Activity of Lysosomal Enzymes in the Bile and Serum of Mice with Intrahepatic Cholestasis

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Lysosomal enzyme activity in the bile and blood serum was compared in mice with experimental intrahepatic cholestasis induced by α-naphthyl isothiocyanate and Triton WR 1339. Triton WR 1339 increases the synthesis of cholesterol (fatty acid precursor) in liver cells. The development of intrahepatic cholestasis was confirmed by the increase in activities of alkaline phosphatase and γ-glutamyltransferase in blood serum. Administration of Triton WR 1339 in a dose of 100 mg/100 g was followed by a 10-fold increase in β-galactosidase activity (hepatocyte lysosomal enzyme) in the bile, but not in the serum of mice. β-Galactosidase activity significantly increased in the bile, but decreased in the serum of mice after treatment with α-naphthyl isothiocyanate in a dose of 200 mg/kg. Our results indicate that intrahepatic cholestasis is manifested in increased secretion of lysosomal glycosidases into the bile. Bile components can aggravate damage to liver cells by affecting the processes of hepatocyte apoptosis and necrosis.

Key Words: *lysosomes; bile; cholestasis; α-naphthyl isothiocyanate; Triton WR 1339*

Functional activity of lysosomes depends on the type of cells. For examples, lysosomes of thyroid gland cells play a role in thyroglobulin secretion. Peribiliary lysosomes of hepatocytes are involved in bile secretion [1,2,3]. Easily solubilized lysosomal glycosidases (β-galactosidase, β-glucuronidase, and N-acetyl-β-D-glucosaminidase) were revealed in the bile of rats. They are released from liver cells into the bile by means of exocytosis [4,8]. Molecular mechanisms of bile production and secretion in mammals were studied by proteomic methods. However, little is known about protein secretion into the bile [5]. It remains unclear which mechanisms mediate the production and secretion of lysosomal enzymes in the bile. The effect of bile components on liver cell damage is poorly understood.

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In the present work, lysosomal enzyme activity in the bile and blood serum was measured during experimental cholestasis. Intrahepatic cholestasis was induced by α-naphthyl isothiocyanate (ANIT) and lysosomotropic agent Triton WR 1339, which increases the synthesis of cholesterol (fatty acid precursor) in liver cells.

MATERIALS AND METHODS

Experiments were performed on male CBA mice weighing 25-30 g and obtained from the vivarium of the Institute of Pharmacology (Siberian Division of the Russian Academy of Medical Sciences, Tomsk). Corn oil solution of ANIT (Aldrich) was injected intraperitoneally in a single dose of 200 mg/kg (0.2 ml per mouse) [6,12,14]. The animals were euthanized 24 h after ANIT injection. Triton WR 1339 (Ruger Chemical Co.) was dissolved in physiological saline and injected intraperitoneally in a

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single dose of 100 mg/100 g [15]. The mice were euthanized 24 and 72 h after Triton injection (strong accumulation of the detergent in lysosomes). Oil solution of CCl_4 (50 mg/kg) was administered intragastrically. The animals were euthanized 24, 48, and 72 h after CCl_4 intoxication. Some animals received Triton 2 h before administration of CCl_4 in specified doses (combined treatment). The mice were deprived of food, but received water *ad libitum* over 15 h before euthanasia. Blood serum was obtained by centrifugation of samples on an Eppendorf 5415 R centrifuge at 3000*g* and 4°C for 20 min. The bile was taken from the gallbladder of euthanized animals using a microsyringe. The samples from 10 mice were combined to measure lysosomal enzyme activity in the bile.

Serum alanine transaminase (ALT) activity (sign of hepatocyte cytolysis) was measured colorimetrically using Lachema kits. Activities of alkaline phosphatase and γ-glutamyltransferase were measured using Vital Diagnostics kits. The increase in activities of alkaline phosphatase and γ-glutamyltransferase reflects the development of intrahepatic cholestasis in mice. Fluorescence study was used to measure the following enzyme activities: lysosomal β-D-galactosidase activity (high specific activity in hepatocytes) in the bile and serum (4-methylumbellipheryl-β-D-galactopyranoside as a substrate, Melford Laboratories Ltd.) [1]; β-hexosaminidase, or β-N-acetylglucosaminidase (4-methylumbellipheryl-2-acetamido-2-deoxy-β-D-glucopyranoside as a substrate, Melford Laboratories Ltd.) [1]; and chitotriosidase (4-methylumbellipheryl-β-D-N,N',N"-triacetylchitotrioside as a substrate, Sigma) [13]. Fluorescence of samples was studied on a Perkin Elmer 650-10S spectrofluorometer at the extinction and emission wavelengths of 360 and 445 nm, respectively. The results were expressed in μmol methylumbellipheryl (MUP) per liter over 1 min.

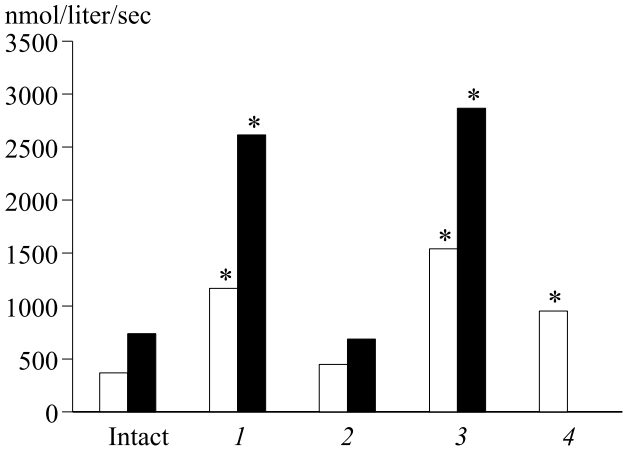

Fig. 1. Activities of alkaline phosphatase (light bars) and γ-glutamyltransferase (dark bars) in serum from CBA mice. Triton WR 1339, 72 h after treatment (*1*); CCl_4, 72 h after treatment (*2*); Triton + CCl_4, 72 h after treatment (*3*); and ANIT, 24 after treatment (*4*). *$p<0.05$ compared to intact animals.

The results were analyzed by the method of parallel series of variational statistics (Student's *t* test). The differences between the means were significant at $p<0.05$.

RESULTS

The development of intrahepatic cholestasis was accompanied by a significant increase in serum activities of alkaline phosphatase and γ-glutamyltransferase (Fig. 1). Gallbladder volume increased in mice with cholestasis (10 μl bile *vs.* 5 μl bile in intact animals after 15-h starvation).

β-Galactosidase activity was similar in the bile and serum of intact mice. Activities of β-hexosaminidase and chitotriosidase in the bile were much lower than in the serum (Table 1). Secretion of hepatocyte lysosomal β-galactosidase into the bile sharply increased (by 10 times) 72 h after overload

TABLE 1. Activity of Lysosomal Enzymes in the Bile and Serum of Mice (*M*±*m*)

Group	β-Galactosidase, μmol MUP/liter/min		Chitotriosidase, μmol MUP/liter/min		β-Hexosaminidase, μmol MUP/liter/min	
	serum	bile	serum	bile	serum	bile
Intact animals	12.30±1.33 (*n*=10)	12.30±1.63 (*n*=8)	20.50±2.79 (*n*=8)	3.40±0.28 (*n*=5)	7.97±0.95 (*n*=10)	0.54±0.07 (*n*=3)
Triton WR 1339, 72 h after treatment	6.07±0.60* (*n*=5)	117.3±1.4* (*n*=5)	8.90±0.81* (*n*=10)	11.65±0.40* (*n*=6)	4.00±0.47⁺ (*n*=5)	2.46±0.20* (*n*=5)
ANIT, 24 h after treatment	4.60±0.32* (*n*=27)	22.20±0.34⁺ (*n*=3)	35.00±2.79* (*n*=27)	17.50±3.56* (*n*=3)	1.52±0.05* (*n*=30)	1.57±0.10* (*n*=3)

Note. *n*, number of animals per group. Study of the bile was performed with combined sample from 10 mice. *$p<0.001$ and ⁺$p<0.05$ compared to intact animals.

of hepatocyte lysosomes with Triton. However, enzyme activity in the serum decreased in these animals (Table 1). Activities of chitotriosidase and β-hexosaminidase increased in bile samples from these animals (Table 1). A significant increase in activity of β-galactosidase (but not of chitotriosidase and β-hexosaminidase) in bile samples was also observed in the later period after Triton administration (48 h postinjection; 32.80±0.66 μmol MUP/liter/min, n=5, p<0.01 compared to intact animals). β-Galactosidase excretion into the bile sharply increased 48 h after combined treatment with Triton and CCl_4 (231.00±2.16 μmol MUP/liter/min, n=5). Hence, high activity of β-galactosidase is the earliest and most reliable criterion for the increased excretion into the bile (as compared to other lysosomal enzymes).

Activities of β-galactosidase, chitotriosidase, and β-hexosaminidase in bile samples increased, while β-hexosaminidase activity (not chitotriosidase activity) in the plasma decreased in animals with ANIT-induced cholestasis (compared to intact mice, Table 1). Glycosidase activity in the bile was high in both models of cholestasis (particularly after Triton injection).

The development of CCl_4-induced hepatitis (after 72 h) was accompanied by hepatocyte cytolysis and increase in plasma ALT activity (9.50±0.42 U/liter/h, n=10; *vs*. 3.50±0.29 U/liter/h in the control, n=10; p<0.01). Cholestasis was not found under these conditions (Fig. 1). The development of cholestasis after combined treatment with Triton and CCl_4 was probably associated with toxic activity of Triton (Fig. 1). Activities of β-galactosidase (116.80± 0.49 μmol MUP/liter/min, n=5) and chitotriosidase (8.40±0.55 μmol MUP/liter/min) in bile samples from treated animals were higher compared to intact mice (Table 1).

Little is known about the role of lysosomal enzymes in bile secretion in mice. Previous studies showed that 24-h "synchronous" exocytosis of lysosomal glycosidases (β-galactosidase, N-acetyl-β-D-glucosaminidase, and β-glucuronidase) into the bile occurs in intact rats [4]. As differentiated from rats, the bile is concentrated in the gallbladder of mice. Functions of lysosomal enzymes in the bile remain unknown. Glycosidases are probably involved in degradation of glucuronides. A sharp increase in β-galactosidase activity and minor changes in β-hexosaminidase and chitotriosidase in the bile of mice with various types of experimental cholestasis probably reflect the increased exocytosis of these enzymes into the bile. Transport disorders and concentration of lysosomal enzymes in the bile can potentiate the effect of lipids and fatty acids, which are excreted into the bile independently of lysosomal enzymes.

Little is known about cellular source of lysosomal enzymes in the serum and bile of mice. The majority of plasma proteins are synthesized in liver cells [4]. Hepatocytes and bile duct cells are probably involved in bile secretion [8]. Chitotriosidase of human serum is of macrophageal origin. This enzyme in mice is localized in cells of the gastrointestinal mucosa [13]. Human plasma contains a high-molecular-weight enzyme precursor (63 kDa) of β-hexosaminidases A and B. The presence of a 38-kDa mature enzyme in liver lysosomes of mammals is related to processing inside the particles [1]. The bile of mice probably contains mature β-hexosaminidase, which is secreted by exocytosis after processing in lysosomes.

The development of cholestasis in humans and experimental animals is associated with metabolic disorders of lipids and lipoproteins and accompanies liver damage of different etiology. Lysosomal enzymes can increase the severity of hepatocyte damage under the influence of toxic components from the bile (fatty acid salts) [5,9]. They have a modulatory effect on apoptosis in liver cells [6-8,10-11]. Administration of ANIT is rapidly followed (8-24 h after-treatment) by reversible damage to the epithelium of bile ducts and serves as an extensively used model of intrahepatic cholestasis in rats and mice. It should be emphasized that the same doses of ANIT induce more severe damage to the liver in mice [3]. ANIT increases the synthesis of cholesterol and impairs its conversion into fatty acids, particularly during the early period after treatment. These changes are followed by the accumulation of free cholesterol in the serum. The advantage of ANIT-induced cholestasis is simplicity and high reproducibility (with no surgical treatment). Single administration of ANIT to mice is followed by the development of intrahepatic cholestasis and abnormalities of the enzymes involved in cholesterol synthesis and degradation in liver cells (similarly to mice). It should be emphasized that the severity of enzyme dysfunction in mice is higher than in rats [3].

Triton causes lipemia and sharp increase in the synthesis of cholesterol in liver cells of various animals, including the rats, mice, rabbits, and dogs [15]. Accumulation of Triton in lysosomes of nonparenchymal and parenchymal cells of the liver occurs over long time (more than 60 days). The detergent is slowly excreted by exocytosis (probably in bile). We showed that Triton increases exocytosis of lysosomal enzymes in the bile (Table 1). These enzymes are probably excreted together with Triton.

Protein concentration in the bile of mammalian species is 3-5%. The concentration or selective excretion of individual proteins can occur in the gall-

bladder [4]. Differences in lysosomal enzymes of the bile in mice and rats are probably related to anatomical features of these animals. The gallbladder is present in mice, but not in rats.

Cholestasis and increased synthesis and exocytosis of lysosomal enzymes can increase the severity of liver damage by affecting the apoptosis in liver cells. Several medicinal preparations (*e.g.*, chlorpromazine and estradiol) cause cholestasis. Some of these drugs increase the synthesis of cholesterol in liver cells. Prevention of cholestasis should include the evaluation and limited use of drugs that increase cholesterol synthesis in liver cells. Our results open new perspectives in studying the relationship between liver disorder and atherosclerosis. Special attention should be given to the use of hypolipidemic drugs (statins), which cause pathological changes in liver cells (undesirable effect).

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