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IS XANTHINE OXIDASE A UNIVERSAL SOURCE OF SUPEROXIDE RADICALS IN LIVER DAMAGE INDUCED BY ISCHEMIA AND REPERFUSION?

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It has now been shown that an essential role in the development of ischemic damage of organs is played by oxygen radicals [5, 8], and the most widely held hypothesis is that a key position in the development of ischemic damage is occupied by superoxide radicals, arising under the influence of xanthine oxidase. The basis for this hypothesis consists of two groups of facts obtained chiefly in experiments on a model of ischemia in vitro. First, ischemia leads to rapid breakdown of high-energy phosphates [3]. This may result in the accumulation of substrates of the xanthine-oxidizing enzyme, namely hypoxanthine and xanthine. Second, during ischemia is various organs (intestine, heart, liver, etc.) the change was observed from xanthine dehydrogenase (the D-form of the enzyme) into xanthine oxidase (the O-form of the enzyme), which can generate superoxide radicals [5]. On this basis it was concluded that the conditions are created during ischemia which may lead during subsequent reperfusion to an increase in the rate of formation of superoxide radicals.

Since the main results relative to the role of the xanthine oxidase system have been obtained on a model of ischemia of the liver in vitro and are not entirely consistent, the aim of the present investigation was to study on a model of total ischemia of the liver in vivo whether substrates and products of the xanthine oxidase reaction accumulate whether the D-form of the enzyme changes into the O-form.

EXPERIMENTAL METHOD

Experiments were carried out on Wistar rats weighing 180-220 g, under hexobarbital anesthesia. Total ischemia of the liver was induced by applying microforceps to the vascular pedicle of the central and left lateral lobes of the liver for a period of 2 h. Reperfusion was carried out by removing the forceps, and after ischemia the nonischemic lobes, amounting to about 30% by volume, were resected.

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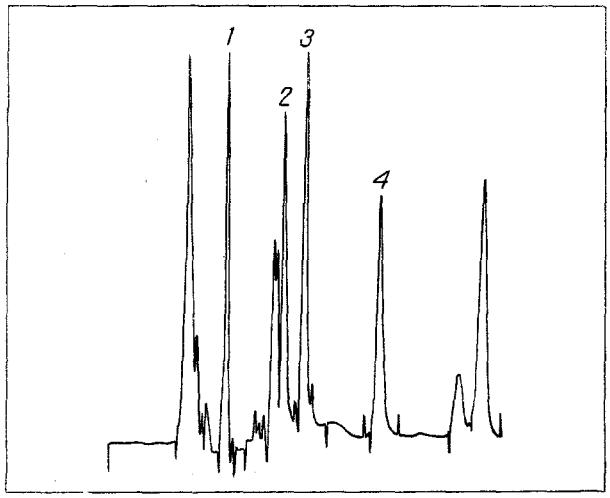

Fig. 1. Chromatogram of intact rat liver. 1) Uric acid, 2) hypoxanthine, 3) xanthine, 4) allopurinol.

TABLE 1. Concentrations of Hypoxanthine, Xanthine, and Uric Acid in Rat Liver after Ischemia and Reperfusion (in moles/g tissue, $M \pm m$; $\times 10^7$).

Exptl. conditions	Hypoxanthine	Xanthine	Uric acid
Control	15,84±2,0	3,36±1,4	8,88±2,04
Ischemia (2 h)	5,64±2,1	16,92±2,1**	31,68±3,0**
Ischemia for 2h + reperfusion for 24 h	18,24±2,4	7,44±2,4*	19,44±2,6**

Legend. Number of experiments = 5; $*p < 0.05$, $**p < 0.01$ compared with control.

Immediately after the end of ischemia (2 h) and reperfusion (24 h) the lobes of the liver were quickly frozen in liquid nitrogen. All subsequent procedures for isolation of the cytosol were conducted at 0-4°C. The liver of intact (anesthetized) rats was used as the control.

Concentrations of hypoxanthine, xanthine, and uric acid in the liver were determined by high-performance liquid chromatography. To prepare the specimens, the liver frozen in liquid nitrogen was homogenized in 10 ml of 4% $HClO_4$, neutralized with 10% KOH (2 ml) to pH 6.0, and centrifuged (105,000g) for 1 h. The supernatant was analyzed on an LKB liquid chromatograph with UV- detection (λ = 254 and 278 nm), using an IBM column (25 × 0.4 cm), and 5 μM silica-gel C_{18}. Ammonium acetate buffer (pH 5.4) containing 2% acetonitrile was used as the eluant. The internal standard was allopurinol (AP). The concentrations of the substrates were determined by measuring areas of peaks on a calibration curve on an LKB integrator.

To determine enzyme activity the homogenate (1:3) was made up in 0.1 M Na-phosphate buffer, pH 7.8, containing 2×10^{-4} M EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM dithiothreitol (DTT), and centrifuged at 105,000g for 60 min. The supernatant was subjected to gel-filtration on a Sephadex G-50 column (1.5 × 23 cm), equilibrated with same buffer, to remove low-molecular-weight impurities interfering with the determination of xanthine oxidase activity. Activity was measured in 0.1 M Na-phosphate buffer, pH 7.8, containing 10^{-4} M EDTA, as uric acid accumulation, spectrophotometrically at λ = 295 nm [4]. The reaction mixture for measurement of oxidase activity contained 0.067 mM xanthine, while that for measurement of total activity of the D- and O-forms of the enzyme contained, besides xanthine, 0.7 mM NAD^+, 0.5 mM sodium pyruvate, and 0.1 activity unit of lactate dehydrogenase. The protein concentration was measured by Lowry's method.

TABLE 2. Oxidase and Total ($D + O$) Activity during Ischemia and Reperfusion of Rat Liver ($M \pm m$, $n = 10$)

Series	Exptl. conditions	Total activity	Oxsidase activity
I	Control	1,31±0,28	0,114±0,031
	Ischemia for 2 h	0,98±0,19	0,13±0,06
	Ischemia for 2 h + reperfusion for 24 h	1,13±0,33	0,143±0,01
II	Control	0,72±0,08	0,064±0,017
	Ischemia for 2 h	0,63±0,17	0,065±0,038
	Ischemia for 2 h + reperfusion for 24 h	1,14±0,013	0,076±0,036

EXPERIMENTAL RESULTS

Changes in concentrations of substrates and products of the xanthine oxidase reaction were determined. Typical chromatograms of the cytosol obtained from normal liver are given in Fig. 1.

The period of ischemia chosen (2 h) was critical and caused death of 60% of the animals. During this time the hypoxanthine was evidently exhausted, for its steady-state concentration fell by almost 67% below normal (Table 1). At the same time the second substrate of xanthine oxidase, namely xanthine, and the reaction product, uric acid, were observed to accumulate. Uric acid accumulation may indicate absence of activation of uricase during ischemia.

During reperfusion of the ischemic liver the concentrations of all metabolites studied were shifted in the normal direction. These results are in agreement with data in [3], showing rapid exhaustion of ATP, ADP, and AMP in the ischemic rat liver, and with data on accumulation of hypoxanthine, xanthine, uric acid, and allantoin in the perfusion fluid of the ischemic rat liver [7].

Normally the liver contains the xanthine-oxidizing enzyme mainly in the D-form [2]. Activity of the O-form in the liver of the intact rats was about 9% of the total activity of the enzyme (Table 2), which is close to the value of 12.3 obtained in [4]. According to [7], activity of the O-form is 20%. It must be noted that the D-form is easily converted into the O-form, in particular as a result of oxidation of SH-groups (reversible transition) or of partial proteolysis (irreversible transition) [2]. The relative content of the O-form of the enzyme, given by different authors may therefore vary depending on the time and conditions of processing of the tissue during isolation of the crystal.

There were two series of experiments, aimed at elucidating the problem of whether irreversible or reversible transition of the D-form of the enzyme into the O-form takes place in vivo during ischemia.

In series I all the solutions during homogenization and gel-filtration contained 10 mM DTT and 1 mM PMSF — conditions protecting against both reversible (DTT) and irreversible (DTT + PMSF) transition from D into O during preparation of the specimen.

As the data in Table 2 show, during ischemia there was a small decrease (by about 1.3 times) in total activity of the enzyme. Since the oxidase activity was close to or a little above normal, this means that the decrease took place on account of inactivation of the D-form. During reperfusion the values of activity of the D- and O-forms were close to their values in the liver of intact animals.

It can be concluded from the results of series I that within the limits of this model of ischemic and subsequent reperfusion injury to the rat liver irreversible transition from dehydrogenase into oxidase does not take place.

To discover whether reversible transition from the D-form into the O-form takes place during ischemia and reoxygenation, in the experiments of series II the cytosol was isolated in the absence of DTT. In this case all stages were carried out as quickly as possible in the cold and the cytosol was not subjected to gel-filtration on Sephadex G-50. The fact will be noted that for intact animals, when the cytosol was isolated without gel-filtration, total activity of the enzyme in it was 1.8 times less than in the cytosol after gel-filtration (Table 2). The cytosol evidently contains a factor reducing the activity of the xanthine-oxidizing enzyme, which is separated during gel-filtration. During ischemia, in the experiments of series II a small decrease also was observed in total activity of the enzyme compared with normal, and no significant increase in oxidase activity was found. It must be pointed out that absolute values of oxidase activity were low, and, as Table 2 shows, the degree of error of their determination was high.

Thus despite changes discovered in purine metabolism characteristic of severe ischemic damage, no appreciable transition from the D-form into the O-form was found in this model after a critical period of ischemia. These results are similar to those given in [7], in which only a small increase (8%) was observed in activity of the O-form in the rat liver during hypoxia in vitro. It is difficult on the basis of the data given above, which were obtained during total ischemia of the liver, to expect any appreciable contribution of superoxide radicals to ischemic and reperfusion damage on account of the xanthine oxidase reaction. Similarly it would be incorrect to regard the xanthine oxidase system as the universal and principal source of O_2^- radicals during ischemia and reperfusion. A critical examination of the data on ischemia of the heart in vitro also shows the absence of any appreciable transition from the D-form into the O-form [1-4]. The electron transport chain and NADPH-oxidase of neutrophils migrating into the reperfused tissue may evidently be more important sources [5].

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