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## Effects of statins on liver fibrosis reversibility and activities of lysosomal exoglycosidases

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
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### Summary

#### Background:

Liver fibrosis is a spontaneously reversible process occurring predominantly after withdrawal of fibrogenic agents. We tested statins as promising antifibrotic agents using an experimental model of thioacetamide (TAA)-induced liver fibrosis reversal. The statins investigated were simvastatin (SS) and fluvastatin (FS). The aim of our study was to evaluate exoglycosidase activities as markers of fibrosis severity in the liver on a rat model of hepatic fibrosis reversal after treatment with statins.

#### Material/Methods:

Hepatic fibrosis (HF) was induced in 50 laboratory rats by TAA administration (200 mg/kg, i.p., 2 times per week) during 3 months. SS (5 and 10 mg /kg b.w.) and FS (10 mg /kg b.w.) were administered i.g. during 2 months after TAA withdrawal.

Severity of liver fibrosis was determined by computer image analysis of liver slides stained according to Azan-Mallory. Activities of  $\beta$ -galactosidase and  $\alpha$ -mannosidase in rat liver supernatant were measured by Zwierz method.

#### Results:

TAA administration resulted in a strongly pronounced liver fibrosis which was partially reversed during 2 months after TAA withdrawal. Activities of both exoglycosidases significantly increased in TAA-treated rats and decreased during fibrosis resolution. Treatment of rats with the highest SS dose (10 mg/kg) decreased fibrosis rate and activity of  $\beta$ -galactosidase, whereas lower doses of SS (5 mg/kg) and FS did not affect the measured parameters.

#### Conclusions:

Activities of liver lysosomal exoglycosidases can serve as markers of liver fibrosis severity and activity of  $\beta$ -galactosidase - as a predictor of liver fibrosis regression.

#### Key words:

**liver fibrosis • statins •  $\beta$ -galactosidase •  $\alpha$ -mannosidase**

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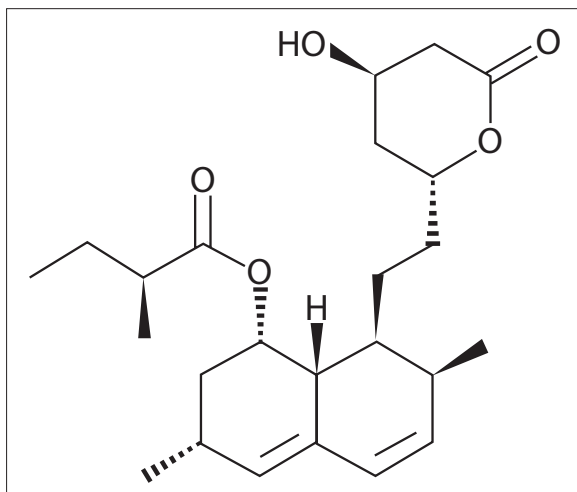
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## BACKGROUND

Hepatic fibrogenesis, the common result of liver injury, is believed to be a critical factor that leads to hepatic dysfunction and may be important in portal hypertension. The liver fibrogenic response is a complex process in which accumulation of extracellular matrix proteins, tissue contraction, and alteration in blood flow are prominent. A critical event in fibrogenesis is activation of resident perisinusoidal cells named "hepatic stellate cells". Stellate cell activation is characterized by enhanced extracellular matrix synthesis and prominent contractility [1].

Statins (Fig. 1), which are HMG-CoA reductase inhibitors, belong to a class of drugs that lowers the level of cholesterol in the blood. Statins probably have also antifibrotic function. Statins block hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), which takes part in liver synthesis of cholesterol. Recent research shows that statins reduce inflammation, what has a beneficial effect on atherosclerosis. Reduction of inflammation by statins is independent of statins ability to reduce cholesterol. Furthermore, anti-inflammatory effects of statins can be seen as early as two weeks after starting statin treatment [2, 3]. Statins exhibit action that goes beyond lipid-lowering activity in the prevention of atherosclerosis. There are four proposed mechanisms for prevention of cardiovascular diseases by statins (all subjects of a large body of biomedical research): 1. Improving endothelial function, 2. Modulating inflammatory responses, 3. Maintaining plaque stability, 4. Preventing thrombus formation. During statin treatment, liver enzyme derangements may also occur, typically in about 0.5% of cases. However, liver enzyme derangements are also seen at similar rates with placebo use and repeated enzyme testing. Generally, liver enzymes return to normal levels either without discontinuance of statins over time, or after briefly discontinuing the drug [4]. Lovastatin has been shown to induce apoptosis in normal and fibrotic lung fibroblasts *in vitro*, as well as to reduce tissue granulation. Reduction in tissue granulation was associated with ultrastructural evidence for fibroblast apoptosis *in vivo* in a guinea pig wound model [5]. Some data on potential antifibrotic effects of statins has been described [6, 7].



**Figure 1.** Lovastatin, the first statin to be marketed.

The lysosomal exoglycosidases take part in catabolism of glycoconjugates. The glycoconjugates: glycoproteins, glycolipids and proteoglycans, are the main components of the intra- and extracellular membranes as well as extracellular matrix.  $\beta$ -galactosidase is one of the lysosomal exoglycosidases which catalyzes removal of galactose residues from the non-reducing end of oligosaccharide chains of glycoconjugates and  $\alpha$ -mannosidase which catalyzes removal of  $\alpha$ -mannose [8,9]. Lysosomal exoglycosidases were used as the markers of tissue remodeling e.g in inflammatory processes [10-12].

The result of cells damages in hepatic fibrosis are disturbances of metabolic pathways which may change the activity of exoglycosidases. The aim of our study was to evaluate the effects of statins on  $\beta$ -galactosidase and  $\alpha$ -mannosidase activity in the liver on a rat model of hepatic fibrosis reversal.

## MATERIAL AND METHODS

All substrates for determination of exoglycosidase activities and Triton X-100 were obtained from Sigma, St. Louis, USA. Other chemicals were supplied by Polskie Odczynniki Chemiczne, Gliwice, Poland.

Hepatic fibrosis (HF) was induced by the thioacetamide (TAA). Male Wistar rats, 180-200 g, were divided into six groups of ten animals each:

- 1 – control – rats were kept on a standard rat chow with free access to tap water,
- 2 – (HF) – TAA (200 mg/kg b.w., i.p.), 2 times a week for 3 months to induce hepatic fibrosis,
- 3 – (HF+R) – 2 months after the TAA withdrawal + placebo (saline) i.g.
- 4 – (HF+10FS) – (HF) + fluvastatine sodium (FS), 10 mg/kg b.w. for 2 months after the TAA withdrawal, i.g.
- 5 – (HF+5SS) – (HF) + simvastatin (SS) 5 mg /kg b.w. for 2 months after the TAA withdrawal, i.g.
- 6 – (HF+10SS) – (HF) + simvastatin (SS) 10 mg /kg b.w. for 2 months after the TAA withdrawal, i.g.

The rats were sacrificed under pentobarbital anesthesia (40 mg/kg, i.p.). The investigation was approved by the Ethical Committee, Institute of Biochemistry, National Academy of Sciences, Grodno, Belarus.

Sampling for histopathology was performed as follows: liver specimens of the right lobe of all rat liver were fixed in 10% formalin and embedded in paraffin. Sections 5- $\mu$ m thick were processed routinely for Azan-Mallory staining and slides were used for morphometric analysis to determine the percentage of liver tissue affected by fibrosis using a computer-assisted automated image analyzer (BIOSCAN, Minsk, Belarus). Results from 15 random fields per slide (fibrosis index) were calculated as a ratio of Azan-Mallory positive area to the total area examined and expressed as a relative square of connective tissue (%).

The liver tissues for exoglycosidases measurements were weighed and homogenized (90 sec) in 3 volumes of ice-cold 0.15 M KCl with 0.2% Triton X-100. The homogenates were centrifuged at 12,000 x g for 30 minutes at 4°C, and the supernatants were used for determination of exoglycosidases activity.

Activity of  $\beta$ -galactosidase and  $\alpha$ -mannosidase were determined by Zwierz et al. method [13]. Absorbances were measured in duplicate and the appropriate standard solutions were analyzed in parallel. Protein was determined by the biuret method [14], with lyophilized bovine serum albumin as a standard.

The results were analyzed by Statistica 7.0 (StatSoft, Cracov, Poland) according to ANOVA test (test NIR) and by nonparametric analysis using Prizm II software. The statistical significance of differences was regarded to be  $p < 0.05$ .

## RESULTS

TAA administration resulted in a significant accumulation of collagen fibers in pericentral areas leading to septal micronodular fibrosis with structural failure of the parenchyma. The fibrosis rate evaluated in these animals by morphometry of slides, stained accordingly to Azan-Mallory, was dramatically higher as compared to the control group (Table 1).

In rats treated with saline for 4 weeks after TAA withdrawal, these findings were markedly lower, but abnormalities of the parenchymal structure remained. The severity of fibrosis evaluated by computer image analysis was significantly lower in this group than in animals treated with TAA for 12 weeks.

The treatment with both FS and 5SS did not modify the fibrotic appearance of the liver. However, parenchymal structure of the liver in these groups was significantly improved: no signs of hepatocyte architecture distortion or inflammatory signs were noted. Fibrotic rate in rats treated with both FS and 5SS was similar to that observed in the TAA + placebo group.

The most pronounced effects on fibrosis resolution in the group treated with 10SS were fibrosis rates significantly lower in comparison to the TAA + placebo group and no observable parenchymal changes.

Rats treated with TAA for 3 months showed an increased liver hydroxyproline content compared with healthy control

**Table 1.** Effects of statins on fibrosis rate (square of the Azan-Mallory stained area, % to total slide square) in the liver with fibrosis regression.

Groups	Fibrosis rate
Control	1.73 ± 0.22
TAA	12.8 ± 1.27*
TAA + placebo	8.3 ± 1.01*
Fluvastatin, 10 mg/kg	9.7 ± 0.81*
Simvastatin, 5 mg/kg	10.8 ± 1.94
Simvastatin, 10 mg/kg	5.5 ± 0.66*•

\*-  $p < 0.05$  to the control group; •-  $p < 0.05$  to the TAA + placebo group

rats (Table 1). This parameter was not changed in all groups after 2 months after TAA withdrawal, independently of statins treatment.

The mean activity values (pKat/kg protein) of  $\beta$ -galactosidase and  $\alpha$ -mannosidase in the liver are presented in Table 2. We observed an increase in the activity of  $\beta$ -galactosidase in rat liver in the HF group as compared to the control group ( $p = 0.008833$ ) and a decrease in the activity of  $\beta$ -galactosidase in the HF + S10 in comparison to the HF group ( $p = 0.000096$ ) and in the HF + SS5 ( $p = 0.015285$ ). We observed also an increase in the activity of  $\alpha$ -mannosidase in rat liver in the HF and HF + R groups in comparison with the control group ( $p = 0.0028$  and  $0.0000$ ) and a decrease in the HF + 10 FS and HF + 5 SS ones in comparison with HF + R ( $p = 0.0068$  and  $0.0460$ ).

## DISCUSSION

Spontaneous resolution of liver fibrosis was demonstrated in several rodent models after elimination of the etiological agent. There are many cogent arguments of human fibrosis regression when injurious stimuli are withdrawn. Resorption of the fibrotic tissue in rats with biliary cirrhosis induced by bile duct ligation was showed after bilioduodenal anastomosis [15]. Reversibility of  $\text{CCl}_4$ -induced liver fibrosis after the recovery period is generally recognized [16]. In rats receiving for 3 months drinking water containing 0.03% TAA, reversal of liver micronodular fibrosis was observed 2 months after TAA withdrawal [17]. A significant decrease of fibrosis rate after 60 days of TAA abolition was found in rats treated intraperitoneally with TAA for 24 weeks [18]. However, other authors found morphological signs of fibrosis regression in

**Table 2.** Activity (nKat/kg of protein) and standard deviation of exoglycosidases in liver of rats with hepatic fibrosis.

	C	HF	HF + R	HF + 10 FS	HF + 5 SS	HF + 10 SS
GAL						
mean	238.06	273.68	246.22	240.94	249.69	222.27
standard deviation	14.659	16.406	23.091	30.331	25.029	35.272
MAN						
mean	52.363	62.525	69.246	58.359	60.479	64.693
standard deviation	1.752	4.834	4.224	7.028	7.637	9.054

the same experimental model 2 weeks after TAA withdrawal, but without significant improvement in hepatic collagen content [19]. In our study, TAA withdrawal leads to partial reversal of liver fibrosis in 2 months with a significant decrease of fibrosis rate.

Our results suggest that liver fibrosis severity, evaluated by morphometry, is in agreement with activities of exoglycosidases, mainly  $\beta$ -galactosidase, however, the activity of both enzymes significantly increased in rats administered with TAA. In animals with fibrosis reversibility, the activity of  $\beta$ -galactosidase normalized whereas  $\alpha$ -mannosidase activity did not change in this group. These findings confirm the literature data on activation of lysosomal enzymes in experimental animals with liver fibrosis. It was found that  $\beta$ -galactosidase activity in liver parenchymal cells from mice with CCl<sub>4</sub>-induced liver fibrosis was significantly higher than in those from untreated animals [20]. Similar changes of exoglycosidases were demonstrated also in the serum [21] and the liver [22] of rats with CCl<sub>4</sub>-induced liver fibrosis.

In non-parenchymal liver cells of rats with liver fibrosis induced by TAA, the catalytic activities of  $\beta$ -N-acetyl-D-glucosaminidase,  $\beta$ -glucuronidase,  $\alpha$ -L-iduronidase and cathepsin D increased significantly during chronic liver damage [23].

Among the investigated compounds, only the highest dose of simvastatine (10 mg/kg) significantly decreased fibrosis rate in the liver. The activity of  $\beta$ -galactosidase also significantly decreased only in animals of the HF+10SS group, as compared with the group with fibrosis reversal.

Prospective antifibrotic properties of statins are described in current literature. It was reported that lovastatin and simvastatin reduced proliferation of hepatic stellate cells and their collagen steady-state levels [6]. Additionally, statins exert antioxidant [24] and immunomodulatory [25] effects. Simvastatin did not prevent liver fibrosis in rats with bile duct ligation [26] but improved distinct fibrosis markers [7]. In our study, simvastatin develops moderate antifibrotic action that characterizes this compound as a promising antifibrotic agent with known extent of clinical safety, whereas we were not able to document the effect of fluvastatin on liver fibrosis.

## CONCLUSIONS

Activities of liver lysosomal exoglycosidases, mainly  $\beta$ -galactosidase, which corresponded to liver fibrosis severity, can serve as a marker of liver fibrosis severity, and a predictor of liver fibrosis regression.

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