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H_2O_2 FORMATION DURING NUCLEOTIDE DEGRADATION IN THE HYPOXIC RAT LIVER: A QUANTITATIVE APPROACH

WERNER SIEMS, HEIKE SCHMIDT, MARIANNE MÜLLER, WOLFGANG HENKE and GERHARD GERBER

Institut für Biochemie, Bereich Medizin (Charité), Humboldt-Universität zu Berlin, 1040 Berlin, Hessische Strasse 3–4, DDR

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In the hypoxic liver an increased rate of cytosolic and peroxisomal H_2O_2 generation is due to the accelerated purine nucleotide degradation. The relative contribution of the oxidase type of xanthine oxidoreductase activity increases in hypoxia by less than 10%, the dehydrogenase type of this enzyme is hardly inhibited by the increased concentration of free NADH. Nevertheless, due to the high hypoxanthine supply the xanthine oxidase related H_2O_2 formation is increased six-fold and together with the peroxisomal uricase-mediated share it accounts for half of the oxygen consumption.

Key words: hydrogen superoxide, nucleotides, rat liver, hypoxia, xanthine oxidoreductase, glutathione, uric acid

INTRODUCTION

During hypoxic perfusion as well as in incomplete ischemia of liver all prerequisites for a burst of activated oxygen species and hydrogen peroxide are fulfilled[1]. Indeed it was confirmed recently that the liver under limited oxygen supply is liable to an oxidative stress originating from the degradation of purine nucleotides and the accompanying formation of H_2O_2 and $O_2^{\cdot-}$ in the reactions catalyzed by xanthine oxidoreductase (E.C. 1.2.3.2) and uricase (E.C. 1.7.3.3.)[2,3]. In normoxia the contribution of cytosolic H_2O_2 formation is of minor significance[4]. Due to the rapid degradation of purine nucleotides[5] and the high activities of xanthine oxidoreductase and uricase the hypoxic liver appears to be an interesting model regarding both of physiological and clinical-experimental aspects concerning a high rate of production of reductive oxygen species. This study was prompted to perform an estimation of this rate; the experiments were carried out at hypothermic conditions (25°C).

MATERIALS AND METHODS

Preparation of the liver for perfusion

Male rats from Wistar H-strain with body weight of 257 ± 15 g and liver weights of 11.4 ± 0.4 g were used. The animals had free access to standard commercial pellet food and tap water. They were anaesthized with an intraperitoneal injection of sodium hexobarbital (25 mg per 100 g b.w.). The abdomen was opened and the tissue surrounding the liver was excised. A thin plastic tube was inserted into vena portae and the vena cava inferior was cutted to prevent a buildup of pressure in the liver. For removal of blood the liver was perfused in situ for five minutes with modified Krebs-Henseleit-bicarbonate buffer, pH 7.4 + 5 mM glucose + 2 mM lactate + 0.2 mM pyruvate precooled to 4°C. The perfusion pressure was maintained below 20 cm water column (2 kPa) to circumvent edema and destruction of liver endothelia.

Perfusion of the liver

The rat liver was perfused hemoglobin-free for three hours at 25°C in a recirculating mode with a solution composed as that used in the initial perfusion. Perfusate flow was about 2 ml × min^{-1} × g w.w.$^{-1}$ and was kept constant throughout the particular experiment. The liver was surrounded by perfusion medium. Oxygenation was performed in a bubble oxygenator with carbogen (95% O_2 + 5% CO_2, pO_2 of the influent: 80 kPa), with air (moderate hypoxia, pO_2 18.6 kPa) and with a nitrogen-air-mixture (severe hypoxia, pO_2 5.2 kPa).

Determination of metabolites

The uric acid concentration of perfusion medium was measured both enzymatically with uricase and catalase (E.C. 1.11.1.6) according to Kageyama[6] and in the course of a HPLC separation of nucleosides and nucleobases. These two methods used gave identical results. The HPLC equipment (DuPont Comp., Wilmington, Delaware, USA) consisted of a 870 pump module 8800 Series gradient controller, 850 Absorbance detector (254 nm), column department and a SP 4100 computing integrator. The columns used were a Permaphase CDS Guard-column and a 4.6 × 25 cm Zorbax ODS. The chromatographic conditions were used according to Hartwick *et al.*[7]: solvent A 0.01 M KH_2PO_4, pH 5.5; solvent B methanol-water (80:20); linear gradient 0 to 25% B, 30 minutes; flow rate 1.5 ml per minutes, temperature ambient. For the estimation of allantoin a modification of the method according to Christman[8] and the enzymatic determination of urea according to Bergmeyer[9] were used. The interference of this assay by purine bases and purine nucleosides was checked and excluded. Oxidized glutathione (GSSG) efflux rate was measured according to Hissin *et al.*[10] as described in[3]. Lactate and pyruvate were determined enzymatically with lactate dehydrogenase (E.C. 1.1.1.27). The shares of type D (dehydrogenase) and of type O (oxidase) of xanthine oxidoreductase activity were determined as described by Della Corte[11,12].

RESULTS AND DISCUSSION

The increase of the extent of hypoxia is accompanied by an increase in uric acid

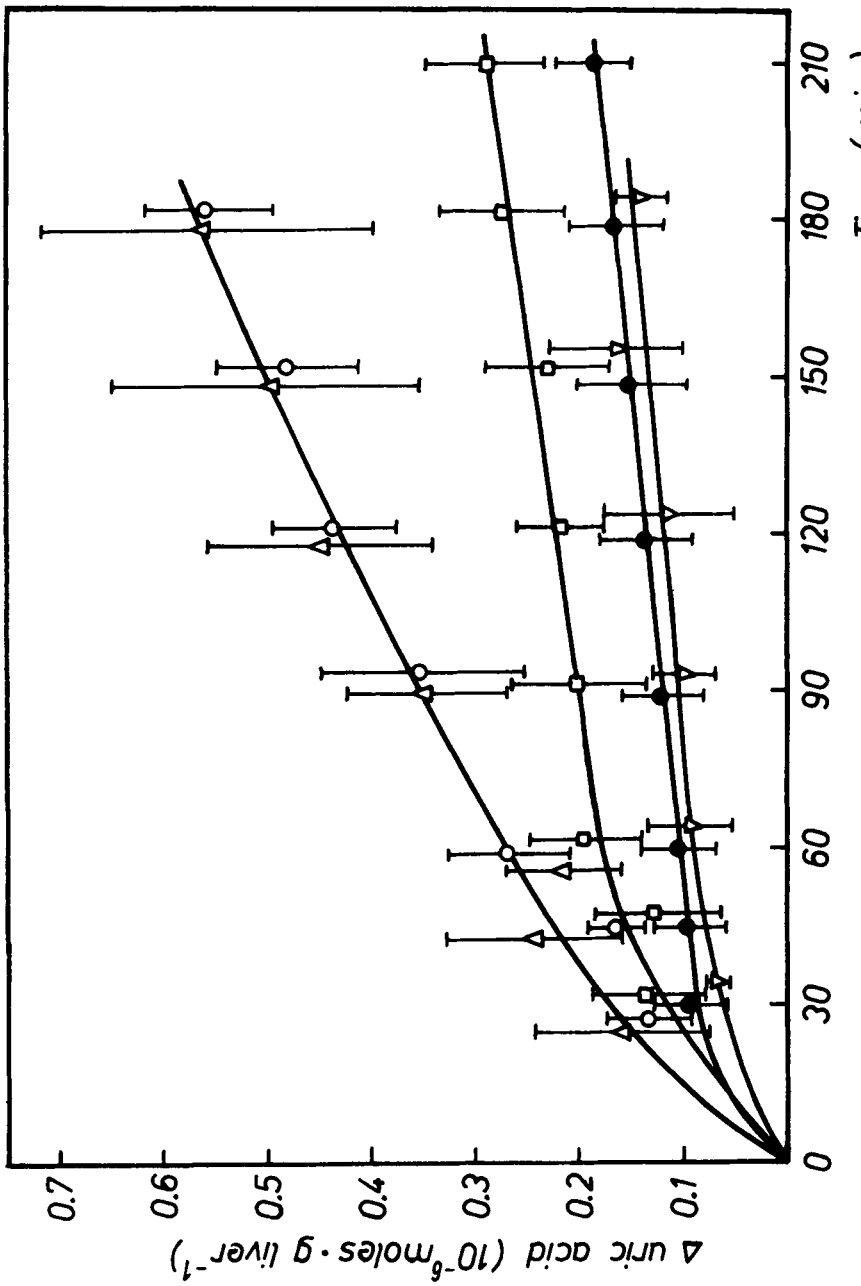

FIGURE 1 Uric acid release of the recirculating perfused rat liver (25°C). Normoxia (●) n = 6; severe hypoxia (○) n = 4; severe hypoxia + formate (△) n = 3; moderate hypoxia (□) n = 4; severe hypoxia + allopurinol (▽) n = 2.

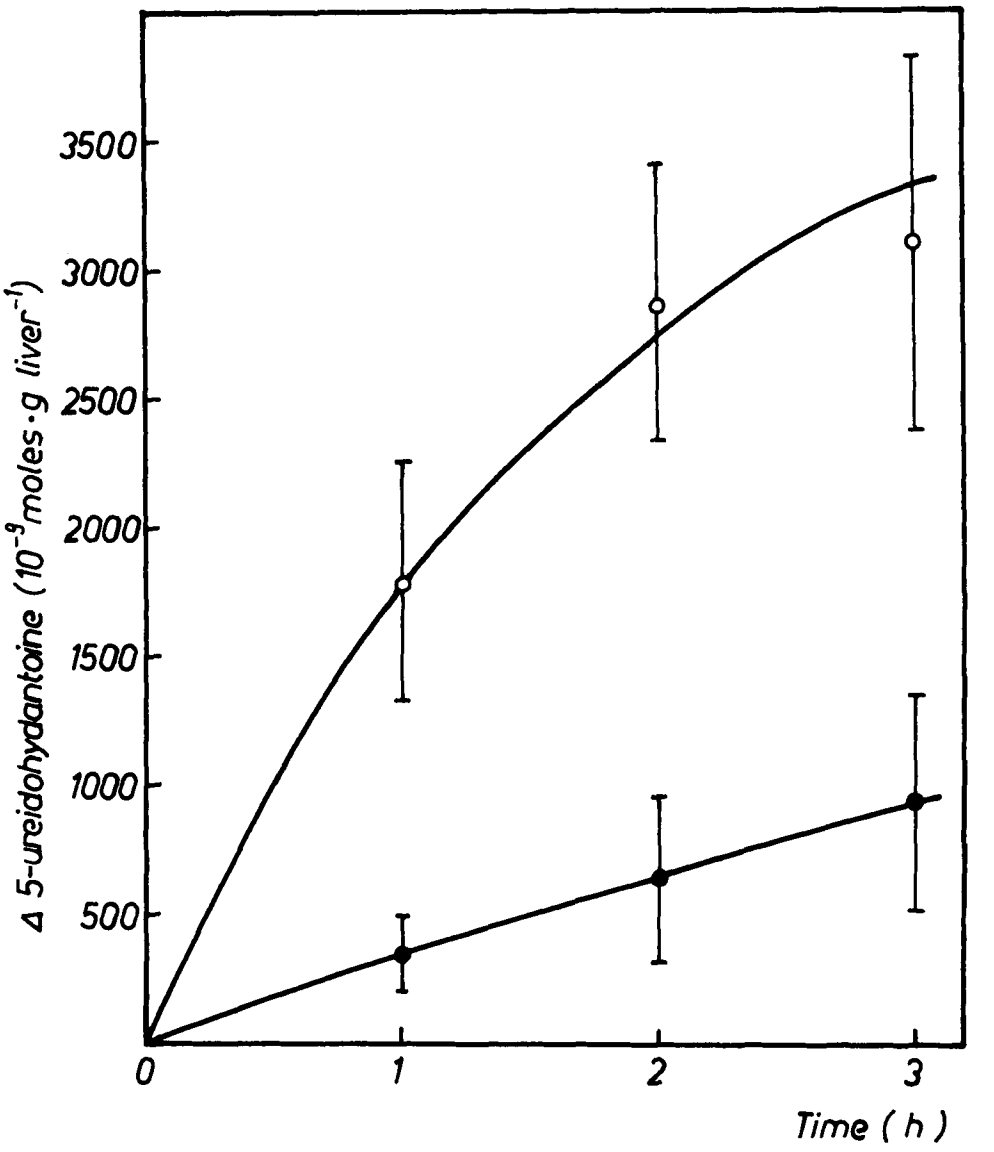

FIGURE 2 5-ureidohydantoine (allantoin) release of the recirculating perfused rat liver (25°C). Normoxia (●) 2 experiments with each 2 to 4 determinations; severe hypoxia (○) 5 experiments with each 2 to 5 determinations.

accumulation in the perfusion solution as may be seen in Figure 1. Formate as scavenger of the hydroxyl radical which reduces the export of glutathione disulfide into bile[3] does not change the production of uric acid in severe hypoxia. Allopurinol, however, as an inhibitor of the xanthine oxidoreductase diminishes the uric acid production. That holds to be true for the accumulation of allantoin, too (Figure 2).

For calculation of the cytosolic generation of H_2O_2 three items were considered:

1) the existence of xanthine oxidoreductase (XOR) in two types[11,12,13]: as an oxidase (XO) and as an NAD-dependent dehydrogenase (XD);

2) the strong inhibition of the XD-type by free NADH not bound to cytosolic protein[13];

3) the production of O_2^- as well as H_2O_2 by the XO-type[14,15].

Owing to the action of superoxide dismutase (E.C. 1.15.1.1) two superoxide radical anions are dismutated to H_2O_2 and O_2 and, therefore, the total amount of H_2O_2 formed is independent of the primary product of the XO-reaction.

Table I shows that the share of the XO-type on the total XOR-capacity increases from 20 to 26% during the period of 2 hours severe hypoxia. The value for normoxia lies slightly higher than that given by Kaminski *et al.*[16]. The sum of XO- and XD-capacity does not change, in agreement with the literature[17]. Using the lactate/pyruvate ratio determined in the perfusate the cytosolic free NADH concentration was calculated (Table II). These calculations are based on the assumption that the lactate/pyruvate ratio in the perfusate represents the cytosolic one[18,19]. The concentration of free NADH in the cytosol was calculated from lactate/pyruvate ratios, taking 504 nmol cytosolic free NAD^+ × g liver w.w.$^{-1}$ and an apparent equilibrium constant for the lactate dehydrogenase system $K_{app} = 10^{-4}$ at cytosolic pH 7.0[20,21]. The value obtained as nmol $NADH_{free}$ × g liver^{-1} was corrected to nmol × ml cytosol^{-1} [21]. The initial lactate/pyruvate ratio was 10 and the initial concentration of $NADH_{free}$ was 1.17 μM. Table II shows an about 25-fold increase of the concentration of free NADH in severe hypoxia.

By introducing the calculated values of free NADH, the portion of XD-capacity,

TABLE I

Contribution of xanthine oxidase (XO) activity in uric acid formation estimated from XD/XO ratio and from the influence of cytosolic $NADH_{free}$ concentration on XD activity.

Perfusion condition	Relative contribution of XO activity			
	1 h	2 h	3 h	n
	%			
Normoxia	20.0	20.0	20.0	4
Hypoxia	25.7	25.6	25.7	3
Severe hypoxia	27.2	27.7	28.2	4
	Uric acid formed by XO form			
	1 h	2 h	3 h	
	μmol × g w.w.$^{-1}$			
Normoxia	0.093	0.060	0.070	6
Severe hypoxia	0.563	0.344	0.097	4

TABLE II

Calculated cytosolic $NADH_{free}$ concentration of perfused liver.
1 μmol $NADH_{free}$ × 1 cytosol^{-1} = 8.55 lactate/pyruvate

Perfusion condition	$NADH_{free}$ (μmol × 1 cytosol^{-1})			
	1 h	2 h	3 h	n
Normoxia	0.88 ± 0.02	0.75 ± 0.04	0.71 ± 0.12	4
Moderate hypoxia	2.50 ± 0.50	2.23 ± 0.74	2.58 ± 0.87	3
Severe hypoxia	7.86 ± 1.63	11.47 ± 1.92	17.21 ± 4.62	4

the $K_{m,NAD}$ + of 22.4 μM[13], the $K_{i,NADH}$ of 2.9 μM[13] and the NAD^+ concentration of 1169 nmol × ml cytosol^{-1} [20] into the equation of competitive inhibition the extent of XD-inhibition by $NADH_{free}$ was estimated (Table III). As may be seen the maximal inhibition does not exceed 10%.

The relative contribution of XO-activity in uric acid formation increases from 20% to only about 28% in severe hypoxia as shown in Table I. But in absolute terms there is an almost six-fold increase in uric acid formation accompanied by H_2O_2 generation during the first two hours of perfusion. In the third hour there is a low rate of uric acid formation apparently owing to exhaustion of the adenine nucleotide pool. Taking into account different temperatures choosen here (25°C) and in experiments of Sies (37°C)[22] a correspondence in the release of uric acid may be pointed out: we found about 10 nmol × g w.w.$^{-1}$ × min^{-1} within the first hour of severe hypoxia and Sies[22] estimated 36 nmol × g w.w.$^{-1}$ × min^{-1} after transition of pO_2 from 0.26 mM to 0.06 mM in perfusion medium. In Table IV a synopsis of H_2O_2 formation via nucleotide degradation is presented. Both the cytosolic as well as the uricase mediated peroxisomal part are tremendously accelerated in severe hypoxia. The XO shares always about one-third of the total H_2O_2 generation.

The efflux of glutathione disulfide reflects 3% of the turnover of cytosolic glutathione peroxidase (E.C. 1.15.1.9.2)[4,23,24]. The increase of GSSG-efflux rate by 0.050 μmol × g liver w.w.$^{-1}$ in the first hour of severe hypoxia compared with normoxia corresponds to 1.65 μmol cytosolic H_2O_2 formation. Therefore, about 60% of the increased GSSG-efflux may be explained by the enhanced function of XO.

In normoxia about one-tenth of the oxygen consumed is used for H_2O_2 formation (Table V[25]). In severe hypoxia the rate of O_2 consumption is decreased to about 20%, but the total H_2O_2 formation seems to be in the same order of magnitude. Thus about

TABLE III
Inhibition of XD activity by the increase of the cytosolic $NADH_{free}$ concentration (%).

Perfusion condition	Inhibition of XD activity 1 h	2 h	3 h
Normoxia	1.8 ± 0.0	1.9 ± 0.0	1.9 ± 0.1
Moderate hypoxia	2.6 ± 0.2	2.5 ± 0.3	2.6 ± 0.4
Severe hypoxia	4.9 ± 0.7	6.4 ± 0.8	8.2 ± 2.2

TABLE IV
H_2O_2 formation via purine nucleotide degradation under normoxic and severe hypoxic conditions and comparison with GSSG efflux rate.

	Normoxia		Severe hypoxia	
	0–60 min	60–180 min	0–60 min	60–180 min
Total H_2O_2 formation via purine nucleotide breakdown	0.551	0.842	2.924	2.181
Cytosolic share[a]	0.186 (34%)	0.260 (31%)	1.126 (39%)	0.882 (40%)
Peroxisomal share[b]	0.365	0.582	1.798	1.299
GSSG efflux rate	0.114	0.054	0.164	0.107

[a] H_2O_2 formed by XO-type of xanthine oxidoreductase (in parentheses: percent of total H_2O_2 formation).
[b] H_2O_2 formed by uricase.
Rates are given as μmol × g w.w.$^{-1}$ × h^{-1}.

TABLE V
Total, cytosolic and peroxisomal H_2O_2 formation in relation to the oxygen consumption. n.d. = not determined.

Conditions	Oxygen consumption	Total H_2O_2 formation	Cytosolic H_2O_2 formation	Peroxisomal H_2O_2 formation
	μmol × g w.w.$^{-1}$ × h^{-1} (% of total H_2O_2 formation)			
Normoxic perfusion	26.4	2.6–3.9[a]	0.2 (5–8)[b]	0.4 (10–15)[c]
Severe hypoxic perfusion	5.5	2.9[b,c]	1.1 (38)[b]	1.8 (62)[c]
Maximally stimulated subcellular fractions[d]	n.d.	9.6	0.5 (5)	3.3 (35)

[a] From the estimate that 10–15% of total oxygen consumption are used for H_2O_2 formation[25].
[b] Only XO form activity.
[c] Only urate oxidase activity.
[d] With additional data about mitochondrial H_2O_2 formation: 1.5 μmol × g w.w.$^{-1}$ × h^{-1} (15% of total) and microsomal H_2O_2 formation: 4.3 μmol × g w.w.$^{-1}$ × h^{-1} (45% of total)[26].

one-half of oxygen is used for H_2O_2 formation. The striking observation may be the enhancement of the cytosolic H_2O_2 formation in severe hypoxia.

References

1. J.M. McCord and R.S. Roy, *Can. J. Phyiol. Pharmacol.*, **60**, 1346, (1982).
2. W. Siems, B. Mielke, M. Müller, C. Heumann, L. Räder and G. Gerber, *Biomed. Biochim. Acta*, **42**, 1079, (1983).
3. L. Räder, W. Siems, M. Müller and G. Gerber, *Cell biochem. Funct.* 3 (accepted for publication).
4. B. Chance, H. Sies and A. Boveris, *Physiol. Rev.*, **59**, 527, (1979).
5. M.-F. Vincent, G. van den Berghe and H.-G. Hers, *Biochem. J.*, **202**, 117, (1982).
6. N. Kageyama, *Clin. Chim. Acta*, **31**, 421, (1971).
7. R.A. Hartwick and P.R. Brown, *J. Chromatogr.*, **126**, 679, (1976).
8. A.A. Christman, P.W. Foster and M.B. Esterer, *J. Biol. Chem.*, **155**, 161, (1944).
9. H.U. Bergmeyer, Methods of enzymatic analysis (Verlag Chemie Weinheim, Academic Press: New York, London, 1974), vol. 4, p. 1791.
10. P.J. Hissin and R. Hilf, *Anal. Biochem.*, **74**, 214, (1976).
11. E. Della Corte and F. Stirpe, *Biochem. J.*, **126**, 739, (1972).
12. F. Stirpe and E. Della Corte, *J. Biol. Chem.*, **244**, 3855, (1969).
13. E. Della Corte and F. Stirpe, *Biochem. J.*, **117**, 97, (1970).
14. P.B. McCay, T. Noguchi, K.-L. Fong, E.K. Lai and J.L. Poyer, in The Radicals in Biology, ed. W.A. Pryor (Academic Press: New York), vol. IV, p. 155.
15. I. Fridovich, *J. Biol. Chem.*, **245**, 4053, (1970).
16. Z.W. Kaminski and M.M. Jezewska, *Biochem. J.*, **181**, 177, (1979).
17. Z.W. Kaminski and M.M. Jezewska, *Biochem. J.*, **200**, 597, (1981).
18. T. Bücher, B. Brauser, A. Conze, F. Klein, O. Langguth and H. Sies, *Eur. J. Biochem.*, **27**, 301, (1972).
19. H. Sies, S.T. Ta, B. Brauser and T. Bücher, *Adv. Enz. Regul.*, **10**, 309, (1972).
20. H. Sies, in Metabolic compartmentation, ed. H. Sies (Academic Press: London, 1982), p. 205.
21. E.A. Siess, D.G. Brocks and O.H. Wieland, in Metabolic compartmentation, ed. H. Sies (Academic Press: London, 1982), p. 235.
22. H. Sies, *Hoppe-Seyler's Z. Physiol. Chem.*, **358**, 1021, (1977).
23. H. Sies and K. Summer, *Eur. J. Biochem.*, **57**, 503, (1975).
24. N. Oshino and B. Chance, *Biochem. J.*, **162**, 509, (1977).
25. N. Oshino, D. Jamieson, T. Sugano and B. Chance, *Biochem. J.*, **146**, 67, (1975).
26. A. Boveris, N. Oshino and B. Chance, *Biochem. J.*, **128**, 617, (1972).

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