

# Lack of conversion of xanthine dehydrogenase to xanthine oxidase during warm renal ischemia

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Received 17 September 1990

Irreversible transformation of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) during ischemia was determined measuring XDH and total enzyme activity in kidneys before and after 60 min of clamp of the renal pedicle. Tissue levels of adenine nucleotides, xanthine and hypoxanthine were used as indicators of ischemia. After 60 min of clamping, ATP levels decreased by 72% with respect to controls whereas xanthine and hypoxanthine progressively reached tissue concentrations of  $732 \pm 49$  and  $979 \pm 15$  nmol·g tissue<sup>-1</sup>, respectively. Both total and XDH activities in ischemic kidneys ( $30 \pm 15$  and  $19 \pm 1$  nmol·min<sup>-1</sup>·g tissue<sup>-1</sup>) were significantly lower than in controls when expressed on a tissue weight basis. The fraction of enzyme in the XDH form was however unchanged indicating that the reduction of the nucleotide pool is not accompanied by induction of the type-O activity of xanthine oxidase.

Xanthine oxidase; Xanthine dehydrogenase; Renal ischemia; Adenine nucleotide; Rat kidney; HPLC

## 1. INTRODUCTION

The existence of xanthine oxidase, enzyme of purine catabolism in mammalian organs, in two structurally distinct forms, either a NAD<sup>+</sup> dependent dehydrogenase (XDH) or an oxidase (XO) that utilizes oxygen as electron acceptor has been well documented [1,2]. However the physiological role of XO is still under discussion. The conversion process from XDH to XO appears to occur first with the reversible transformation of the enzyme by a sulphhydryl oxidase. The modified enzyme is readily reconverted into the dehydrogenase by thiols. A subsequent irreversible modification occurs instead via proteolysis of the enzyme protein. The role of XO in ischemic damage was first proposed by Granger et al. [3] and confirmed by Roy et al. [4,5] who postulated that cellular injury occurring during reperfusion subsequent to tissue ischemia may be correlated to the transformation of XDH into irreversible XO; the process should be caused by the decrease in energy supply, followed by the enhancement of calcium concentration which activates the calcium-dependent protease(s) responsible for the degradation of the enzyme protein.

Ischemic process induced in vitro in liver and kidney showed that conversion of XDH to irreversible XO occurs only after a long incubation time [6,7] which might cause cell modifications and even death. Moreover results obtained in in vivo ischemia are conflicting about the central role of XO in causing ischemic damage [8–10].

In order to clarify this point we determined the effects of ischemia on xanthine oxidase. Activity of the enzyme was determined by an HPLC method allowing the simultaneous measurement of uric acid and NADH from which total and XDH activities are calculated, respectively [11]. The fraction of the enzyme in the XDH form was unmodified in kidneys where ATP levels were significantly reduced by one-hour clamping of the renal pedicle with respect to the contralateral control kidneys collected immediately after anesthesia.

## 2. EXPERIMENTAL

### 2.1. Chemicals

Xanthine, hypoxanthine, dithiothreitol (DTT), NAD<sup>+</sup>, NADH, ATP, ADP, AMP were obtained from Boehringer (Mannheim, FRG). Tetrabutylammonium phosphate (Pic A) was from Millipore (MA, USA). Acetonitrile was of HPLC grade Merck (Darmstadt, FRG).

### 2.2. Animals

For in vivo ischemia, male Sprague–Dawley rats (150–170 g b.wt) were anesthetized (sodium pentobarbital 50 mg/kg i.p.). Kidneys were exposed through a midline incision and ischemia was induced by clamping the renal pedicle of the right kidney to completely occlude renal vein and artery. The contralateral kidney was clamped similarly and immediately removed. Abdomen of the rats was then closed and animals were kept under constant body temperature for 60 min. The removed kidneys, both controls and ischemic, were quickly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processing either for nucleotide analysis or for xanthine oxidase isolation and determination of the enzyme activity. Basal values of both total and type-D activity of xanthine oxidase were determined in kidneys of unanesthetized rats. For this purpose animals were sacrificed by decapitation and the kidneys were immediately collected and frozen as above.

### 2.3. Preparation of the enzyme fraction and measurement of total and XDH activities of xanthine oxidase

Frozen kidneys were weighed and homogenized (1:5, w:v) in 0.1 M

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Tris-HCl buffer (pH 8.1) [12], centrifuged at  $800 \times g$  for 20 min and then at  $100\,000 \times g$  for 60 min. The supernatant was dialyzed for at least five hours against the same buffer [13] at  $4^\circ\text{C}$ . The enzyme preparations ( $0.25\text{ ml} = 40\text{ mg}$  tissue) were preincubated for 30 min at  $37^\circ\text{C}$  in the presence of  $10\text{ mM}$  DTT [2]. The assay of both XDH and total xanthine oxidase (XO + XDH) activity was carried out as reported [11] with some modifications. Briefly, the DTT-activated enzyme preparations were incubated in Tris-HCl buffer (pH 8.1) at  $25^\circ\text{C}$  for 10 min after addition of  $60\text{ }\mu\text{M}$  xanthine and  $0.67\text{ mM}$   $\text{NAD}^+$  [14], final volume  $0.1\text{ ml}$ . In blank samples, which were incubated under the same conditions, xanthine was omitted. The incubations were stopped by the addition of  $1\text{ ml}$  ethanol, the samples were centrifuged at  $1000 \times g$  for 5 min and the supernatants dried under nitrogen flow. The residues were stored at  $-40^\circ\text{C}$  and were resuspended in  $0.6\text{ ml}$  of  $0.1\text{ M}$  ammonium phosphate (pH 7). Under these conditions samples were stable for two days. Irreversible XO activity was calculated by subtracting XDH activity from total activity.

#### 2.4. Evaluation of adenine nucleotides, xanthine and hypoxanthine levels by HPLC analysis

Frozen kidneys were quickly weighed and homogenized for one min in cold  $3.6\%$  perchloric acid ( $1:5$ , w:v) with the Ultraturax shaft previously cooled in liquid nitrogen. After again cooling the homogenizer in liquid nitrogen, the tissue was homogenized for one additional min. Vials containing the tissue were kept in ice during the homogenization step. Lack of hydrolysis of ATP under the above conditions was demonstrated analyzing extracts of homogenates prepared in the presence of added ATP. The homogenate was centrifuged at  $10\,000 \times g$  for 5 min. The supernatant was brought to pH 6–7 with  $2\text{ M}$   $\text{K}_2\text{CO}_3$  in  $2\text{ M}$  KOH. The samples were centrifuged at  $10\,000 \times g$  for 5 min and the supernatants stored at  $-80^\circ\text{C}$ . HPLC analysis was carried out on Lichrosorb RP18 reversed phase column (Merck,  $25\text{ cm}$ ,  $7\text{ }\mu\text{m}$ ,  $0.5\text{ cm}$  I.D.), with  $0.1\text{ M}$   $\text{NH}_4\text{H}_2\text{PO}_4$  adjusted to pH 5.5 with  $3\text{ N}$   $\text{NH}_4\text{OH}$  as the mobile phase to evaluate ATP and ADP [15]. For AMP, xanthine and hypoxanthine the mobile phase was  $0.22\text{ M}$   $\text{KH}_2\text{PO}_4$ ,  $5\text{ mM}$  Pic A (tetrabutylammonium phosphate) taken to pH 6.9 with phosphoric acid in  $1\%$  methanol.

#### 2.5. Statistical analysis

All data are reported as means  $\pm$  SE. The significance of the differences between groups was evaluated by either paired or unpaired Student's *t*-test.

### 3. RESULTS AND DISCUSSION

The present study was undertaken on the basis of the following observations: (1) warm ischemia induced by clamping the renal artery causes a dramatic decrease of adenine nucleotides with consequent accumulation of xanthine and hypoxanthine which are substrates for xanthine oxidase [16]; (2) reactive  $\text{O}_2$  species formed by the type-O activity of xanthine oxidase during reoxygenation of ischemic tissue, produce severe cell damages [5,17]; (3) allopurinol a potent inhibitor of XO

Table II

Effect of one hour ischemia on renal xanthine oxidase activity

Ischemia (min)	Enzyme activity ( $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g tissue}^{-1}$ )		
	Total	XDH	% XDH
0 (16) <sup>a</sup>	$46 \pm 2$	$34 \pm 2$	$73 \pm 2$
0 (7) <sup>b</sup>	$42 \pm 2$	$29 \pm 1$	$68 \pm 1$
60 (7)	$30 \pm 15^*$	$19 \pm 1^*$	$66 \pm 1$

Enzyme activity was computed either from the amount of uric acid (total activity) or the amount of NADH (XDH activity) formed in 10 min of incubation at  $25^\circ\text{C}$  of the enzyme fraction previously treated for 30 min at  $37^\circ\text{C}$  with  $10\text{ mM}$  dithiothreitol. Results are means  $\pm$  SE of the number of kidneys indicated in brackets, which were analyzed in triplicate. <sup>a</sup>non-ischemic control kidney obtained from rats killed by decapitation. <sup>b</sup>non-ischemic kidney (left) from anesthetized rats. In the same animals the right kidney was clamped for 60 min to induce ischemia.  $^*P < 0.01$  vs non-ischemic control (a) by the paired Student's *t*-test.

decreases reoxygenation injury and prevents in kidneys the decrease of ATP levels after 30–60 min in vivo ischemia [16].

Since according to Roy et al. [4] the decrease of ATP level during ischemia might be responsible for cell injury inducing transformation of XDH to irreversible XO, we evaluated the decrease of adenine nucleotides and the activities of total xanthine oxidase and XDH after 60 min of ischemia in vivo. Levels of ATP, ADP and AMP which were of the same order of magnitude as those reported by other authors in control kidneys [16] decreased by 72, 85 and 44% respectively after 60 min ischemia (Table I). Correspondingly xanthine and hypoxanthine were elevated dramatically.

During the isolation of xanthine oxidase from the kidney, addition of thiols such as  $\beta$ -mercaptoethanol or dithiothreitol were omitted in the homogenization buffer but the enzyme preparations were preincubated in the presence of  $10\text{ mM}$  DTT at  $37^\circ\text{C}$  for 30 min [2] in order to transform the reversible form of type-O xanthine oxidase into the D-type enzyme. To remove the renal endogenous substrates from the enzyme preparations, dialysis [13] instead of Sephadex G-25 column [6–8,10] was carried out because in our hands the latter procedure gave higher basal levels. After 1 h of ischemia, total and XDH activities significantly decreased with respect to controls (Table II), nevertheless the fraction of the enzyme in the type-D form was unchanged.

Table I

Effect of one hour ischemia on renal adenine nucleotides

Ischemia (min)	ATP	ADP	AMP ( $\text{nmol} \cdot \text{g tissue}^{-1}$ )	Xanthine	Hypoxanthine
0	$706 \pm 40$	$1020 \pm 70$	$1260 \pm 70$	n.d.	n.d.
60	$201 \pm 16^*$	$147 \pm 21^*$	$713 \pm 49^*$	$732 \pm 49$	$979 \pm 115$

Results are means  $\pm$  SE of 21 and 9 kidneys for the 0 and 60 min of ischemia, respectively.

$^*P < 0.01$  vs non-ischemic control by the Student's *t*-test. n.d. = not detectable (lower than  $10\text{ nmol} \cdot \text{g tissue}^{-1}$ ).

As already reported by other authors [18] the ischemic period caused a significant increase ( $P < 0.01$ ) in the wet weight of the kidney with respect to the weight of the contralateral non-ischemic control ( $1.07 \pm 0.03$  vs  $0.77 \pm 0.02$  g). Enzyme activity expressed as  $\text{nmol} \cdot \text{min}^{-1}$  per kidney was therefore  $32 \pm 3$  and  $33 \pm 3$  for total XO activity in the ischemic kidney and in contralateral non-ischemic kidney respectively, with no modification induced by ischemia. The same was for the XDH activity which became  $21 \pm 2$  and  $22 \pm 2$   $\text{nmol} \cdot \text{min}^{-1}$  per organ.

The unchanged activity of the irreversible type-O enzyme after 60 min in vivo ischemia, argues against xanthine oxidase giving origin to the oxygen radicals suggested as the main cause of cellular injury. Results obtained by Joannidis et al. [10] using a different experimental approach and published at the time when we had already carried out our experiments, are in full agreement with our conclusions regarding the lack of modification by 45 min in vivo renal ischemia of both total and type-O xanthine oxidase activity in the whole organ. Increase of kidney weight during ischemia reported by these authors was also in agreement with that found by us. Other findings reported very recently by Linas et al. [19] accord with our results as far as the irreversible type-O enzyme is concerned, which remains about 20% of the total even after 30 min of warm ischemia. In addition to the irreversible form of the oxidase, both Joannidis et al. [10] and Linas et al. [19] considered the modifications of the type-O reversible form during ischemia. The obtained results are contrasting since the former authors found no modification whereas the latter reported a dramatic increase of this enzyme form during ischemia. Reversible and irreversible XO are evaluated from the difference of activity determined in enzyme preparations in which dithioerythritol is either absent or present. The different results by the two groups [10,19] cannot be attributed to the different concentrations of the thiol because Linas et al. [19] used a lower concentration (5 mM) than that reported by Joannidis et al. [10] (10 mM). In our experiments we did not consider the reversible oxidase formation because, as previously reported [12], we found that XDH is very easily converted to the reversible type-O enzyme even during storage at low temperature unless a protecting thiol is added to the enzyme fraction. In our opinion the physiological

significance of the reversible type-O enzyme is therefore hard to be evaluated as reported [10,19].

From the results obtained in the present study it might be concluded that no transformation from XDH to XO occurs during 60 min of in vivo renal ischemia which renders unlikely the proposed role of the enzyme as being responsible for the production of oxygen radicals during ischemia.

*Acknowledgements:* This work was supported by a grant of the Ministry of Public Education (60%) to Prof. G. Cighetti. The skillful technical assistance of Mr Andrea Lorenzi is acknowledged.

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