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Short communication

Activity of liver proteases in experimental methanol intoxication

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Intoxication of rats with methanol (1.5 and 3.0 g/kg body weight) led to a significant, time- and dose-dependent decrease in the activities of cathepsins A, B and C, while the activity of cathepsin D was unaffected. The decrease was associated with a different partial release of individual cathepsins to the post-lysosomal fraction.

Methanol is oxidized, in about 80%, to formaldehyde and formate in the liver [1], with a concomitant increase in NADH level. Methanol metabolites are highly toxic. Formaldehyde reacts both with small- (amino acids, peptides, urea) and high-molecular (nucleic acids, proteins) compounds [2–4]. Formic acid causes, among others, metabolic acidosis and decreasing ATP synthesis [5, 6]. Metabolic acidosis and the increased NADH concentration can lead to intensive production of hydroxyl radicals and superoxide anions [7]. Damage to lysosomes and changes in distribution and activity of lysosomal proteolytic enzymes may be accompanied by formation of the above mentioned metabolites. The aim of the experiments reported in this paper was to examine this hypothesis.

MATERIAL AND METHODS

Male Wistar rats (about 230 g body weight) were fed a standard diet containing 0.55% of cysteine and methionine. Two groups of 36 animals each were intoxicated. Rats were given a 50% solution of methanol in isotonic saline orally through a plastic tube by syringe. The first group received 1.5 g, and the second — 3.0 g methanol/kg body weight. An equivalent volume of saline was given orally to six control rats.

At 6, 12 and 24 h, and 2, 5 and 7 days after methanol administration the animals were killed under ether anaesthesia, then livers were removed quickly and placed in ice-cold 0.15 M NaCl, perfused with the same solution in order to remove completely blood cells,

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Abbreviations: CBZ, *N*-carbobenzoxy; Bz, α-*N*-benzoilo; pNA, *p*-nitroanilid.

then blotted on filter paper, weighed and homogenized in ice-cold 0.25 M sucrose without and with 0.2% Triton X-100 (1:9, w/v) in a glass-teflon homogenizer. The homogenates were centrifuged at 18000 × *g* at 4°C for 20 min. The supernatant of homogenate treated with Triton X-100 included lysosomal enzymes, while that of non-treated was devoid of lysosomes (post-lysosomal fraction). In the latter the presence of acid phosphatase, a marker lysosomal enzymes did not exceed 8% of the total acid phosphatase activity. Also addition of Triton X-100 at the final concentration of 0.2% practically had no effect on the activities of lysosomal enzymes in the post-lysosomal fraction. The supernatants were kept frozen until assayed for enzymatic activities (not longer than 2 days). Cathepsin A was assayed with CBZ-Glu-Tyr at pH 5.0 by measuring the released tyrosine by the ninhydrin method [8]. The activity of cathepsin B was determined with Bz-DL-Arg-pNA, as a substrate, at pH 6.0 [9], and that of cathepsin C with Gly-Phe-pNA, at pH 6.0, by measuring *p*-nitroaniline at 405 nm [10]. All three substrates were from Sigma (St. Louis, U.S.A.). Cathepsin D activity was measured with the urea-denatured hemoglobin (Difco, Detroit, U.S.A.) at pH 4.0 by measuring tyrosine by the modified Folin-Ciocalteau method [11]. The quantity of released tyrosine, attributed to the activity of cathepsin E did not exceed 5%.

The activity of cathepsins in the post-lysosomal fraction was referred to that of control rats.

Protein concentration was determined according to Lowry *et al.* [12].

The results were expressed as means ±S.D. Statistical analysis was performed using Tukey's multiple-range test at $P < 0.05$.

RESULTS AND DISCUSSION

Determination of enzymatic activities, including cathepsins in hepatic tissues is a standard procedure in examination of hepatotoxicity of many substances [13, 14]. In this study changes in total cathepsin activities were monitored in rat liver during 7 days after methanol intoxication with special consideration of the effect of methanol on lysosomes. The activities of cathepsins in the post-lysosomal supernatant of control rats varied from 9% (cathepsin B) to 15% (cathepsin C) of their total hepatic activities (Fig. 1).

Intoxication of rats with methanol resulted in a significant decrease of total activities of cathepsin A, B and C noticeable 24 h after administration of methanol (Table 1). On the second day of intoxication this decrease was from 13% (cathepsin A) to 23% (cathepsin C), but on the 7 day the activities of these cathepsins were fully recovered. The effect of methanol was dose-dependent.

In the intoxicated rats the decrease in total cathepsin activities was associated with the increase in the post-lysosomal fraction (Fig. 2). The release from lysosomes above the control value (Fig. 1) was dose-dependent. varied for individual enzymes and

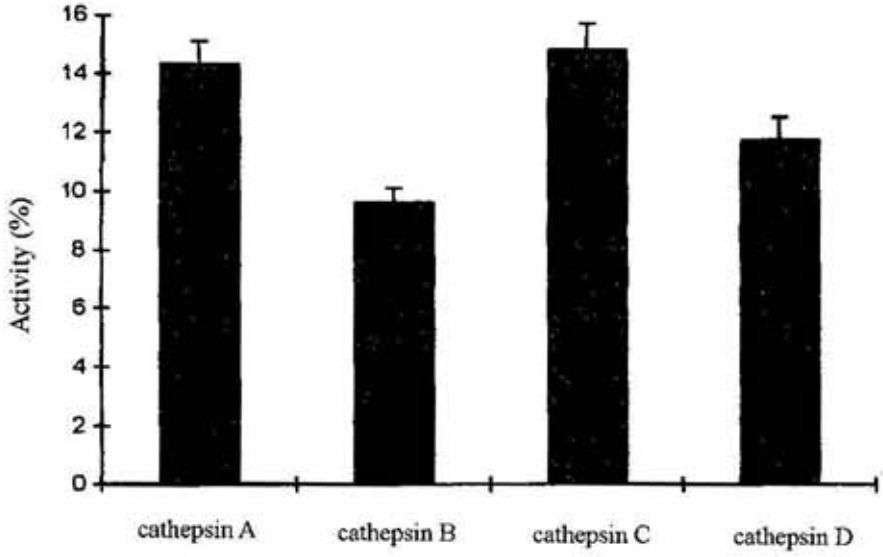

Figure 1. The activity of cathepsins in the post-lysosomal fraction in percents of the total activity in control rats liver

Table 1. The total activity of lysosomal proteases in the liver of control rats and rats intoxicated with methanol

Mean values ± S.D.

Cathepsin	Methanol	Control	Intoxication time					
	g/kg b.wt.		6 h	12 h	24 h	2 d	5 d	7 d
Cathepsin A	1.5	8.58 ± 0.34[a]	8.55 ± 0.47[a]	8.21 ± 0.48[a]	8.04 ± 0.39[a]	7.45 ± 0.44[a,b]	8.03 ± 0.44[a]	8.21 ± 0.38[a]
Tyr, nmol/g tissue per 2 h	3.0		8.67 ± 0.44[a]	7.94 ± 0.39[a]	7.50 ± 0.49[a,b]	7.50 ± 0.46[a,b]	7.90 ± 0.42[b]	8.23 ± 0.39[a]
Cathepsin B	1.5	2.21 ± 0.14[a]	2.13 ± 0.16[a]	2.07 ± 0.16[a]	1.94 ± 0.17[a]	2.00 ± 0.15[a]	2.13 ± 0.15[a]	2.11 ± 0.13[a]
pNA, nmol/g tissue per 2h	3.0		2.19 ± 0.16[a]	2.01 ± 0.17[a]	1.88 ± 0.17[a,b]	1.78 ± 0.15[a,b]	2.02 ± 0.16[a]	2.07 ± 0.15[c]
Cathepsin C	1.5	1.41 ± 0.10[a]	1.36 ± 0.11[a]	1.35 ± 0.11[a]	1.18 ± 0.11[a]	1.22 ± 0.10[a]	1.32 ± 0.10[a]	1.36 ± 0.09[a]
pNA, nmol/g tissue per 2h	3.0		1.37 ± 0.11[a]	1.31 ± 0.12[a]	1.20 ± 0.10[a]	1.08 ± 0.10[a,b,c]	1.19 ± 0.09[a]	1.32 ± 0.09[d]
Cathepsin D	1.5	3.04 ± 0.17[a]	2.96 ± 0.17[a]	2.97 ± 0.18[a]	2.95 ± 0.19[a]	2.87 ± 0.19[a]	2.92 ± 0.18[a]	2.96 ± 0.17[a]
Tyr, nmol/g tissue per 2h	3.0		3.02 ± 0.19[a]	2.94 ± 0.18[a]	2.89 ± 0.19[a]	2.81 ± 0.19[a]	2.85 ± 0.19[a]	2.95 ± 0.18[a]

The same letters in raws indicate lack of significant differences at $P < 0.05$ in the Tukey multi-range test. Lack of differences in columns referring to each of cathepsins is marked in the same way.

amounted for cathepsin A, B, C and D 28, 16, 20 and 22% after 3.0 g methanol dose, respectively. The response to methanol dose was significantly differentiated in case of cathepsin A and D to a less extent in case of cathepsin C and almost no effect on the release of cathepsin B was noted.

Partial release of cathepsins on methanol administration indicates damage to lysosomes confirmed by ultrastructural microscopic examination of liver (unpublished). Administration of methanol to rats caused a distinct increase in the number of lysosomes after 12 h of intoxication, while after 24 h and

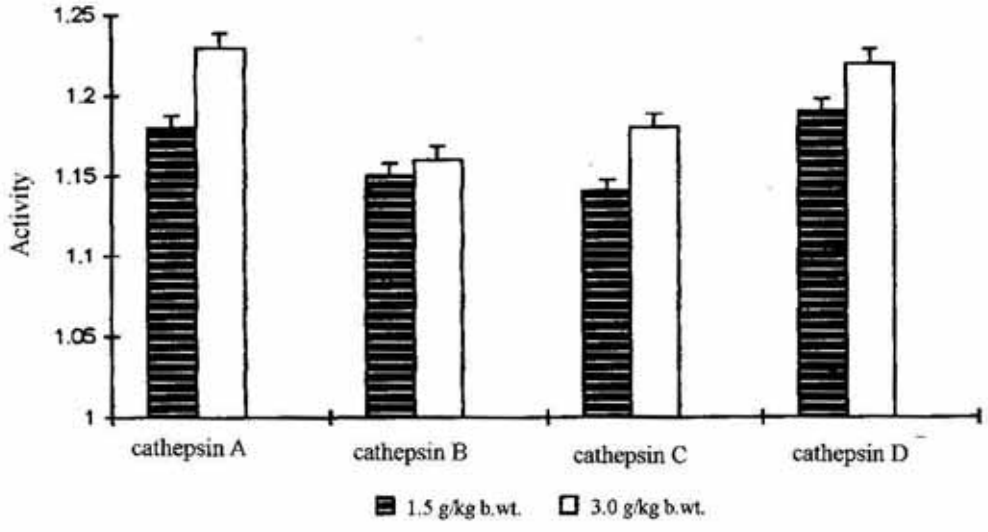

Figure 2. Effect of methanol dose on the activity of cathepsins in the post-lysosomal fraction of liver rats as referred to control rats (control value is equal 1).

48 h blurring of the limiting membrane structure was evident. At higher methanol dose these changes were intensified. This is due to peroxidation of membrane lipids by oxygen free radicals produced during methanol metabolism [7, 15]. Moreover the decrease in ATP content, revealed in methanol intoxication, is another lysosomal membrane destabilization factor. Both these factors lead to the damage to liver cell membranes and in consequence to the escape of cell constituents, including enzymes into the vessel lumen (unpublished). This might be an additional reason for the decrease of cathepsin activity in hepatocytes.

Moreover the cathepsins may be inactivated by formaldehyde and free radicals. Formaldehyde reacts with the residues of amino acids with free amino, sulphhydryl or hydroxyl groups [4, 16]. This and oxidative modifications may cause changes in the conformation of the enzymes molecules and especially in the structures of active center [17]. Cysteine residues present in the active centres of cathepsin B and C are susceptible to oxidative modification by free radicals. This may explain a stronger response of these cathepsins to methanol intoxication (Table 1, Fig. 2) as compared to cathepsin D, a carboxyl protease.

Not all of the observations made could be explained pointing only to possible differences in binding to lysosomal membranes, permeability and/or regulation of cathepsin activities.

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