and NADH, in 100 mM potassium phosphate buffer, pH 7.4, were mixed and purged with a continuous stream of nitrogen over 5 min before addition (arrow) of a similarly purged solution of XOR (1 U, 0.71 mg) in the same buffer. At the time point indicated (arrow) the flow of nitrogen was replaced by air.

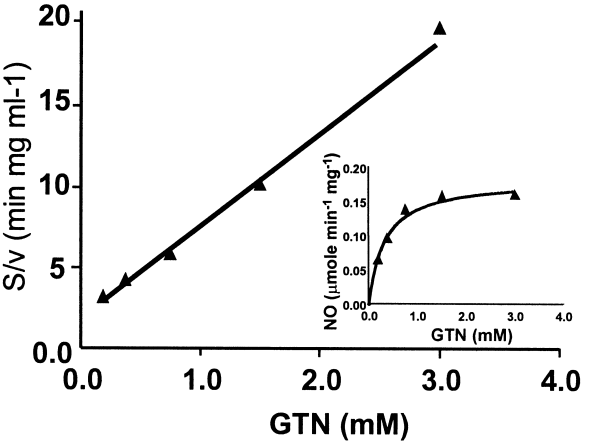

Fig. 2. Hanes-Woolf plot of NO production catalysed by XOR in the presence of GTN and 300 μM NADH. Experimental conditions are described in the legend to Fig. 1 for a given concentration of GTN; progress curves were derived as outlined in Section 2. Inset shows the Michaelis-Menten curve fitted to the data.

3.2. XOR-catalysed reduction of inorganic nitrate and nitrite to NO

XOR catalysed the reduction of both inorganic nitrate and nitrite to NO in the presence of NADH. Reduction of nitrate followed Michaelis-Menten kinetics (Fig. 4), giving apparent K_m and V_{max} values of 0.29 ± 0.6 mM and $9.7 \pm 0.3 \times 10^{-8}$ mol/min/mg, respectively. In the case of nitrite reduction, the data, fitted to a Michaelis-Menten hyperbola (Fig. 5, inset), gave operational apparent K_m and V_{max} values of 22.9 ± 8.1 mM and $3.73 \pm 0.72 \times 10^{-6}$ mol/min/mg, respectively. However, the Hanes-Woolf linear plot (Fig. 5) shows systematic deviation from Michaelis-Menten kinetics.

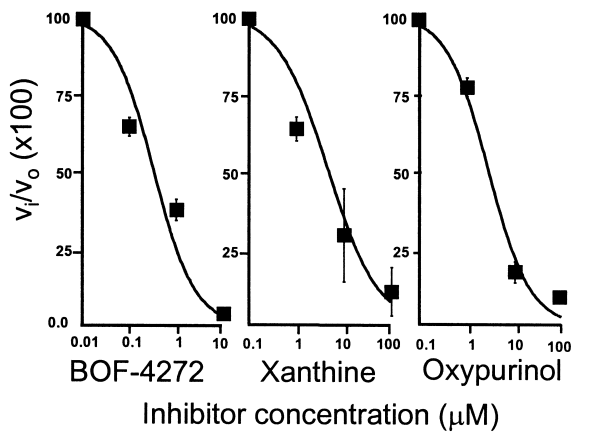

Fig. 3. Inhibition, by oxypurinol, (−)BOF-4272, and xanthine of NO production catalysed by XOR in the presence of 3 mM GTN and 300 μM NADH. Experimental conditions are described in the legend to Fig. 1 except that inhibitor, at the appropriate concentration, was added immediately prior to addition of enzyme. Inhibitions are displayed as semilogarithmic plots of percentage inhibition vs. inhibitor concentration; v_i is the inhibited rate and v_o is the rate in the absence of inhibitor. The solid lines are drawn using parameters obtained from the fits of inhibition data as described in Section 2. Error bars on experimental points represent ±S.E.M. of duplicate determinations from each of two experiments.

4. Discussion

We show that, under conditions of low oxygen tension, XOR catalyses the reduction of the organic nitrate, GTN, to NO in the presence of NADH.

The ability of NADH to act as a reducing substrate for XOR has long been known [24] but has been little studied and is not generally recognised. In air-saturated medium, both dehydrogenase and oxidase forms of XOR show NADH oxidase activity [25], generating superoxide anion and hydrogen peroxide at maximal rates of NADH oxidation of approximately 3×10^{-7} mol/min/mg. This compares with a maximal rate of 1.8×10^{-7} mol NO/min/mg XOR determined at 1% oxygen in the present studies. Considering a XOR concentration of 10^{-4} mg/mg endothelial cell protein (Harrison, unpublished data), this rate appoximates to 20 pmol NO/min/mg cell XOR protein; a rate comparable with that determined by Feelisch et al. [26] in endothelial cells exposed to GTN. In view of the likely competition between GTN and molecular oxygen for NADH-generated reducing equivalents on XOR, it is to be expected that NO production will increase as oxygen tension falls further. Conversely, it appears from our results that, as normoxic conditions are approached, oxygen competes increasingly for available electrons.

By analogy with the nitrate reductases [20] it might be anticipated that GTN acts at the molybdenum site of XOR. This is consistent with our demonstration of inhibition of NO production by oxypurinol [22], (−)BOF-4272 [23]. It is well established that NADH donates electrons and molecular oxygen accepts electrons at the FAD site of XOR [14].

Regarding the pathway of NO production from GTN, the present data are consistent with initial reduction to inorganic nitrite, although determination of the latter by the commonly used Griess reaction [27] was not feasible in the presence of NADH as electron donor. Certainly, XOR was found readily to catalyse the reduction of nitrite to NO. Rates were 30–50-fold faster than those determined with GTN or inorganic nitrate as reducing substrates, suggesting that reduction of nitrate is the rate-limiting step in NO production in these latter cases.

On the basis of these considerations, a plausible case can be made for a role for XOR in metabolism of GTN and of other organic nitrates in vivo (we also observed conversion of ISDN to NO by XOR; data not shown). Endothelial cells have been identified as a site of bioactivation of organic nitrates to NO [27], a process deemed to be enzymically catalysed [27]. XOR is known to be present at relatively high levels in endothelial cells [28], in which its enzymic activity has been shown to be increased in hypoxia [29,30]. Under conditions of ischaemia, NADH levels will rise, and as oxygen tension falls NO generation will be increasingly favoured. It is relevant that organic nitrates are generally more effective in the venous circulation than in large coronary arteries or (still less effective) in arterioles [1,31], a comparison that reflects the distribution of XOR activity [32]. It is worth noting that hypoxanthine inhibits XOR-catalysed generation of NO, albeit less effectively than does xanthine. Concentrations of hypoxanthine are generally assumed to be elevated in ischaemia [33,34] and, depending on the actual levels attained, rates of NO production via this route could be affected. However, hypoxanthine levels, most commonly determined in complete ischaemia, are uncertain under conditions of reduced blood flow [34]. Moreover, rates of NO generation clearly depend upon oxygen tension, itself subject to wide variation in the pathological state. It is, accordingly, difficult to predict the magnitude of these effects in vivo.

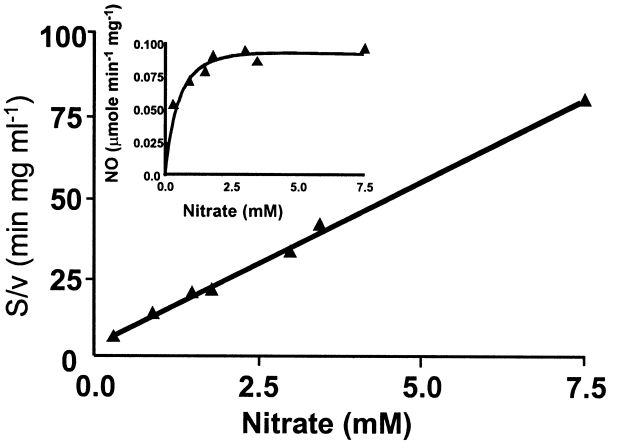

Fig. 4. Hanes-Woolf plot of NO production catalysed by XOR in the presence of inorganic nitrate and 300 μM NADH. Experiments were carried out and analysed as described in the legend to Fig. 2. Inset shows the Michaelis-Menten curve fitted to the data.

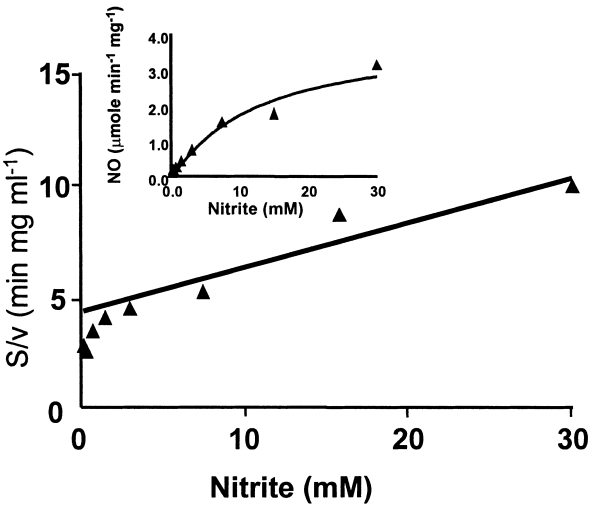

Fig. 5. Hanes-Woolf plot of NO production catalysed by XOR in the presence of inorganic nitrite and 300 μM NADH. Experiments were carried out and analysed as described in the legend to Fig. 2. Inset shows the Michaelis-Menten curve fitted to the data.

XOR-catalysed generation of NO under hypoxic conditions can be seen as complementary to the activity of NO synthase, which requires oxygen [3]. Thus, in ischaemic blood vessels, XOR catalyses the metabolism of GTN to NO, which mediates vasodilation and relief from angina. Under these hypoxic conditions meanwhile, NO synthase is induced [35–37] and, being dependent on molecular oxygen for its function, is set to take over NO production as the oxygen tension rises and XOR-catalysed NO production shuts down. This complementarity is also relevant in the absence of ingested organic nitrates, when circulating inorganic nitrates and/or nitrites could act as oxidising substrates for XOR-catalysed generation of NO under ischaemic conditions. Such a role for XOR would be consistent with our findings and with those of Zweier et al. who recently reported formation of NO in biological tissues that was independent of NO synthase [38].

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