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Deteriorating effect of fluvastatin on the cholestatic liver injury induced by bile duct ligation in rats

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Abstract. Antiinflammatory effect of statins mediated by the reduction of cytokine IL-6 in hepatocytes have been reported. Contrary to beneficial effect, statins can increase susceptibility to mitochondrial dysfunction. Extrahepatic biliary obstruction is associated with oxidative stress, pro-inflammatory response and hepatocyte mitochondrial dysfunction. The aim of our study was to verify the effect of fluvastatin on cholestatic liver injury.

Cholestasis was induced in Wistar rats by bile duct ligation. Fluvastatin (1 or 5 mg/kg) was administered after surgery and then daily for 7 days. The dose of 5 mg/kg led to the deterioration of hepatocellular injury. Despite lower production of IL-6, decrease in GSH content, rise of TGFß and inhibition of respiratory complex I in mitochondria were determined. The mRNA expressions of canalicular transporter Mdr1b and basolateral transporter Mrp3 increased in cholestatic liver. Fluvastatin administration then led to the attenuation of this change. Analogously, mRNA expression of conjugative enzyme Ugt1a1 was diminished by fluvastatin administration to cholestatic rats.

We can conclude that decrease in the antioxidative status and mitochondrial dysfunction could at least in part participate on the deteriorating effect of fluvastatin. Whether these processes can be a consequence of the alteration in metabolism and transport of potentially toxic substances remains to verify.

Key words: Statin — Cholestasis — Bile duct ligation

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BDL, bile duct ligation; CYP450, cytochrome P450; GGT, gamma-glutamyl transferase; GSSG, oxidized glutatione; GSH, reduced glutatione; IFNγ, interferon gamma; IL-1, interleukin 1; IL-6, interleukin 6; LAP, laparotomy; Mdr, multidrug resistence protein; Mrp, multidrug resistence-associated protein; TGFß, transforming growth factor ß; TNFα, tumor necrosis factor α; Ugt1a1, uridine diphosphate-glucuronosyltransferase 1a1.

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Introduction

Extrahepatic biliary obstruction induces variety of changes leading to liver injury. There was documented that liver injury induced experimentally by cholestasis is associated with oxidative stress, the depletion of glutathione content and decrease in the antioxidative capacity, respectively. Oxidative stress seems to be rather a consequence than a cause of liver injury (Muriel and Suarez 1994) and does not play such an important role in the cholestatic liver injury (Barón and Muriel 1999). Neutrophil infiltration in the liver, considered to be a potential source of liver injury during acute biliary obstruction (Koeppel et al. 1997), was observed within the first three hours after the experimental bile duct ligation (BDL) (Saito and Maher 2000). Interruption of enterohepatic circulation of bile acids leads to the increase in the gut permeability and supports portal endotoxemia. Obstructive cholestasis induced by BDL sensitizes the animals to endotoxin, both intense pro-inflammatory response with increase in the production of cytokines and organ injury develop (Kennedy et al. 1999; Fujiwara et al. 2001; Lázár et al. 2002). Literature data support that hepatocyte mitochondrial dysfunction and the reduction of ATP synthesis are also involved in the development of obstructive jaundice (Rolo et al. 2002; Younes et al. 2007). Cholestasis leeds to the accumulation of potentially toxic substances such as bilirubin. Adaptive anticholestatic mechanisms are developed in hepatocytes to limit a loss of biliary excretory function and liver impairment caused by these compounds (Lee et al. 2001; Bohan et al. 2003; Paumgartner 2006). Adaptive responses comprise changes in the expression of proteins important for the transport of potentially toxic substances (Zollner and Trauner 2006).

Some studies have tested the treatment attenuating the development of cholestatic liver injury by antioxidative substances vitamin C, E and N-acetylcysteine. Unfortunately, their results are not ambiguous. While the treatment lasting for 28 days caused the improvement of the antioxidative liver capacity, markers of liver injury and cholestasis were not attenuated (Soylu et al. 2006; Tahan et al. 2007). The results of experiments with anti-inflammatory and immunosuppressive agents have brought more convincing results. After 7 and 14 days of their treatment, the improvement of markers of liver injury, cholestasis, antioxidative liver capacity and histological finding were reported (Eken et al. 2006; Karaman et al. 2006). The problem of clinical usage of these agents could result from their unwanted adverse effects. That is why the agents potentially affecting the activity of Kupffer cells, neutrophils and thus the production of pro-inflammatory cytokines could be more promising (Demirbilek et al. 2006; Yilmaz et al. 2007). Statins are widely used for lowering hypercholesterolemia, but increase in the evidence of their anti-inflammatory effects could extend the spectrum of their therapeutic usage. The sporadic studies have documented statins to reduce the development of cholestatic liver injury (Demirbilek et al. 2007; Dold et al. 2009; Awad and Kamel 2010). Contrary to these findings statins have a potential for adverse effects that could be mediated by induction of mitochondrial dysfunction (Velho et al. 2006; Nadanaciva et al. 2007; Hattori et al. 2009; Lee et al. 2010). Due to those controversial effects of statins, the aim of this study was to shed light on the modulatory effects of statins on the development of cholestatic liver injury.

Materials and Methods

Animal experiments

Male albino Wistar rats (BioTest, Konárovice, Czech Republic) were housed at 23 ± 1°C, 55 ± 10% relative humidity, air exchange 12–14 times/h, and 12-hour light/dark cycle periods (6:00 a.m. to 6:00 p.m.). The animals had free access to standard laboratory rat chow (DOS 2B, Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set by the institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

Extrahepatic cholestasis was induced by method of common BDL. Fluvastatin (Lescol, Novartis) was administered immediately after surgery and then daily by intragastric tube. Animals were sacrificed 7 days after surgery. Fluvastatin at the dose of 1 and 5 mg/kg was applied. Animals without fluvastatin treatment received equal amount of aqua *per injection*. Blood was collected from abdominal aorta and the liver was removed for biochemical and histological examination.

Biochemical analysis

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total and conjugated bilirubin and albumin were determined using automatic biochemical analyser Modular (Roche, Basel, Switzerland).

Determination of glutathione

Liver homogenates were used for the analysis of reduced (GSH) and oxidized (GSSH) glutathione; glutathione was analyzed by modified fluorometric method (Hissin and Hilf 1976; Kandár et al. 2007). Glutathione was allowed to react with *o*-phthalaldehyde in phosphate buffer (RT, 15 min, pH 8), and the fluorimetric detection was carried out by excitation and emission wavelengths of 350 nm and 420 nm, respectively. The final concentrations of glutathione were obtained after comparison of the signal in a sample to the calibration curve.

Determination of tissue cytokines

Liver samples were homogenized in RIPA buffer and centrifuged (10 000 × *g*). Concentration of liver IL-6 and TGFß were measured by ELISA kits (Bender MedSystems, Austria) according to the manufacturer´s instructions.

Measurement of oxygen uptake by isolated mitochondria

Rat liver mitochondria were prepared from liver homogenates by conventional differential centrifugation (Hogeboom and Schneider 1951; Bustamante et al. 1977). Oxygen consumption by isolated mitochondria was measured using High Resolution Oxygraph 2K (Oroboros, Austria). For evaluation of oxygen uptake Oroboros software (DatLab 4.2) was used.

Liver histology

Liver samples were taken immediately after the rats were sacrificed and fixed in 10% formaldehyde. Paraffin sections were stained with haematoxylin-eosin to detect cholestasis, ductular proliferation, inflammation, necrosis and fibrosis. The evaluation was performed in 10 randomly selected fields *per* slide from all animals in each group.

Real time RT-PCR analysis

Expressions of Mdr1b, Mrp3 and Ugt1a1 mRNA were examined using qRT-PCR as described previously (Brcáková et al. 2009) on 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). Total RNA was isolated from liver tissue samples using Qiagen RNeasy Mini Kit (Bio-Consult Laboratories, Czech Republic) and converted into cDNA *via* High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). Reaction mixture contained 30 ng of analyzed cDNA. The amplification of each sample was performed in triplicate using TaqMan® Fast Universal PCR Master Mix and pre-designed TaqMan® Gene Expression Assay for Mdr1b (Abcb1b, Rn00561753_m1), Mrp3 (Abcc3, Rn00589786_m1) and Ugt1a1 (Rn00754947_m1) all provided by Applied Biosystems. The time-temperature profile used in the "fast" mode was: 95°C for 3 min; 40 cycles: 95°C for 7 s, 60°C for 25 s. For normalization, two reference genes were selected – GAPDH (4352338E, Applied Biosystems, Foster City, USA) and Ywhaz (GENERI BIOTECH s.r.o., Hradec Králové, Czech Republic) – using the geNorm according to Vandesompele et al. (2002). Expression values of each sample were obtained as described previously (Radilova et al. 2009). Briefly, the expression data were normalized by the geometric mean of GAPDH and Ywhaz expressions. Finally, the relative expression between control and affected tissue was determined by comparison of normalized data.

Statistical analysis

All values are expressed as means ± SD. One-way ANOVA test was used to determine the statistical significance. Tukey-Kramer´s *post hoc* test was used for multiple comparisons between groups (GraphPad Instant 3.06 for Windows, GraphPad Software, CA, USA).

Results

Fluvastatin at dose of 1 mg/kg did not alter the development of cholestatic liver injury, only the effects of 5 mg/kg were noticed.

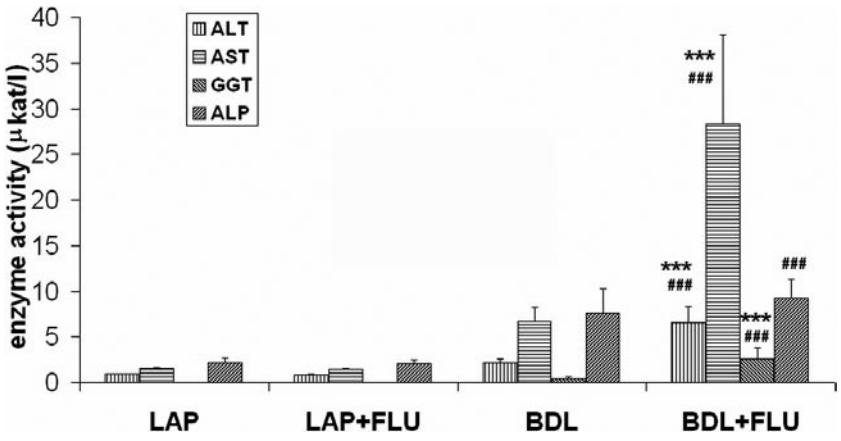

Figure 1. ALT, AST, GGT and ALP activity in the serum of sham-operated rats (LAP), sham-operated rats with administration of 5 mg/kg fluvastatin (LAP+FLU), bile duct ligated rats (BDL) and bile duct ligated rats with administration of fluvastatin (BDL+FLU). ### $p < 0.001$ compared to LAP+FLU, *** $p < 0.001$ compared to BDL.

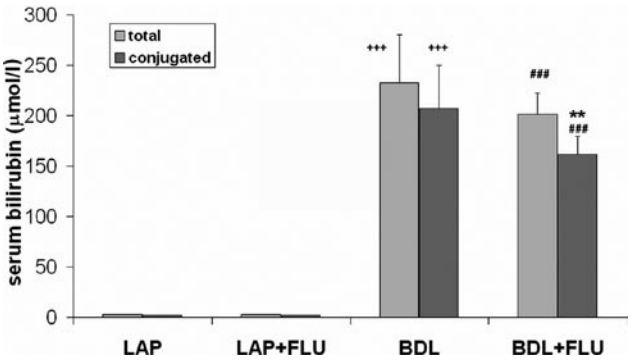

Figure 2. Concentration of total and conjugated bilirubin in the serum. $^{+++}$ $p < 0.001$ compared to LAP, $^{###}$ $p < 0.001$ compared to LAP+FLU, ** $p < 0.01$ compared to BDL (other symbols see legend to Fig. 1).

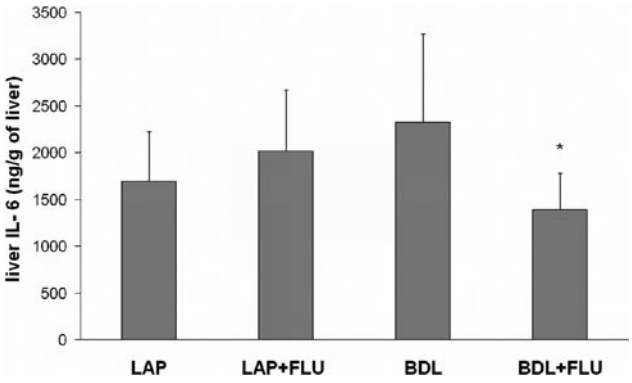

Figure 4. IL-6 production in the liver. * $p < 0.05$ compared to BDL (other symbols see legend to Fig. 1).

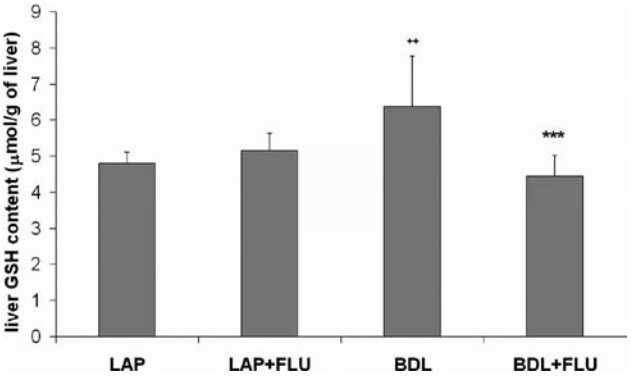

Figure 3. Glutathione content in the liver. $^{++}$ $p < 0.01$ compared to LAP, *** $p < 0.001$ compared to BDL (other symbols see legend to Fig. 1).

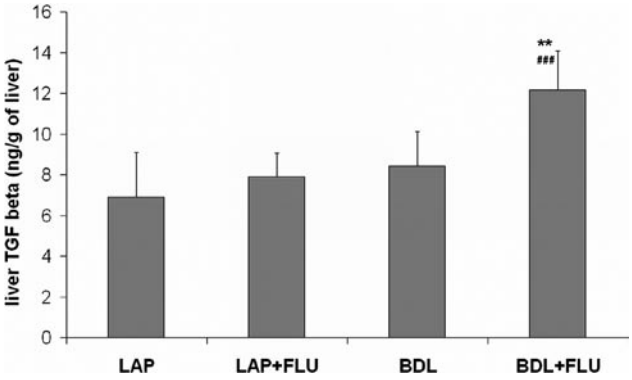

Figure 5. TGFß production in the liver. $^{###}$ $p < 0.001$ compared to LAP+FLU, ** $p < 0.01$ compared to BDL (other symbols see legend to Fig. 1).

Serum ALT, AST, GGT and ALP activities

ALT, AST, GGT and ALP (Fig. 1) activities in the serum of rats undergoing BDL raised, but nonsignificantly, as compared to sham-operated group. In BDL rats, treatment with fluvastatin led to the increase in ALT, AST, GGT ($p < 0.001$) and ALP ($p < 0.01$) contrary to sham-operated rats treated with fluvastatin. Administration of fluvastatin in BDL rats caused further increase in the activities of ALT, AST and GGT compared to BDL group without treatment ($p < 0.001$).

Concentration of total and conjugated bilirubin

BDL was accompanied with increase in both total and conjugated bilirurubin (Fig. 2) as compared to control animals ($p < 0.001$). Administration of fluvastatin to BDL rats led to nonsignificant decrease in the total and significant decrease in the conjugated bilirubin compared to untreated BDL rats ($p < 0.01$). Nevertheless, the concentration of bilirubin was still higher than in controls treated with fluvastatin ($p < 0.001$).

Liver glutathione content

Figure 3 documents that GSH content significantly increased in BDL rats compared to sham-operated untreated controls ($p < 0.01$). In BDL rats treated with fluvastatin GSH content decreased as compared to nontreated BDL group ($p < 0.001$). Analogous changes were determined in GSH/GSSG ratio (data not shown).

Production of IL-6 in the liver

BDL did not cause any significant change in the production of IL-6 in the liver (Fig. 4). Treatment with fluvastatin in BDL

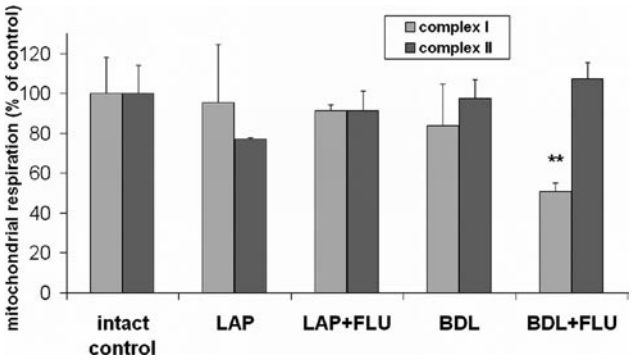

Figure 6. Activity of respiratory complex I (gray) and II (black) in mitochondria. ** $p < 0.01$ compared to intact control (other symbols see legend to Fig. 1).

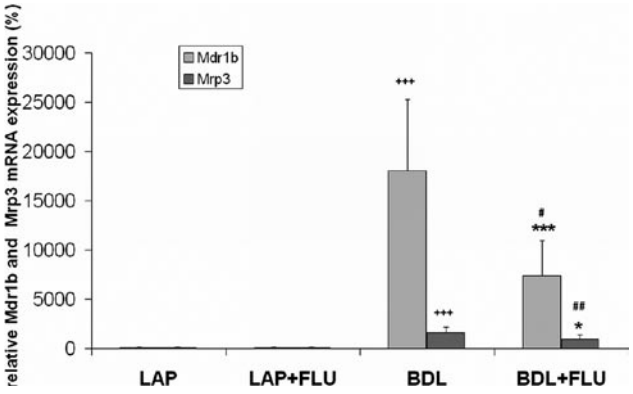

Figure 7. mRNA expressions of Mdr1b and Mrp3 in the liver. $^{+++}$ $p < 0.001$ compared to LAP, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared to LAP+FLU, *** $p < 0.001$ compared to BDL (other symbols see legend to Fig. 1).

rats led to the significant decrease in the concentration of IL-6 in the liver as compared to BDL group ($p < 0.05$).

Production of transforming growth factor ß in the liver

Concentration of TGFß (Fig. 5) in the liver of BDL rats was not changed. Fluvastatin administered to BDL rats led to the increase in TGFß production that was significantly higher than in both sham-operated controls treated with fluvastatin and BDL rats ($p < 0.001$, $p < 0.01$).

Activities of mitochondrial respiratory complex I and II

Cholestasis induced by BDL did not significantly change the respiratory activity of both complex I and II in the liver mitochondria (Fig. 6). In BDL rats treated with fluvastatin the activity of complex I was significantly decreased as compared to intact control ($p < 0.01$).

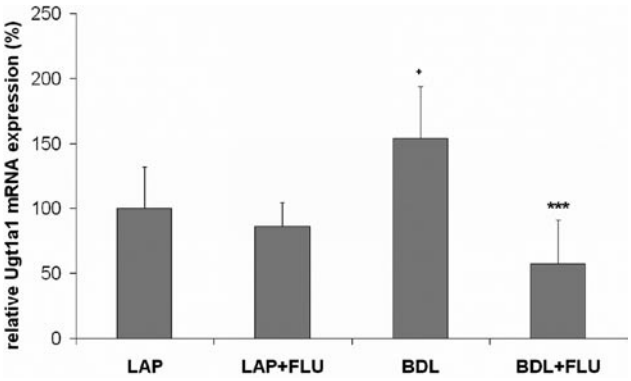

Figure 8. mRNA expression of Ugt1a1 in the liver. $^{+}$ $p < 0.05$ compared to LAP, *** $p < 0.001$ compared to BDL (other symbols see legend to Fig. 1).

Mdr1b mRNA expression

The mRNA levels of Mdr1b in the liver are shown in Fig. 7. Mdr1b expression increased in BDL animals compared to controls ($p < 0.001$). In BDL rats treated with fluvastatin the expression decreased compared to BDL rats ($p < 0.001$), nevertheless the expression remained higher as compared to sham rats treated with fluvastatin ($p < 0.05$). Fluvastatin did not alter the mRNA expression of Mdr1b in control treated with fluvastatin.

Mrp3 mRNA expression

The mRNA levels of Mrp3 in the liver are shown in Fig. 7. BDL led to increase in Mrp3 expression ($p < 0.001$). Decrease in the expression was evaluated in BDL rats treated with fluvastatin as compared with BDL group ($p < 0.05$). This level was increased ($p < 0.01$) compared to sham operated animals treated with fluvastatin. Fluvastatin did not alter the mRNA expression of Mrp3 in control treated with fluvastatin.

Ugt1a1 mRNA

The mRNA levels of Ugt1a1 in the liver are shown in Fig. 8. The mRNA expression of Ugt1a1 significantly increased after BDL compared to sham rats ($p < 0.05$) Administration of fluvastatin to rats after BDL led to the decrease of Ugt1a1 mRNA expression compared to BDL group without fluvastatin treatment ($p < 0.001$). Fluvastatin did not alter the mRNA expression of Ugt1a1 in control treated with fluvastatin.

Histopathological finding

BDL was accompanied with cholestasis, ductular proliferation, inflammation and necrotic changes contrary to

controls. No significant results were found after fluvastatin administration in bile ligated rats, only sporadic progression of fibrosis was determined.

Discussion

In our study, the rats were subjected to BDL for 7 days. Changes of the enzyme activities ALT, AST, GGT, ALP and levels of total and conjugated bilirubin in the serum give evidence for the presence of cholestasis and advancement of hepatocellular damage in the liver. Fluvastatin at dose of 1 and 5 mg/kg was administered daily to retard the development of cholestatic liver injury. Contrary to the expected, while the treatment using 1 mg/kg of fluvastatin did not lead to any change (data not shown), the dose of 5 mg/kg further deteriorated the markers of liver injury.

Cholestasis is accompanied with intense pro-inflammatory response mediated by cytokine production. An abrupt increase in pro-inflammatory cytokines in serum of BDL rats was paralleled with enhanced activation of the transcription factor NF-kB (Liu et al. 2001).

In patients with chronic liver diseases, elevated levels of inflammatory mediators including TNFα, IL-6, IL-1 and IFNγ are found in the serum (Tilg et al. 1992). Gene expression of pro-inflammatory cytokines has also been described in the liver. Unfortunately, the correlation between the increase of pro-inflammatory cytokines in plasma and the extent of liver damage is not known and needs to be further investigated (Lacour et al. 2005). There are controversial reports documenting changes in plasma and hepatic cytokine levels induced experimentally by BDL. Twenty-eight days after BDL in rats, plasma and liver TNFα was markedly increased. Simultaneously plasma IL-6 was significantly increased while hepatic IL-6 was not changed (Fernandéz-Martinéz et al. 2006). Plebani et al. (1999) did not observe increase in hepatic levels of both TNFα and IL-6 throughout the 28 day BDL interval in rats. The finding of direct anti-inflammatory effect of statins mediated by the modulation of IL-6 activity in hepatocyte culture (Arnaud et al. 2005) led us to evaluate the production of IL-6 in the liver. Our measurement documents stable IL-6 level in the liver after BDL. Administration of fluvastatin then attenuated the production of hepatic IL-6. However the production of IL-6 was suppressed after fluvastatin treatment, liver injury induced by BDL was deteriorated. Cytokine TGFß is one of the main pro-fibrogenic factors used to analyze the fibrogenic response (Czaja et al. 1989). In our study, BDL for 7 days did not alter the production of TGFß in the liver but increase in the production was observed in the liver of BDL rats treated with fluvastatin. In agreement with this finding, sporadic progression of fibrosis in cholestatic liver was found after fluvastatin administration; however, our histological analysis documents nonsignificant changes.

Cholestatic liver injury is characterized by accumulation of hydrophobic bile acids in hepatocytes where they can exert their cytotoxic effect associated with oxidative stress. Antioxidant status of the liver in our study was determined by the measurement of GSH and GSSG content in liver homogenates. Literature data regarding changes in GSH level in the liver of animals with experimentally induced cholestasis are contradictory (Lu 2009; Yang et al. 2009). Our result documents increase in the GSH content. This is in agreement with Purucker et al. (1998) that have described a dynamic change in hepatic glutathione system – an increase in reduced GSH up to 23 days after BDL and then continuous decline of GSH. The changes could be explained by a transient increase in GSH synthetic enzymes early after BDL followed by a fall to about 50% of baseline levels (Yang et al. 2009). In our study fluvastatin treatment in BDL rats was associated with the depletion of glutathione in the liver. GSH content decreases in experimental fibrosis and in human fibrotic diseases (Vendemiale et al. 2001). Unfortunately, mechanism and biological significance of GSH depletion in the development of fibrosis remain unclear (Liu and Gaston Pravia 2010). As mentioned above, pro-fibrogenic TGFß rised in our study after fluvastatin administration in cholestatic rats. Experimental results have documented the role of TGFß in GSH depletion that is suggested by the inhibition of GSH synthesis (Tiitto et al. 2004).

Bile acids accumulated in the liver as a result of cholestasis seem to induce hepatocyte injury *via* a mitochondrial-dependent mechanism (Krähenbühl et al. 1995; Gores et al. 1998; Rolo et al. 2001, 2002). Mitochondrial permeability transition (characterized by permeabilization of the inner mitochondrial membrane to compounds with molecular weight to 1.5 kDa), mitochondrial swelling and a collapse of mitochondrial membrane potential have been described. Bile acids depressed respiration in isolated mitochondria and inhibited the activity of respiratory complexes II, III and IV. Statins, like other drugs, have a potential for adverse effects. There is a rising evidence that mitochondrial dysfunction can lead to cell injury and cell death thus contributing to the deleterious effects of statins (Velho et al. 2006; Nadanaciva et al. 2007; Hattori et al. 2009; Lee et al. 2010). Statins have been demonstrated to induce Ca^{2+} -dependent increase in the permeability of mitochondrial inner membrane (Velho et al. 2006). We have tested the effect of fluvastatin on mitochondria in the cholestatic liver with regard to the activity of the first two entering respiratory complexes I and II, respectively. Fluvastatin treatment was associated with a selective inhibition of the respiratory complex I while the activity of complex II was not influenced. Our finding corresponds with data documenting that statins can inhibit respiratory complexes (Nadanaciva et al. 2007). In the study of Nadanaciva and co-workers fluvastatin incubated with isolated mitochondria caused inhibition of complex V and activity

of the complex I was not changed. Fluvastatin administered to control rats in our experiment did not cause impairment of the complex I activity that was measured in isolated mitochondria. Nevertheless, fluvastatin in BDL rats caused the attenuation of the activity of this complex. We speculate that inhibition of the mitochondrial respiratory complex I could at least in part participate on the deleterious effect of fluvastatin under the present experimental conditions.

We have mentioned that cholestasis induces an adaptive response comprised of changes in the expression of proteins important for the transport of potentially toxic substance (Zollner and Trauner 2006). P-glycoprotein Mdr1 localized in the canalicular membrane of hepatocytes plays a major role in the biliary excretion of many unrelated hydrophobic compounds and metabolites. While the expression of Mdr1b, an inducible form of Mdr1 increased in BDL group, lower expression accompanied fluvastatin administration to BDL rats. McRae et al. (2003) has recently shown that exposure of IL-6 decreases Mdr1 levels. Unfortunately, whether low IL-6 production in rats treated with fluvastatin after BDL may result in the decrease of Mdr1 expression is not clear. Upregulation of basolateral efflux protein Mrp3 which also contribute to the protection of the liver from the accumulation of the potentially toxic biliary compounds during cholestasis has been determined in hepatocytes (Ogawa et al. 2000). Our result in nontreated cholestatic rats corresponds with this finding. Treatment with fluvastatin then led to a slight decrease in the mRNA expression of Mrp3 transporter. Prior to transport of basolateral efflux proteins from hepatocytes, many xenobiotics are metabolized by cytochrome P450 and thereafter conjugated to become more hydrophilic and better excreted (Paumgartner 2006). Conjugative enzyme uridine 5′-diphosphate-glucuronosyltransferase (Ugt) catalyzes the glucuronidation reaction important for excretion of bilirubin and drugs from hepatocyte into the bile. In accordance with literature data we observed increase in the Ugt1a1 expression after BDL. Fluvastatin administration to cholestatic rats was associated with decrease in the expression of Ugt1a1. Deterioration of conjugative function in the liver may explain the reduction of conjugated bilirubin in the serum of BDL rats treated with fluvastatin. We suppose that fluvastatin administered under cholestatic conditions is likely to alter the metabolism and elimination of endogenous and exogenous compounds and may lead to their cytotoxicity. All the same, we have to consider that the dose aggrevating the cholestatic liver injury in our study exceeds the doses recommended in clinical practice. The lower dose in the present study is equivalent to maximum doses used clinically and did not induce any adverse effect.

Interestingly, our finding of the deteriorating effect of fluvastatin on the cholestatic liver injury is not in accordance with results obtained by some authors. Protective effects of fluvastatin (Demirbilek et al. 2007), simvastatin (Dold et al. 2009) and rosuvastatin (Awad and Kamel 2010) have been described. The effect of simvastatin was studied in mice ligated for 12 hours, rosuvastatin and fluvastatin were administered to Sprague-Dawley rats starting three days after BDL. Thus these experiments differ in animal species and timing.

We can conclude that fluvastatin administration to cholestatic rats can induce a deleterious effect in the liver depending on the dose. Although the production of pro-inflammatory cytokine IL-6 was suppressed by fluvastatin, other evaluated markers of liver injury were further deteriorated. Decrease in antioxidative capacity of the liver and mitochondria dysfunction can contribute to this aggravating effect of fluvastatin. Whether these processes can be a consequence of the alteration in metabolism and transport of potentially toxic substances shoud be verified.

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