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Boldine Attenuates Cholestasis Associated With Nonalcoholic Fatty Liver Disease in Hereditary Hypertriglyceridemic Rats Fed by High-Sucrose Diet

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Summary

The aim of the current study was to clarify the effect of high sucrose diet (HSD) on bile formation (BF) in rats with hereditary hypertriglyceridemia (HHTg). Potentially positive effects were studied for boldine, a natural choleretic agent. Administration of HSD to HHTg rats led to increased triglyceride deposition in the liver. HSD reduced BF as a consequence of decreased biliary secretion of bile acids (BA) and glutathione. Responsible mechanism was down-regulation of hepatic transporters for BA and glutathione, Bsep and Mrp2, respectively. Moreover, gene expressions of transporters for other constituents of bile, namely Abcg5/8 for cholesterol, Abcb4 for phospholipids, and Oatp1a4 for xenobiotics, were also reduced by HSD. Boldine partially attenuated cholestatic effect of HSD by promotion of biliary secretion of BA through up-regulation of Bsep and Ntcp, and by increase in biliary secretion of glutathione as a consequence of its increased hepatic disposition. This study demonstrates mechanisms of impaired BF during nonalcoholic fatty liver disease induced by HSD. Altered function of responsible transporters suggests also potential for changes in kinetics of drugs, which may complicate pharmacotherapy in subjects with high intake of sucrose, and with fatty liver disease. Sucrose induced alterations in BF may be alleviated by administration of boldine.

Key words

Nonalcoholic fatty liver disease • High-sucrose diet • Boldine • Bile flow • Bsep

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Introduction

Nonalcoholic fatty liver disease (NAFLD) has become serious clinical problem affecting 30-40 % of population in some developed countries (Ali and Cusi 2009). NAFLD is closely associated with features of the metabolic syndrome such as obesity, dyslipidemia and insulin resistance (Dowman *et al.* 2010). The course of disease consist of initial stage, simple steatosis, which may last unrecognized for prolonged period, and increases vulnerability of the liver tissue to various toxic insults (Kucera *et al.* 2014). The situation may progress to more serious form of NAFLD, nonalcoholic steatohepatitis (NASH) with ongoing liver inflammation and fibrosis.

One of the mechanisms, which may contribute to increased sensitivity of liver tissue during NAFLD is the accumulation of endo-, and xenobiotics resulting from their impaired secretion into bile (Schrieber *et al.* 2008, Canet *et al.* 2015, Ferslew *et al.* 2015). The effect is ascribed to alterations in transporting proteins in the liver. Commonly described are especially upregulations of

MRP efflux transporters at basolateral membrane of hepatocytes (Hardwick *et al.* 2011, Ferslew *et al.* 2015), downregulations of basolateral uptake transporters and variable changes of apical efflux transporters, especially Multidrug resistance-associated protein 2 (MRP2), the rate limiting for bile acid independent bile flow based on secretion of glutathione (Geier *et al.* 2005, Fisher *et al.* 2009, Canet *et al.* 2014). Clear statement about character of changes in individual transporters and definition of their clinical impact is however precluded by limited availability of human samples, by the variability between individuals, and by discrepancies between animal models used to study NAFLD (Canet *et al.* 2014). This status suggests that changes in transporting proteins may be determined by individual predisposition and by cause of NAFLD including composition of diet.

Recently, it has been stated that NAFLD with associated obesity is tightly related with increased dietary sugars income (Saab *et al.* 2015). The impact of high-sugar diet-induced NAFLD on the liver transporting proteins has not been tested yet. Several works documented that non-obese strain of hereditary hypertriglyceridemic rats (HHTg), which were selected from Wistar rats (Vrana and Kazdova 1990), may serve as suitable model of human hypertriglyceridemia (Klimes *et al.* 1995), and are very sensitive to administration of sucrose. High-sucrose diet (HSD) in this strain induces typical hallmarks of metabolic syndrome including mild weight gain, hypertension, insulin resistance with hyperinsulinemia, signs of oxidative stress (Vrana *et al.* 1993), and also increases liver weight and steatosis (Skottova *et al.* 2004). Data about liver histological status, bile formation and involved transporting processes and their modulation by HSD in this strain of rats are not available so far.

Many promising approaches exist to NAFLD therapy. One of them is stimulation of Bile salts export pump (Bsep), the rate limiting transporter for bile acid dependent bile flow (Halilbasic *et al.* 2013). The principle comes from knowledge that mice with low levels of Bsep due to absence of its main transcriptional regulator, Farnesoid X receptor (FXR), develop spontaneously hepatic steatosis, and hypertriglyceridemia with insulin resistance (Thomas *et al.* 2008, Wu *et al.* 2015). On the contrary, mice overexpressing Bsep have increased biliary lipid excretion and are protected from steatosis when fed an atherogenic diet or methionine-choline-deficient diet (Figge *et al.* 2004, Sundaram *et al.* 2005). Similar positive effect on hepatic steatosis was consequently achieved by administration of FXR receptor agonists in mice and humans (Zhang *et al.* 2009, Sanyal 2015). We have recently reported that boldine, the major alkaloid from the Chilean Boldo tree, is also agonist of FXR and produces sustained mild bile acid (BA)-dependent choleresis by upregulation of Bsep (Cermanova *et al.* 2015). Moreover, it is also effective as an antioxidant and possess significant hepatoprotective potential in various models of toxic liver injury (Lanhers *et al.* 1991, Fernandez *et al.* 2009), but its effect on NAFLD has not been tested yet. Therefore, the aim of the present work was to characterize changes in mechanisms of bile production and biliary drug excretion during NAFLD induced by high-sucrose diet in hereditary hypertriglyceridemic rats. In addition, potential for positive modulation of these changes was studied for boldine.

Methods

Animals and experimental design

Two types of rats were used throughout the study: female Wistar rats (220-270 g, n=7, Velaz, Konarovice, CR) and female hereditary hypertriglyceridemic (HHTg) rats (195-300 g, n=6-7, IKEM, CR). The animals were housed under controlled environmental conditions (12-hour light-dark cycle; temperature, 22±1 °C) with a food and water freely available, and received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by U.S. National Institutes of health (NIH publication, 1996). The study protocol was approved by the animal welfare committee of the Charles University in Prague, Faculty of Medicine in Hradec Kralove.

HHTg rats were fed for 6 weeks with either STD (standard diet; H-S rats) or HSD (high-sucrose diet containing 50 % of sucrose; H-H rats). One group of HHTg rats received also HSD containing 0.2 % of boldine (H-H-B rats). Wistar rats fed with STD served as controls (W-S rats). The diet was isocaloric and contained equal amounts of proteins (19.6 cal%), fat (10.4 cal%), carbohydrate (70 cal%) as starch (STD) or sucrose (HSD). Bile collection study was performed in all experimental groups after overnight fasting. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), fixed in a supine position on a heated platform to maintain body temperature at 37 °C, and carotid artery (for blood sampling), jugular vein (saline administration), and bile duct (for bile collection) were cannulated. All animals received continuous intravenous infusion of saline at 6 ml/h/kg to replace fluid losses by sampling.

Bile was collected in preweighted tubes at 30-min intervals over 90 min. At the end of the experiment, rats were sacrificed by exsanguination from carotid artery, and samples of serum, bile and livers were snap frozen in liquid nitrogen and stored at –80 °C until analysis.

Serum biochemistry and bile acids and glutathione measurement

The concentrations of glucose, bilirubin, cholesterol, HDL, TAG in serum and activities of ALT and AST in serum were measured by routine laboratory methods on Cobas Integra®800 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Bile acids (BA) in serum and bile were assayed using a commercial kit (Diazyme). Liver triglyceride concentrations were determined by commercial kits Triglycerides 250 S (Erba-Lachema s.r.o.) as described previously (Hirsova *et al.* 2012). Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were analyzed separately using validated HPLC method with fluorescence detection (Hirsova *et al.* 2013).

Quantitative real-time RT-PCR

Gene expression was examined as previously described (Cermanova *et al.* 2014). All chemicals including TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits were identical with those used in our former work (Kolouchova *et al.* 2011), and all were purchased from Life Technologies. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reference for normalizing the data (Life Technologies).

Western blot

Crude membranes were prepared from rat liver homogenates, and were separated by SDS-PAGE electrophoresis (15 µg of protein), transferred to a PVDF membrane (Millipore) and incubated with appropriate antibodies as previously described (Hirsova *et al.* 2013). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were from GE Healthcare. The immunoreactive bands on the autoradiography films were scanned with calibrated densitometer ScanMaker i900 (UMAX, Prague, CZ) and quantified using the QuantityOne imaging software (Bio-Rad Laboratories, Hercules, CA). Expressions of proteins were normalized to β-actin levels.

Histology

Livers were collected immediately after death, fixed in 10 % neutral buffered formalin, embedded in paraffin, and 10 % cut to 4-5 µm thick sections. These were stained with hematoxylin-eosin and evaluated with BX-51 light microscope (Olympus) at x100 of original magnification. The liver architecture and the presence of lipid accumulation, and cellular inflammatory infiltration were assessed by the same specialist.

Table 1. Effect of HSD and boldine-enriched HSD on selected morphometric and serum liver biochemical parameters of HHTg rats. Wistar rats fed with STD served as controls.

	W-S	H-S	H-H	H-H-B
Glucose (mmol/l)	8.1 ± 1.2	9.3 ± 1.3	15 ± 5.0**††	14 ± 2.6**††
Bilirubin (µmol/l)	0.8 ± 0.5	1.5 ± 0.9	2.0 ± 0.8**	1.4 ± 0.5
ALT (µkat/l)	0.7 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1
AST (µkat/l)	1.6 ± 0.7	1.8 ± 0.5	1.6 ± 0.3	1.9 ± 0.5
Cholesterol (mmol/l)	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.1 ± 0.1
HDL cholesterol (mmol/l)	0.9 ± 0.2	0.6 ± 0.2*	0.8 ± 0.1	0.9 ± 0.1††
TAG (mmol/l)	0.3 ± 0.1	2.0 ± 1.6*	2.7 ± 1.1**	1.5 ± 1.0*
BA (µmol/l)	3.6 ± 1.4	6.0 ± 1.7*	5.5 ± 2.5*	2.7 ± 0.7††‡
Liver weight (g)	7.7 ± 1.1	7.4 ± 1.0	10 ± 1.1***†††	9.8 ± 0.7***†††
Body weight (g)	250 ± 16.3	225 ± 14.0**	284 ± 17.2***†††	281 ± 15.7***†††
Triglycerides (µmol/g liver)	1.6 ± 0.3	2.6 ± 0.8	4.9 ± 2.0***††	4.3 ± 1.6**†

Data are presented as means ± SD from groups of 6-7 animals. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine. Significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05).

Statistical analysis

Data are expressed as Mean ± SD. Comparison on multiple groups was done by one-way ANOVA followed by the Newman-Keuls *post-hoc* test. Differences were considered significant at $P<0.05$ value. All analyses were performed using GraphPad Prism 6.0 software (San Diego, USA).

Results

Biochemical analysis of serum showed increased concentrations of triglycerides in all HHTg rats, compared to W-S group of rats (Table 1). Administration of HSD to HHTg rats increased serum concentrations of glucose without influence on any other evaluated biochemical parameter. HHTg animals on STD as well as on HSD presented with increased level of bile acids (BA) in serum. Addition of boldine to diet reduced bile acid serum concentrations toward levels seen in control Wistar animals on STD diet. Boldine did not change serum glucose or triglyceride concentrations.

All rats fed with HSD with or without boldine had significantly greater body and liver weights (Table 1). Histological evaluation showed normal architecture in Wistar and HHTg rats on STD (Fig. 1A/B). Addition of sucrose to diet induced significant accumulation of lipids in hepatocytes localized in pericentral region of liver lobule which presented as typical enlargement of hepatocytes and macrovesicular clarifications in the cytoplasm (Fig. 1C/D). This was in agreement with increased concentration of triglycerides in liver tissue of rats on HSD diet (Table 1). There were no signs of increased inflammatory cells infiltration induced by HSD. Boldine in diet had no influence on HSD-induced changes.

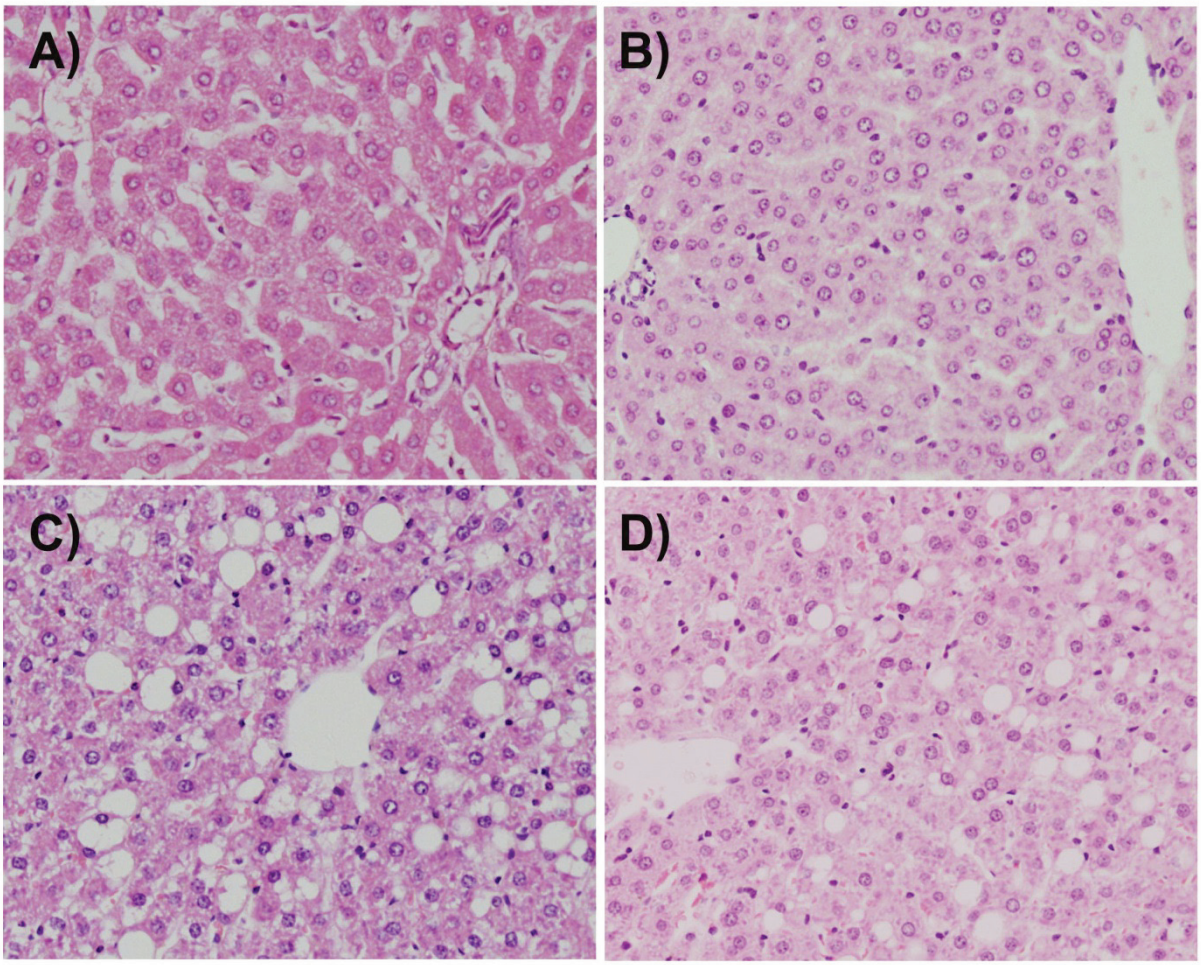

Fig. 1. High-sucrose diet-induced steatosis in HHTg rats. Hematoxylin and eosin-stained formalin-fixed paraffin-embedded liver sections from HHTg rats fed with HSD diet (**C**) for 6 weeks developed characteristic steatotic features with enlargement of pericentral hepatocytes, macrovesicular lipid deposits, but the absence of inflammation. Boldine did not modulate these changes (**D**). Wistar rats (**A**) and HHTg rats (**B**) fed with the control diet had healthy livers with no evidence of NAFLD. Original magnification, x100.

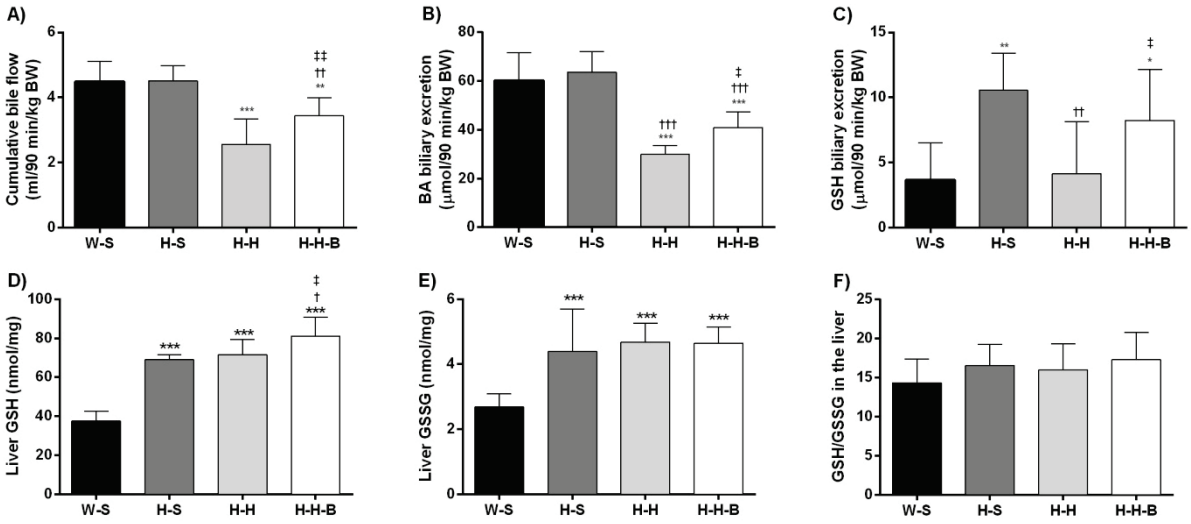

Fig. 2. Effect of HSD and boldine added to HSD on parameters associated with bile production in HHTg rats. Wistar rats fed with STD served as controls. Cumulative bile flow (**A**), biliary secretion of bile acids (**B**), and glutathione biliary excretion (**C**) was evaluated over 90 min. Related plasma concentrations of reduced (GSH – **D**), and oxidized (GSSG – **E**) glutathione (in nmol per mg of liver protein), and their ratio (**F**) in the liver were measured at 90th minute. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine. Data are presented as means ± SD from groups of 6-7 animals; significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05, ‡‡ P<0.01).

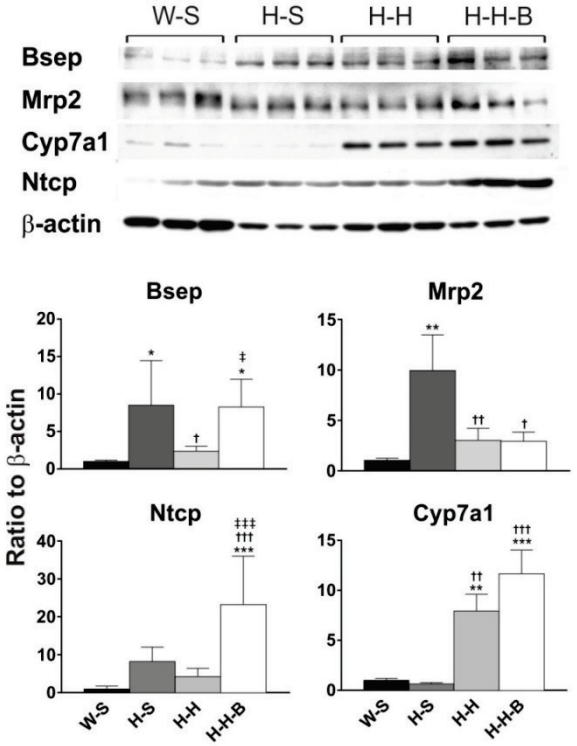

Fig. 3. Effect of HSD and boldine enriched HSD on the protein expression of Bsep, Mrp2, Ntcp, and Cyp7a1 in the liver of HHTg rats. Wistar rats fed with STD served as controls. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine. Data are presented as means ± SD from groups of 6-7 animals; significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05, ‡‡‡ P<0.001).

HHTg rats on STD have cumulative bile flow identical with standard Wistar rats (Fig. 2). Only difference was increased biliary excretion of glutathione related to its increased concentrations in liver tissue of all HHTg rats. Administration of HSD led to significant reduction of cumulative bile flow by 43 % as a result of decreased biliary secretion of bile salts, and glutathione (Fig. 2A/B/C). Boldine significantly raised biliary secretion of both bile constituents, which resulted into increased net cumulative bile flow. Boldine also increased liver concentrations of glutathione in reduced form (Fig. 2D).

Analysis of liver gene expression of major transporters responsible for bile production showed constitutive upregulation of Oatp2 and Mrp3 mRNA and downregulation of Mrp2 in HHTg rats on STD in comparison with Wistar rats (Table 2). HSD produced downregulation of Oatp2, Ntcp, Abcg5/8, Mdr2, and Mrp3 transporters, and upregulation of Cyp7a1 mRNA. Addition of boldine to HSD increased mRNA of transporters for bile acids, Bsep, and Ntcp. In order to detect inflammatory reaction, we measured also mRNA expression of key mediators (Table 2). Interestingly, NAFLD induced by HSD diet led to paradoxical reduction of Mcp-1 and TNF-α, and boldine had no effect on these molecules.

Hepatic expression of crucial molecules for bile formation was evaluated also at protein level (Fig. 3).

Interestingly, HHTg on STD presented with increased expression of both rate limiting transporters for biliary excretion of bile acids and glutathione, Bsep and Mrp2, respectively. HSD diet downregulated both, Bsep and Mrp2, and induced protein content of Cyp7a1, the rate limiting enzyme for synthesis of bile acids from cholesterol. Addition of boldine to HSD caused upregulation of Bsep and Ntcp protein, but did not change Cyp7a1 or Mrp2 expression.

Table 2. Effect of HSD and boldine enriched HSD on liver mRNA expression of the main molecules involved in bile formation and regulation of inflammatory reaction in HHTg rats. Wistar rats fed with STD served as controls. W-S, control Wistar rats fed with STD; H-S, HHTg rats fed with STD; H-H, HHTg rats fed with HSD; H-H-B, HHTg rats fed with HSD enriched by 0.2 % boldine.

Target gene	W-S	H-S	H-H	H-H-B
Abcb11 (Bsep)	100 ± 39	72 ± 22	48 ± 17**	93 ± 19 ‡‡
Abcc2 (Mrp2)	100 ± 19	64 ± 18***	51 ± 10***	47 ± 8***
Abcg5	100 ± 37	160 ± 105	23 ± 13*†††	56 ± 39††
Abcg8	100 ± 59	96 ± 67	20 ± 14*††	33 ± 36*††
Abcb1a (Mdr1a)	100 ± 36	156 ± 76	104 ± 36	106 ± 48
Abcb1b (Mdr1b)	100 ± 44	354 ± 367	171 ± 190	63 ± 35
Abcb4 (Mdr2)	100 ± 25	114 ± 51	57 ± 29*†	70 ± 16†
Abcg2 (Bcrp)	100 ± 70	355 ± 105***	149 ± 74†††	169 ± 61†††
Slc47a2 (Mate2)	100 ± 49	159 ± 137	132 ± 121	137 ± 115
Slc10a1 (Ntcp)	100 ± 20	98 ± 18	72 ± 20*†	94 ± 21 ‡
Slc22a1 (Oct1)	100 ± 34	118 ± 46	91 ± 22	91 ± 31
Slco1a4 (Oatp2)	100 ± 36	205 ± 104**	101 ± 41††	99 ± 38†
Slc22a7 (Oat2)	100 ± 31	99 ± 29	73 ± 13	67 ± 18
Abcc3 (Mrp3)	100 ± 63	286 ± 185**	89 ± 29††	109 ± 38††
Abcc4 (Mrp4)	100 ± 40	83 ± 35	51 ± 21*	53 ± 15*
Cyp7a1	100 ± 35	43 ± 30	202 ± 73**†††	174 ± 60*††
TGF-β1	100 ± 27	142 ± 68	88 ± 32*	75 ± 22*
Acta2	100 ± 25	153 ± 77	204 ± 101*	144 ± 41
IL-6	100 ± 101	71 ± 60	32 ± 38	17 ± 10
Ccl2 (Mcp-1)	100 ± 55	98 ± 86	30 ± 40*†	21 ± 12*†
TNF-α	100 ± 64	98 ± 64	19 ± 27*†	18 ± 10*††

Data are presented as means ± SD from groups of 6-7 animals; significant difference from W-S animals (* P<0.05, ** P<0.01, *** P<0.001); significant difference from H-S animals († P<0.05, †† P<0.01, ††† P<0.001); significant difference from H-H animals (‡ P<0.05, ‡‡ P<0.01).

Discussion

Bile formation is a unique function of the liver which is vital to survival of the organism. Among other functions, bile is major excretory route for potentially toxic exogenous lipophilic substances including drugs, as well as for endogenous compounds such as bile salts and bilirubin (Boyer 2013). Any impairment of bile formation may therefore led to retention of such substances in the liver, where they can inflict damage, activate inflammation, fibrosis, and eventually carcinogenesis which all aggravate the underlying pathology (Cuperus *et al.* 2014). Especially bile acids are known for direct toxic effect on hepatocytes and initiation of inflammatory response in the liver, if they are retained, what can be typically seen in different type of cholestasis. Increased serum concentrations of bile acids have been recently indeed demonstrated in patients with NASH (Ferslew *et al.* 2015), which suggest that mechanism of bile formation may contribute to pathophysiology of NAFLD and that cholestasis might promote disease progression (Sorrentino *et al.* 2005). For obvious ethical reason the

bile production cannot be measured in humans. Thus available data on influence of NAFLD on bile production are scarce and are taken from different animal models.

Initial results come from obese Zucker rats and demonstrate that even simple liver steatosis without inflammation may reduce bile production as a result of decreased biliary secretion of bile acids (BA) and glutathione, the main osmotic constituents serving as driving force for bile formation. However, systemic serum concentrations of BA or bilirubin might be not affected (Pizarro *et al.* 2004, Geier *et al.* 2005). These changes were ascribed to reduced protein expression of Oatp2 (Oatp1a4), an uptake transporter for numerous endo and xenobiotic including BA, and Mrp2, the transporter for organic anions such as glutathione, bilirubin, and conjugated BA, because other transporters for BA, like Bsep or Ntcp were not affected by this model of NAFLD. Similar conclusion was presented by Kong *et al*. (2012) who showed reduced bile flow in female C57BL/6 mice fed with high-fat diet as a consequence of transcriptional downregulation of Mrp2. In contrast, other experiments with simple liver steatosis induced by high-fat diet administered in rats yielded either unchanged (Fisher *et al.* 2009) or even increased bile production (Lickteig *et al.* 2007) but results were presented without information about biliary secretion of BA or glutathione. Parallel status in protein expression of responsible transporters was absence of change in efflux Mrp2/3/4, Pgp, and Bcrp transporters, and downregulation of basolateral uptake transporters Oatp1a1/4, or Oatp1b2 (Lickteig *et al.* 2007, Fisher *et al.* 2009, Canet *et al.* 2014).

The consequence of NASH, an advanced form of NAFLD, for bile production has been characterized only in one work which showed no alteration (Lickteig *et al.* 2007). All other data focus mainly the changes in the expression of individual transporters in the liver. Commonly reported is upregulation of Mrp2/3/4 and downregulation of uptake Oatp1a1, Oatp1a4, Oatp1b2 or Ntcp at protein level (Lickteig *et al.* 2007, Cheng *et al.* 2008, Fisher *et al.* 2009, More and Slitt 2011, Canet *et al.* 2014). These results are in agreement with available human data, where the protein content of efflux MRP2/3/4/5 is induced only in NASH but not in simple steatosis. However, MRP2 function is probably hampered because of its internalization from apical membranes of hepatocytes (Hardwick *et al.* 2011). The conditions for altered bile formation are therefore met also in humans. The knowledge about BA dependent bile flow, and about protein expression of its rate limiting BSEP transporter is still missing despite described increase in BA concentrations in serum of patients with NASH (Ferslew *et al.* 2015).

Our data are highly compatible with concept of reduced bile formation in NAFLD. The experimental model based on steatosis induced by high-sucrose diet in sensitive HHTg rats was used for the first time to study relationship between NAFLD and bile formation, despite association between sugar intake, obesity and NAFLD is well known. HSD diet in HHTg rats reproduced the situation of transition between simple steatosis and NASH. We observed centrilobular and macrovesicular steatosis typical for NASH (Takahashi and Fukusato 2014) but without marks of NASH such as cellular infiltration or activation of inflammatory mediators. Compared to available data about bile production during NAFLD, we have detected more complex changes based on significant reduction of bile production as a consequence of posttranscriptional downregulation of crucial efflux proteins for biliary secretion of BA and glutathione, Bsep and Mrp2, respectively. In line with previous findings (Geier *et al.* 2005), Oatp2 uptake transporter for BA and other compounds including drugs, was also transcriptionally reduced. In addition, HSD also markedly transcriptionally increased protein expression of Cyp7a1, the rate limiting enzyme for BA synthesis. Because the main regulator of these proteins is FXR, which upon stimulation suppress expression of Cyp7a1, and induces Bsep, the changes in our study suggest that FXR activity is reduced by HSD. In agreement, recently has been described that expression of CYP7A1 is increased in obese NAFLD patients as a consequence of inhibitory effect of free fatty acids on FXR signaling (Bechmann *et al.* 2013). However, despite such complex influence on liver BA homeostasis, HSD did not further increase serum concentrations of BA, because HHTg rats on STD already presented elevated serum levels of these solutes. This effect may be related to increased expression of Mrp3, the sinusoidal efflux transporter. Absence of change in biliary excretion of BA despite upregulation of Bsep in HHTg rats on standard diet support assumption that BAs are excreted back to blood. On the other hand, reduced biliary secretion of BA in HSD animals together with their increased synthesis suggest that BA may accumulate within the steatotic liver and increase vulnerability of the tissue despite no further increase of their concentration in serum. Simultaneously, neither serum liver biochemical tests nor the expression

of proinflammatory cytokines has been changed in HHTg or HSD-HHTg rats. This effect could be ascribed to markedly higher concentration of glutathione in the liver of HHTg rats. The situation deserves further research.

Addition of boldine to HSD fed HHTg rats partly restored impaired bile production by increasing biliary secretion of both BA and glutathione. The effect on BA may be explained by moderate agonistic activity of the boldine at FXR receptor with consequent induction of Bsep (Cermanova *et al.* 2015), which in our study led to important reduction of BA levels in serum. Recently described triglyceride-lowering effect of liver FXR receptor showed in knockout mice (Schmitt *et al.* 2015) was however not achieved in our study perhaps due to low bioavailability of the compound (unpublished observation). These data comply with absence of changes in triglyceride serum concentrations after administration of similar dose of boldine in streptozotocin-treated diabetic rats (Lau *et al.* 2013). On the other hand, boldine increased liver concentrations of glutathione in reduced form. Such effect may originate from its strong antioxidant capability with proven hepatoprotective potential in various models of toxic liver injury (Lanhers *et al.* 1991, Fernandez *et al.* 2009). Because Mrp2 transporter was not changed by boldine, the stimulation of biliary excretion of glutathione may be related to its increased hepatic disposition. The mechanism of Ntcp induction by boldine has not been described yet, and requires further elucidation.

In conclusion, this study presents another model of NAFLD based on administration of high-sucrose diet to hypertriglyceridemic rats. The diet led to significant cholestasis resulting from decreased biliary secretion of bile acids and glutathione. Molecular background of these changes was downregulation of Bsep and Mrp2, and induction of Cyp7a1. The data may significantly contribute to explanation of increased serum bile acids in humans with NAFLD, and to increased sensitivity of liver tissue to endo-, and xenobiotics during NAFLD. Altered excretory function of evaluated pathways may complicate pharmacotherapy in sensitive subjects with high intake of sucrose, and with fatty liver disease. Impairment in bile production was alleviated by administration of boldine, which confirms usefulness of FXR agonists as novel therapeutic strategy for NAFLD.

Conflict of Interest

There is no conflict of interest.

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