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INTERRELATIONSHIPS BETWEEN PURINE NUCLEOTIDE DEGRADATION AND RADICAL FORMATION DURING INTESTINAL ISCHAEMIA AND REPERFUSION: AN APPLICATION OF ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOTIDES, NUCLEOSIDES AND NUCLEOBASES[a,b]

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SUMMARY

There are peroxidative changes during the reperfusion of the rat small intestine following a 1-h period of total ischaemia. That is demonstrated by the increases of the concentrations of glutathione disulphide and of thiobarbituric acid-reactive substances. An important source of the active oxygen species leading to peroxidations is the degradation of purine nucleotides. The nucleotides and their derivatives were measured by an ion-pair reversed-phase high-performance liquid chromatographic separation in a single analysis within 40 min. Modification of the elution gradient resulted in a high resolution of nucleosides and nucleobases, including allopurinol and oxypurinol. The decrease of the nucleoside triphosphate concentration and the increase of nucleoside monophosphate concentration, followed by accumulations of nucleosides and nucleobases in the course of the ischaemia were measured. During reperfusion the nucleotide pools are filled up. Restoration of adenosine triphosphate and guanosine triphosphate can be accelerated by application of the xanthine oxidoreductase inhibitor allopurinol. Pretreatment of the animals with allopurinol also diminished the formation of glutathione disulphide and thiobarbituric acid-reactive substances.

[a]This contribution was presented at the 10th International Symposium on Biomedical Applications of Chromatography and Electrophoresis in Žinkovy, Czechoslovakia, April 27–30, 1988.
[b]This paper is dedicated to Professor Carl-Henric de Verdier, University Hospital Department of Clinical Chemistry, Uppsala, Sweden on the occasion of his 65th birthday.

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INTRODUCTION

There is a multitude of pathophysiologically interesting conditions connected with rapid changes of nucleotide concentrations and related biochemical and functional distortions of cells. Reoxygenation of the ischaemic intestine of rats is shown to result in damage due to oxygen radical formation. An important source of superoxide radicals and hydrogen peroxide generated during post-ischaemic reoxygenation is the accelerated degradation of purine nucleotides [1–3]. Ischaemia results in an accumulation of hypoxanthine and a conversion of the xanthine dehydrogenase form into the xanthine oxidase form of the enzyme xanthine oxidoreductase (XOR) [1]. The scheme in Fig. 1 demonstrates the changes of flux rates within purine nucleotide breakdown and related superoxide radical formation during transition from normoxia (a) to oxygen deficiency (b) [4]. The XOR conversion is very fast in the intestine [1], but it needs long ischaemic periods in other organs, such as in the liver [5].

In this study the concentrations of nucleotides, nucleosides and nucleobases were measured during ischaemia and reperfusion by means of high-performance liquid chromatography (HPLC). Furthermore, criteria for peroxidative changes were determined. A control group of rats was compared with a group treated with allopurinol (4-hydroxypyrazolo-3,4-pyrimidine). Allopurinol is a well known inhibitor of XOR, which rapidly converts the inhibitor into oxypurinol [6].

The separation and determination of nucleotide pools can be realised with various HPLC techniques [7]. These include ion-exchange chromatography of nucleotides [8], with the disadvantage of highly concentrated elution buff-

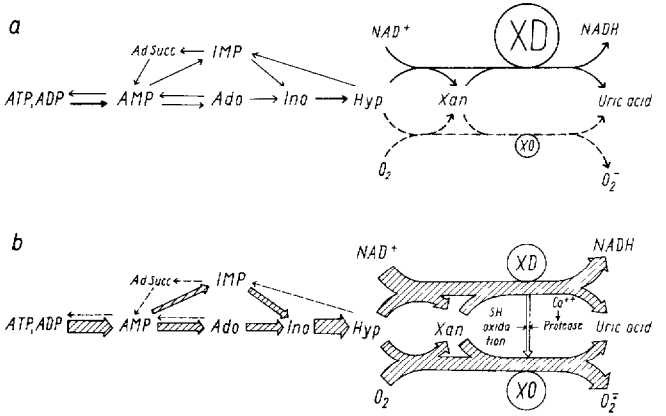

Fig. 1. Schematic representation of flux rates in the purine degradation and related superoxide radical formation and their changes during the transition from normoxic conditions (a) to oxygen deficiency (b) (from Gerber and Siems [4]). XD = dehydrogenase form of the xanthine oxidoreductase; XO = oxidase form of the enzyme.

ers, the separation of nucleosides and nucleobases by reversed-phase chromatography [9], the separation of purine nucleotides and their derivatives by reversed-phase chromatography [10,11] and ion-pair reversed-phase chromatography for the separation of purine and pyrimidine nucleotides, nucleosides and nucleobases [12,13]. For the studies presented here, ion-pair reversed-phase HPLC was applied with the advantage of a high resolution of nucleobases obtained by the use of concave elution gradient instead of the linear gradient elution used previously [13]. Furthermore, the separation technique applied offers the possibility of investigating simultaneously the pharmacokinetics of allopurinol and oxypurinol, which was measured by others by means of separate assays [14–16].

EXPERIMENTAL

Chemicals

All reference standards of the highest analytical grade available were obtained from Boehringer (Mannheim, F.R.G.). Ammonium dihydrogenphosphate was purchased from Fisher (Fair Lawn, NJ, U.S.A.) and acetonitrile was from Merck (Darmstadt, F.R.G.). Tetrabutyl ammonium phosphate (TBA, PIC reagent A) was from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC analyses was glass-distilled twice.

5,5′-Dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Sigma (St. Louis, MO, U.S.A.), N-ethylmaleimide (NEM) and *o*-phthaldialdehyde from Calbiochem (San Diego, CA, U.S.A.) and reduced glutathione (GSH) and oxidized glutathione (GSSG) from Boehringer. Allopurinol (Sigma) was kindly supplied by the Company Henning Berlin (Berlin-West).

High-performance liquid chromatography

An apparatus from Perkin-Elmer (Norwalk, CT, U.S.A.) was used, consisting of an M 410 pump system, an LC 95 variable-wavelength detector, an LCI-100 integrator and a Rheodyne injector system. The columns were from Perkin-Elmer: a C_{18} Sil-X-5 (250 mm × 4 mm I.D.) analytical column and a 25 mm × 4.6 mm I.D. guard-column. As eluents, the buffers A and B were used: buffer A was 10 m*M* $NH_4H_2PO_4$ containing 2 m*M* PIC (tetrabutyl ammonium phosphate) and buffer B was a 80:20 (v/v) mixture of buffer A and acetonitrile. The elution profile was the following: a 12-min concave gradient from 100% buffer A to 80% buffer B–20% buffer A; 25 min of isocratic elution with a 80:20 (v/v) mixture of buffer B and buffer A; in 2 min to 100% buffer A. At the end of gradient elution, the system was flushed with buffer A for 5 min. The flow-rate was 1.3 ml/min. The temperature was ambient (18–22 °C). The deduction wavelength was 254 nm. The peak identification and the exact quantitation were carried out as described previously [11]. The method was calibrated with various amounts of standard mixtures.

Tissue preparation

Male Wistar H-strain rats with a body mass of ca. 350 g were used for the experiments. Allopurinol was injected twice intraperitoneally into animals of one group, 24 h and 30 min before the experiment (50 mg/kg body mass). The animals were anaesthetized with diethyl ether and the intestine was prepared for ischaemia and reperfusion. An initial sample was taken, then the superior mesenteric arteria was ligated by means of a microvascular clamp. After 1 h the clamp was loosened and the small intestine was reoxygenated. All samples were taken immediately into liquid nitrogen and kept there until homogenization. The preparation of samples for HPLC separation of nucleotides, nucleosides and nucleobases included deproteinization with chilled 10% (v/v) perchloric acid, centrifugation for 10 min at 1200 *g*, neutralization with triethanolamine–potassium carbonate and filtration. After filtration, 50 μl of the supernatant were analysed by HPLC. The concentrations of GSH and of GSSG were measured according to Beutler et al. [17] and according to Hissin and Hilf [18], and those of thiobarbituric acid-reactive substances (TBA-RS) according to Ohkawa et al. [19].

RESULTS

For the determination of nucleosides and nucleobases the separation was improved in comparison with the separation technique described previously [11,13]. This is due to the use of a concave gradient starting after nearly isocratic conditions under which the elution is carried out through ca. 7 min. The separation of oxypurinol, allopurinol and uric acid is possible in this mode. A disadvantage of the method is the unsatisfactory separation of IMP and GMP. The nucleotide profile of an extract of the rat intestine is seen in the Fig. 2, which shows the elution profile of purine and pyrimidine compounds of the intestine before ischaemia of an allopurinol-treated animal. The figure demonstrates that the separation method developed is useful for observations of nucleotide metabolism in the small intestinal tissue.

Tables I and II give a synopsis of the concentrations of the nucleotides and their derivatives measured immediately before ischaemia, after 30 and 60 min of ischaemia and after 20 min of reperfusion in control animals (Table I) and in allopurinol-treated animals (Table II). The nucleoside triphosphates show drastic changes, which are presented separately in the Figs. 3 and 4 for the ATP and GTP. ATP, GTP and UTP are rapidly degraded during ischaemia. The decreases of ATP and GTP concentrations lead to temporary increases of the AMP, ADP and GDP pools of the intestinal tissue. The resynthesis of ATP, GTP and UTP is higher in the presence of allopurinol after 20 min of reperfusion. There is a low ATP and GTP resynthesis in control animals. The hypoxanthine concentration is increased ca. ten-fold during ischaemia. At

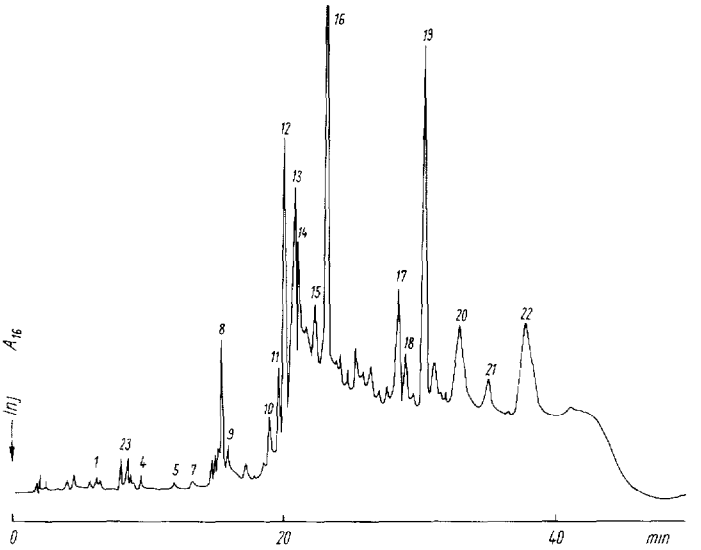

Fig. 2. Chromatogram of an extract of rat small intestine (control value = before ischaemia). The separation was obtained by the use of a gradient elution (see Experimental). Peaks: 1 = uracil; 2 = hypoxanthine; 3 = uridine; 4 = xanthine; 5 = oxypurinol; 7 = uric acid; 8 = inosine; 9 = guanosine; 10 = adenine; 11 = adenosine; 12 = NAD^+; 13 = IMP, 14 = GMP; 15 = UMP; 16 = AMP; 17 = GDP; 18 = UDP; 19 = ADP; 20 = GTP; 21 = UTP; 22 = ATP. Peak identities of the biological samples were confirmed by coelution with each standard substance.

TABLE I

CONCENTRATIONS OF NUCLEOTIDES, NUCLEOSIDES AND NUCLEOBASES IN THE RAT SMALL INTESTINE AT NORMOXIA, DURING ISCHAEMIA AND DURING THE REPERFUSION PERIOD: DATA FOR THE CONTROL GROUP

Metabolite	Concentration (mean ± S.D.) (nmol/mg of protein)			
	Initial values	30 min ischaemia	60 min ischaemia	20 min reperfusion
Hypoxanthine	0.04 ± 0.01	0.21 ± 0.09	0.29 ± 0.09	0.21 ± 0.08
Uric acid	3.8 ± 1.8	3.5 ± 2.7	3.8 ± 3.3	4.0 ± 1.8
IMP	2.7 ± 1.5	1.9 ± 1.4	2.2 ± 1.3	5.2 ± 1.3
AMP	2.6 ± 0.6	4.7 ± 2.0	3.7 ± 3.0	6.6 ± 1.9
GDP	2.3 ± 0.9	7.3 ± 2.3	2.9 ± 1.5	3.9 ± 1.4
ADP	3.2 ± 1.5	6.0 ± 2.8	6.0 ± 1.8	6.3 ± 2.2
GTP	9.3 ± 2.3	5.6 ± 1.9	5.3 ± 2.8	2.7 ± 2.2
UTP	5.2 ± 2.7	3.5 ± 0.8	4.0 ± 2.9	7.4 ± 1.9
ATP	10.4 ± 1.9	3.7 ± 1.0	3.7 ± 1.8	3.7 ± 2.7
Adenosine	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.3	1.2 ± 0.3
Adenine	1.5 ± 0.3	1.4 ± 0.9	1.0 ± 0.3	2.7 ± 0.8
Xanthine	0.4 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	0.2 ± 0.1
Uracil	0.09 ± 0.04	0.09 ± 0.03	0.11 ± 0.04	0.09 ± 0.04
Uridine	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1

TABLE II

CONCENTRATIONS OF NUCLEOTIDES, NUCLEOSIDES AND NUCLEOBASES IN THE RAT SMALL INTESTINE AT NORMOXIA, DURING ISCHAEMIA AND DURING THE REPERFUSION PERIOD: DATA FOR ALLOPURINOL-TREATED ANIMALS

Metabolite	Concentration (mean ± S.D.) (nmol/mg of protein)			
	Initial value	30 min ischaemia	60 min ischaemia	20 min reperfusion
Hypoxanthine	0.2 ±1.9	2.3 ±1.7	1.7 ±1.1	0.7 ±0.6
Uric acid	3.1 ±1.9	3.0 ±0.8	3.2 ±3.5	1.0 ±0.8
IMP	6.1 ±2.6	7.2 ±3.7	6.4 ±4.0	3.3 ±2.7
UMP	4.7 ±1.9	4.8 ±1.9	4.7 ±2.4	2.9 ±1.9
AMP	5.1 ±2.2	11.3 ±5.0	4.6 ±4.2	2 2 ±1.4
GDP	7.0 ±2.8	4.2 ±3.1	3.2 ±1.7	3.7 ±1.0
UDP	3 6 ±1.4	2.6 ±2.0	2 7 ±1.3	1.9 ±0.9
ADP	5.5 ±2.0	5.2 ±2.4	4.0 ±1.4	3.1 ±1.4
GTP	9.0 ±3.4	4.1 ±3.1	2.6 ±1.9	7.6 ±1.9
UTP	7.2 ±5.4	1.0 ±0.8	1.4 ±0.8	5.4 ±3.2
ATP	11.2 ±4.6	2.9 ±2.2	2.2 ±1.6	8.1 ±4.1
Adenosine	2.4 ±1.2	0.9 ±0.6	1.2 ±0 6	0.9 ±0.3
Adenine	1.7 ±0.6	1.0 ±0.7	1.6 ±0.7	0.8 ±0.3
Xanthine	0.09 ± 0.04	1.0 ±0.8	0.7 ±0.4	1.3 ±0.8
Uracil	0.07 ± 0.03	0.17 ± 0.04	0.10 ± 0.04	0.09 ± 0.04
Uridine	0.5 ±0.4	1.5 ±0.6	0.2 ±0.1	0.3 ±0.1
Inosine	0.9 ±0.4	3.0 ±1.7	3.1 ±1.4	0.8 ±0.6
Oxypurinol	3.6 ±1.9	2.8 ±1.7	3.7 ±2.7	3.2 ±1.8

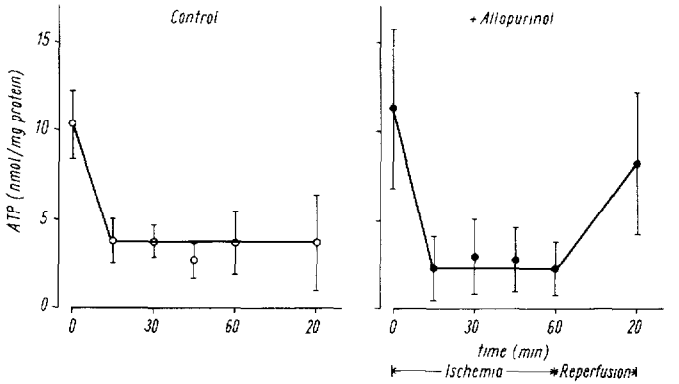

Fig. 3. Change of ATP concentration during ischaemia and reperfusion of rat small intestine. Comparison of control animals with rats pretreated with allopurinol ($n = 3$ for each group).

reoxygenation, hypoxanthine is used for resynthesis of nucleotides and for uric acid formation connected with oxygen radical generation.

As criteria of oxidative stress, the concentrations of reduced and oxidized

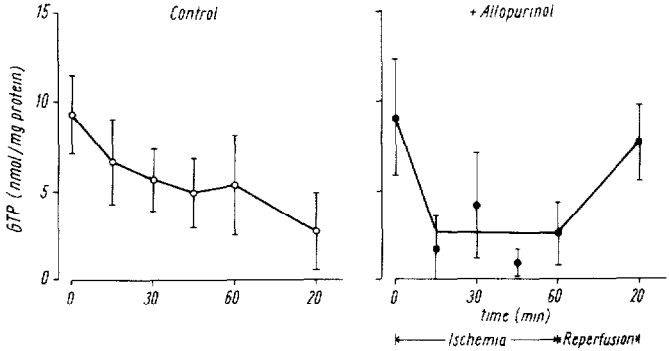

Fig. 4. GTP concentration in the small intestine of rats in the course of ischaemia/reperfusion experiments: 60 min of complete ischaemia and 20 min of reperfusion. Comparison of control animals and rats pretreated with allopurinol ($n=3$ for each group).

TABLE III

GSSG CONCENTRATION OF INTESTINAL TISSUE BEFORE, DURING AND AFTER ISCHAEMIA

Sample	Concentration (nmol/mg of protein)					n
	Before ischaemia	Ischaemia		After reoxygenation		
		30 min	60 min	10 min	20 min	
Control	0.50	0.65	0.67	0.99	0.66	6
Allopurinol-treated	0.47	0.43	0.58	0.62	0.55	6

TABLE IV

RATIO OF 2 GSSG TO (GSH + 2 GSSG) IN SMALL INTESTINAL TISSUE BEFORE, DURING AND AFTER ISCHAEMIA

The concentration of GSSG and GSH were determined as nmol sulphydryl units per mg protein.

Sample	Ratio					n
	Before ischaemia	Ischaemia		After reoxygenation		
		30 min	60 min	10 min	20 min	
Control	0.100	0.121	0.117	0.175	0.152	6
Allopurinol-treated	0.091	0.097	0.113	0.114	0.096	6

TABLE V

CONCENTRATION OF THIOBARBITURIC ACID-REACTIVE SUBSTANCES IN SMALL INTESTINAL TISSUE BEFORE, DURING AND AFTER ISCHAEMIA

Sample	Concentration (nmol/mg of protein)					*n*
	Before ischaemia	Ischaemia		After reoxygenation		
		30 min	60 min	10 min	20 min	
Control	0.067	0.078	0.067	0.111	0.075	6
Allopurinol-treated	0.038	0.051	0.048	0.031	0.023	6

glutathione, the ratio of GSSG to total glutathione and the concentration of TBA-RS were measured. The concentration of total glutathione shows no significant difference between control and allopurinol-treated animals and no changes during ischaemia and reperfusion [20] (data not shown). There is an elevation of the GSSG concentration in the control animals 10 min after the onset of reperfusion. This increase of the GSSG is attenuated by allopurinol (Table III). The changes of the GSSG concentration cause a significant increase of the ratio of GSSG to glutathione within 10 min of reperfusion (Table IV). The concentration of TBA-RS (Table V) is lower in allopurinol-treated animals than in control animals throughout the experiments.

DISCUSSION

Ion-pair reversed-phase high-performance liquid chromatography

The ionic nature of the nucleotides results in interactions with the cationic ion-pair reagent. The separation is influenced by at least two factors: (i) the existence of a dynamic ion exchanger and (ii) the retention of ion pairs. The formation and retention of ion pairs causes the nucleoside monophosphates to elute before the nucleoside diphosphates, which in turn elute before the nucleoside triphosphates. The concentration of the buffer salt ($NH_4H_2PO_4$) in the eluent was restricted to 10 m*M* to prevent a competition with the nucleotides for the ion-pair reagent, to guarantee a sufficient amount of free ion-pair cations [7].

Interrelationships between nucleotide and oxygen radical metabolism

The oxidase type of XOR generates superoxide radicals and hydrogen peroxide. Therefore, an increased supply of hypoxanthine, e.g. during and after severe hypoxia or ischaemia, is accompanied by enhanced free radical production [1,3,4,21,22]. The small intestine is distinguished from other tissues by a high activity of XOR, which is rapidly converted from the dehydrogenase form

into the oxidase type during ischaemia [1]. Some of the enzymic and non-enzymic antioxidative systems of the small intestine are weak (see the glutathione peroxidase activity), and the organ demands large amounts of oxygen and is therefore exceptionally vulnerable to oxidative stress [4]. The importance of oxygen reduction products for intestinal injury during oxygen deficiency and reoxygenation has been demonstrated by others, especially by means of albumin clearance and morphological observations [23–25]. Our study was aimed at the biochemical characterization of intestinal ischaemia and reperfusion, including the measurement of a great number of purine and pyrimidine metabolites.

There is a rapid degradation of purine nucleotides during ischaemia of the small intestine. Furthermore, during reperfusion an oxidative loading was found. The general findings that (i) during ischaemia substrates of the xanthine oxidase accumulate, (ii) these substrates are rapidly metabolized in the presence of oxygen, resulting in an oxidative burst, were reported for the small intestine of the cat by Younes et al. [26]. These authors quantified as changes of purine pools only the ATP, ADP, AMP and hypoxanthine concentrations; many other metabolites were included in the study presented here. It should be remarked, that our initial values for the ATP/ADP and ATP/AMP ratios and also for the GSSG/glutathione ratio are in good agreement with those of Younes et al. [26]. The absolute concentrations of ATP, ADP and AMP are slightly higher in the rat intestine than in the intestinal tissue of the cat. An important property of the small intestine in both species seems to be the slow restoration of the ATP level [23] in control animals. In our experiments the accelerated restoration of ATP in the presence of allopurinol was shown (see Table II). Another interesting feature of the experiments with cat intestine [26] is the discrepancy between the peak of conjugated dienes that are formed as a first step in the peroxidation of polyunsaturated fatty acids immediately after starting the reperfusion and the continuously increasing GSSG concentration during the whole reperfusion period. In our experiments the temporary increases of both GSSG and TBA-RS levels were observed simultaneously 10 min after starting the reoxygenation. The quantification of the superoxide radical and hydrogen peroxide formation via the xanthine oxidase-catalysed reactions is quite difficult because of the efflux of large amounts of the hypoxanthine and xanthine by fast transport processes into the surrounding compartments of the intestine during ischaemia. One can suggest that calculation of superoxide radical formation via xanthine oxidase on the basis of differences between the hypoxanthine concentrations at the end of ischaemia and after different time intervals of reperfusion leads to extreme underestimation, because the extraintestinal hypoxanthine pools reflow during the reperfusion at least partially into the enterocytes. Therefore, for a detailed calculation of radical formation via purine breakdown the measurement of the

intestinal and extraintestinal concentrations of hypoxanthine seems to be necessary.

In summary, the reoxygenation rather than ischaemia itself is responsible for the postischaemic damage. Oxygen radicals play an important role in the damage of the intestine during and after reperfusion. An important source of oxygen radicals formed after intestinal ischaemia is the purine degradation, as demonstrated by the effect of the xanthine oxidase inhibitor, allopurinol.

Effects of allopurinol on nucleotide and oxygen radical metabolism

Allopurinol (A in Fig. 5) is used in the treatment of hyperuricaemia. The active metabolite which acts by inhibition of xanthine oxidase (XO) is oxypurinol (O in Fig. 5). The conversion is catalysed by xanthine oxidase itself, with a product inhibition by oxypurinol. Xanthine oxidase is inhibited by the competitive binding of the hypoxanthine analogue to xanthine oxidase and thus the prevention of hypoxanthine conversion into xanthine and uric acid. The production of free radicals under reperfusion conditions is decreased by the inhibition of these enzymic reaction steps.

The rate-limiting enzyme of purine biosynthesis is the amidophosphoribosyltransferase (amido-PRT) [27]. The intracellular level of phosphoribosylpyrophosphate (PRPP) is diminished by conversion of allopurinol and oxypurinol into the corresponding ribonucleotides (allopurinol ribonucleotide = AR; oxypurinol ribonucleotide = OR) [6]. Allopurinol and

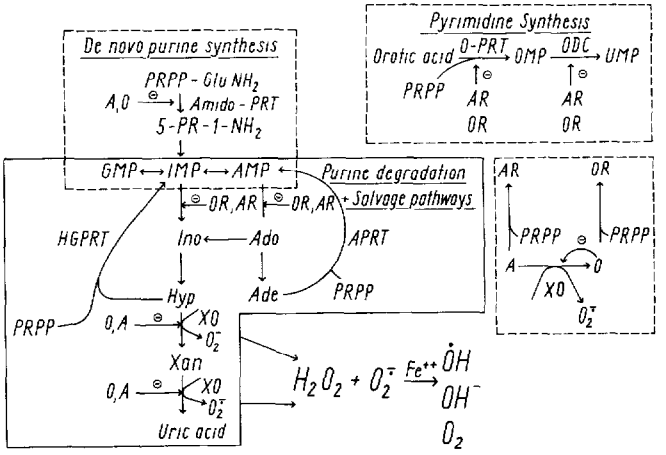

Fig. 5. Scheme of the influences of allopurinol, its derivative oxypurinol and their ribonucleotides on pathways of the nucleotide and oxygen radical metabolism. A = allopurinol; O = oxypurinol; AR = allopurinol ribonucleotide; OR = oxypurinol ribonucleotide. Enzymes: Amido-PRT = amidophosphoribosyltransferase; O-PRT = orotatephosphoribosyltransferase; ODC = orotidine-5′-phosphatedecarboxylase; HGPRT = hypoxanthine-guaninephosphoribosyltransferase; APRT = adeninephosphoribosyltransferase; XO = xanthine oxidase.

oxypurinol reduce the de novo purine biosynthesis by inhibition of amido-PRT and depletion of the substrate PRPP. The PRPP concentration is also rate-limiting for the salvage pathway of guanine, hypoxanthine, xanthine and adenine via the phosphoribosyltransferases [28]. The 5′-nucleotidase responsible for degradation of IMP or AMP to inosine and adenosine, respectively [29], is inhibited by the allopurinol and oxypurinol ribonucleotides [30]. The inhibitory influence of allopurinol-derived metabolites on the nucleotide metabolism also occurs at the level of pyrimidine synthesis via the orotate-phosphoribosyltransferase (O-PRT) and orotidine-5′-P-decarboxylase (inhibition by oxypurinol ribonucleotide and allopurinol ribonucleotide, OR and AR).

Fig. 5 shows the presently known influences of oxypurinol and allopurinol and their corresponding ribonucleotides on the nucleotides metabolism, and gives therefore a framework for explanation of the observations during ischaemia of the small intestine and the changes of the nucleotide levels. The better recovery of ATP and GTP concentrations after reperfusion of rat small intestine pretreated with allopurinol could be explained by the inhibition of nucleotide degradation via 5′-nucleotidase and accumulation of purine bases in the surrounding tissue (inhibition of XO) during ischaemia. The allopurinol was very rapidly converted into oxypurinol.

ACKNOWLEDGEMENT

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