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RADICAL FORMATION IN THE RAT SMALL INTESTINE DURING AND FOLLOWING ISCHEMIA

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Oxidative loading during the reperfusion of the proximal jejunum of rats following a one hour-period of complete ischemia was demonstrated in *in vivo*-experiments by the increases of the GSSG: total glutathione ratio and the concentration of TBA-RS. The pretreatment of the animals with the xanthine oxidoreductase inhibitor allopurinol diminished the accumulation of GSSG and of TBA-RS. It was concluded that the purine nucleotide degradation is an important source of oxygen reduction products in reoxygenated small intestine. The tissue concentrations of nucleotides, nucleosides and nucleobases were measured by an ion-pair reversed-phase HPLC separation. There occurred fast declines of ATP and GTP concentrations during ischaemia leading to temporary increases of nucleoside mono- and diphosphate pools. The hypoxanthine concentration is increased about twentyfold during oxygen deficiency. The ATP and GTP restoration during the reperfusion was accelerated in presence of allopurinol. The shares of the beneficial allopurinol effects are not yet clarified.

KEY WORDS: purine nucleotides, rat small intestine, post ischemic damage, xanthine oxidoreductase, allopurinol, glutathione.

ABBREVIATIONS: GSH, reduced glutathione; GSSG, oxidized glutathione: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NEM, N-ethylmaleimide: TBA-RS, thibarbituric acid-reactive substance;HPLC, high-performance liquid chromatography.

INTRODUCTION

A reduced oxygen supply leads to changes of nucleotide concentrations of cells. There occur drastic changes of flux rates within the purine nucleotide breakdown during transition from normoxia to oxygen deficiency.[1] At normoxia the rate of ATP degradation is very small. The xanthine oxidoreductase is existing predominantly as the dehydrogenase form and the contribution of oxidase type mediated urate production is low. In contrast to normoxia the purine degradation is very fast at oxygen deficiency and the dehydrogenase type of xanthine oxidoreductase is converted partially into the oxidase type. The enzyme conversion from the D-type into the O-type needs long ischemic periods in the liver[2–4] – we measured 27% O-type mediated urate production after three hours[3] – but it is faster in the intestine as known from the literature even if the rate measured in previous reports[5–7] was reevaluated by Parks *et al.*[8] Both organs – liver and small intestine – possess the highest activities of

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xanthine oxidoreductase compared with other organs.[1,9,10] That was a reason to carry out experiments with the small intestine especially the proximal jejunum in continuation of the experiments on liver and liver cells.[3,4,11–14] In this study criteria for peroxidative changes in combination with the concentrations of nucleotides and their degradation products of the small intestine were determined in *in vivo* experiments. A control group of animals was compared with a group of allopurinol-treated rats. Allopurinol (4-hydroxypyrazolo-3,4-pyrimidine) is a well known inhibitor of the xanthine oxidoreductase. This inhibitor is rapidly converted to oxypurinol by the same enzyme.[15] The combination of parameters measured was chosen because of the contentions on close interrelationships between purine nucleotide degradation, radical formation via xanthineoxidase reactions and ischemic reperfusion injury to small intestine.

MATERIAL AND METHODS

Tissue preparation

Male Wistar H-strain rats with a body weight of about 350 g were used for the experiments. Allopurinol (50 mg/kg b.w.) was injected i.p. twice, 24 hours and 30 minutes, before the experiment. The animals were anaesthetized with diethyl ether and the intestine was prepared for ischemia and reperfusion. The complete ischaemia was induced by ligation of the superior mesenteric arteria by means of a microvascular clamp. For reperfusion the clamp was loosened after one hour. The samples taken from the proximal jejunum were given immediately into liquid nitrogen. The preparation of samples for HPLC separation of purines and pyrimidines included the deproteinization with perchloric acid, centrifugation, neutralization with triethanolamine-potassium carbonate and a filtration.

The concentrations of GSH and of GSSG were measured according to Beutler *et al.*[22] and fluorimetrically according to Hissin and Hilf.[23] The GSH autoxidation was prevented by addition of 50 mM NEM. TBA–RS were determined according to Ohkawa *et al.*[24]

High-performance liquid chromatography

An ion-pair reversed-phase HPLC separation using a concave gradient elution was applied to determine nucleotides, nucleosides and nucleobases. An equipment from Perkin Elmer (Norwalk, CT, U.S.A.) was used consisting of a M 410 pump system, a LC 95 variable wavelength detector, a LCI-100 integrator, a Rheodyne injector system. The column was a C-18 Sil-X-5, 250 mm × 4.6 mm i.d. with a precolumn 25 mm × 4.6 mm i.d. As eluents the buffers A and B were used/ Buffer A is 10 mM $NH_4H_2PO_4$ containing 2 mM PIC (tetrabutylammonium phosphate) and buffer B is 80% buffer A + 20% acetonitrile (v/v%). The elution profile was the following: 12 minutes a concave gradient from 100% buffer A to 80% buffer B/20% buffer A; 25 minutes isocratic 80% buffer B/20% buffer A; in 2 minutes to 100% buffer A. At the end of gradient elution the system was flushed with buffer A for 5 minutes. The flow rate was 1.3 ml/min. The peak identification and the exact quantitation were performed as described.[25]

Reagents

All reference standards for HPLC separation 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.). Tetrabutylammoniumphosphate (PIC reagent A) was from Waters Assoc. (Milford, MA, U.S.A.). DTNB and allopurinol were purchased from Sigma Chemical Corp. (St. Louis, MO, U.S.A.) and NEM and o-phthaldialdehyde from Calbiochem (San Diego, CA, U.S.A.), GSH and GSSG from Boehringer (Mannheim, F.R.G.).

RESULTS AND DISCUSSION

Comparing the elution profiles of the nucleotides, nucleosides and nucleobases of the intestinal tissue before and after ischemia one can see the decline of nucleoside triphosphates and furthermore the increase of other metabolites like hypoxanthine. The initial values for the ATP, GTP and UTP concentrations of the small intestine of control animals were 11.1, 5.4, and 3.4 nmol/mg protein which is in good agreement with values measured by others. Figure 1 demonstrates the fast degradation of ATP. In Figure 2 the time course of GTP concentration is depicted. The resynthesis of ATP as well as of GTP is higher after 20 minutes of reperfusion in animals treated with allopurinol. The ATP and GTP restoration in control animals is very slow. In hepatocyte suspensions such effects could not be observed.[26] In our opinion those differences between cell suspensions and whole organs at least partically depend on differences of substrate supply under *in vivo*- and *in vitro*-conditions important for the AMP resynthesis from IMP. Figures 3, 4 and 5 demonstrate the ATP/ADP, ATP/AMP and GTP/GDP ratios during ischemia and reperfusion. The initial values of these ratios cannot be normalized within 20 minutes of reperfusion following a one hour period of total ischemia. But the ATP/ADP and ATP/AMP ratios are nearly normalized during 20 minutes of reoxygenation in animals pretreated with the xanthine oxidase inhibitor. This increase is not significant for GTP/GDP. Table 1 shows that the hypoxanthine concentration is increased about twentyfold during ischemia.

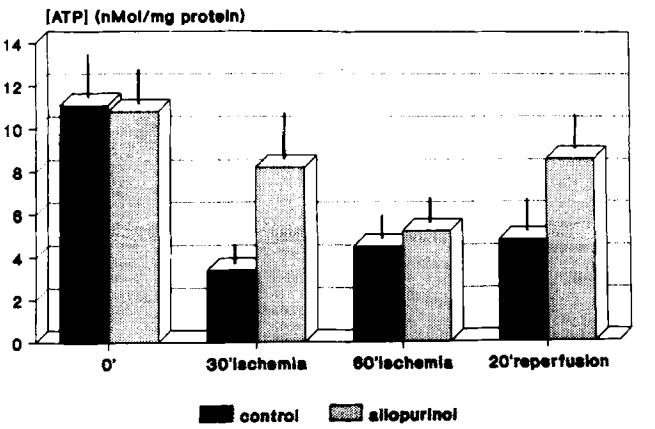

FIGURE 1 ATP content of rat intestine (proximal jejunum). 7 animals in each group.

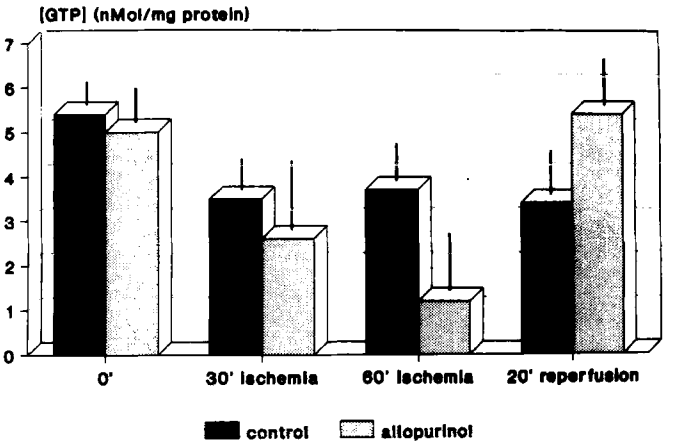

FIGURE 2 GTP content of rat intestine (proximal jejunum). 7 animals in each group.

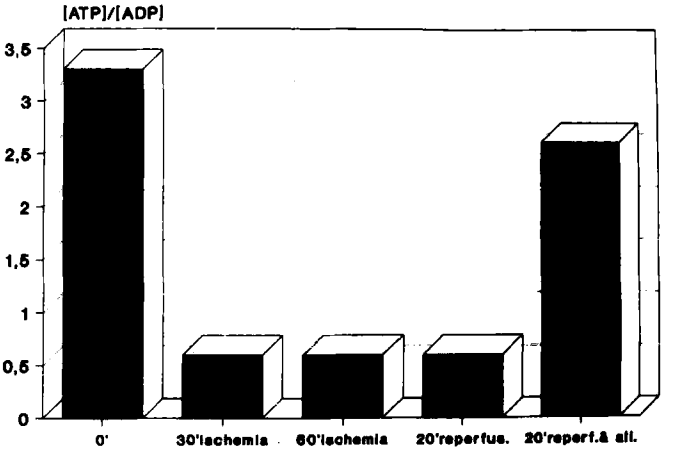

FIGURE 3 ATP/ADP ratio of rat intestine (proximal jejunum). 7 animals in each group.

It should be mentioned that the decline of hypoxanthine concentration during reperfusion in presence of allopurinol obviously is not equal to the increase of ATP concentration. One has to take into account the fast transport of hypoxanthine between tissue and surrounding compartments, especially the peritoneal cavity. Furthermore, one can suggest that the calculation of superoxide radical formation by xanthine oxidase on the basis of differences between the hypoxanthine concentrations leads to an underestimation. That is also due to a reflow of extraintestinal pools during the reperfusion into the mucosal tissue. Such methodological uncertainties for the calculation of superoxide radical formation via xanthine oxidase reactions could be avoided in liver experiments.[3]

As criteria of the oxidative stress, the concentrations of GSH, GSSG and TBA-reactive substances were measured (Table 2). The total concentration of glutathione

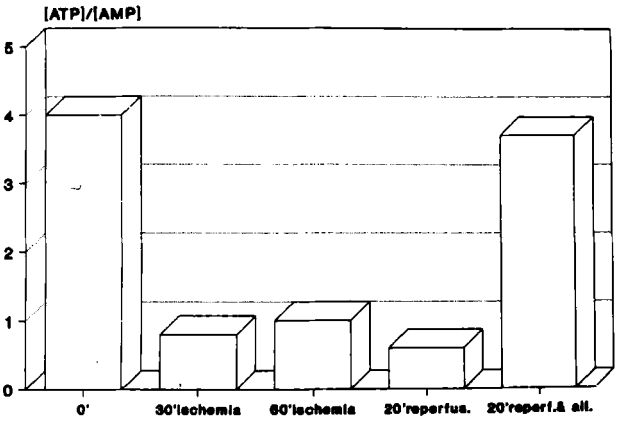

FIGURE 4 ATP/AMP ratio of rat intestine (proximal jejunum). 7 animals in each group.

shows no significant differences between controls and allopurinol-treated animals and no changes during ischaemia and reperfusion. There is an elevation of the GSSG concentration in the control animals ten minutes after the onset of reperfusion. This increase of the GSSG concentration is attenuated by allopurinol. Table 2 shows also the changes in the GSSG glutathione ratio, especially the increase of this ratio within 10 minutes of reperfusion due to the corresponding increase of oxidized glutathione. The concentration of TBA-reactive substances is lower in allopurinol-treated animals than in control rats during the whole duration of the experiments, and there is a temporary increase of these oxidative loading. This is in agreement with results of Younes *et al.*,[27,28] who demonstrated the postischaemic oxidative burst of the cat intestine by means of conjugated dienes. From the increase of TBA-RS one should assume the formation of hydroxyl radicals during the reoxygenation of the intestine. The increases of GSSG and of TBA-RS demonstrate that the oxygen radical induced component is involved in the biochemical changes during reperfusion of the jejunum.

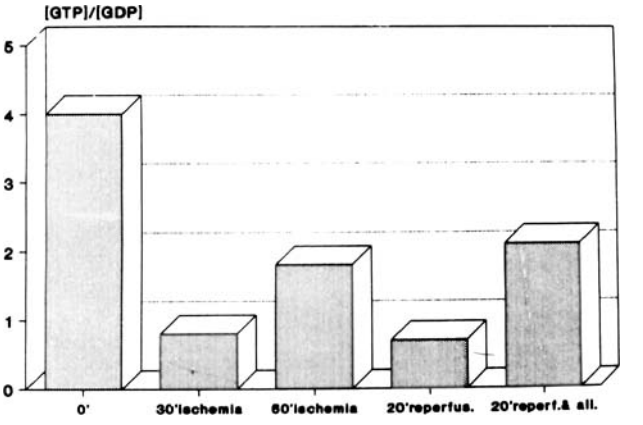

FIGURE 5 GTP/GDP ratio of rat intestine (proximal jejunum). 7 animals in each group.

TABLE 1
Hypoxanthine content in the rat small intestine at ischaemia and reperfusion (nmol/mg protein, n = 7; mean ± S.E.M.)

Initial value	30 min ischaemia	60 min	10 min reperfusion	20 min
0.04 ± 0.01	0.55 ± 0.09	0.81 ± 0.26	0.51 ± 0.15	0.40 ± 0.08

TABLE 2
Contents of reduced, oxidized, total glutathione and of thiobarbituric acid-reactive substances and the GSSG: total glutathione ratio of rat small intestine at ischaemia and reperfusion Data from 6 control animals, 6 allopurinol-pretreated animals.

	GSH	GSSG	Total glutathione	2 GSSG:total glutathione	TBA-RS
Time	nmol/mg protein			-	nmol/mg protein
Control					
0 min	8.94 ± 1.78	0.50 ± 0.16	9.94 ± 2.11	0.100 ± 0.034	0.067 ± 0.017
30 min ischaemia	9.50 ± 1.90	0.65 ± 0.16	10.80 ± 2.24	0.121 ± 0.031	0.078 ± 0.024
60 min ischaemia	10.05 ± 1.68	0.67 ± 0.19	11.39 ± 2.07	0.117 ± 0.034	0.067 ± 0.020
10 min reperfusion	9.40 ± 2.20	0.99 ± 0.41	11.38 ± 3.04	0.175 ± 0.074	0.111 ± 0.043
20 min reperfusion	7.43 ± 1.20	0.66 ± 0.21	8.75 ± 1.62	0.152 ± 0.047	0.075 ± 0.021
Allopurinol pretreated animals					
0 min	9.33 ± 1.61	0.47 ± 0.08	10.27 ± 1.77	0.091 ± 0.016	0.038 ± 0.006
30 min ischaemia	7.97 ± 1.42	0.43 ± 0.07	8.83 ± 1.55	0.097 ± 0.015	0.051 ± 0.018
60 min ischaemia	9.06 ± 1.10	0.58 ± 0.07	10.22 ± 1.24	0.113 ± 0.014	0.048 ± 0.011
10 min reperfusion	9.69 ± 2.09	0.62 ± 0.13	10.93 ± 2.36	0.114 ± 0.024	0.031 ± 0.008
20 min reperfusion	10.34 ± 2.24	0.55 ± 0.08	11.44 ± 2.40	0.096 ± 0.014	0.023 ± 0.006

The useful effects of the xanthine oxidase inhibition demonstrate that purine degradation is an important source of the oxygen radicals formed. Besides the purine breakdown at least one another important source of oxygen reduction products has to be taken into account: the radical formation from immigrated neutrophils.[29,30] There exist close interrelationships between xanthine oxidase-mediated radical formation, the neutrophil infiltration and mucosal injury during the reoxygenation. The reduced oxygen species generated in the purine breakdown may be cytotoxic to the reperfused tissue. Injured cells release neutrophil activators and chemoattractants that recruit circulating leukocytes into the tissue.[29,30] Other chemoattractants are produced by reactions of activated species of oxygen with plasma components.

It seems to be important to clarify in further studies the shares of the different allopurinol effects including the inhibition of xanthine oxidase and including especially the radical scavenging properties[26,31-33] during the reoxygenation of the small intestine and other organs with the aim to optimize the cell protective influences of this drug during and following oxygen deficiency.

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