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Bile acid retention and activation of endogenous hepatic farnesoid-X-receptor in the pathogenesis of fatty liver disease in *ob/ob*-mice

Ina V. Martin[1,2,a,b], Johannes Schmitt[2,b], Alexander Minkenberg[1], Joachim C. Mertens[2], Bruno Stieger[3,5], Beat Mullhaupt[2,4] and Andreas Geier[1,2,4,5,*]

[1] Department of Internal Medicine III, University Hospital Aachen (UKA), D-52074 Aachen, Germany
[2] Department of Internal Medicine, Division of Gastroenterology and Hepatology, University Hospital Zurich (USZ), CH-8091 Zurich, Switzerland
[3] Department of Internal Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital Zurich (USZ), CH-8091 Zurich, Switzerland
[4] Swiss Hepatopancreatobiliary (HPB)-Center, University Hospital Zurich (USZ), CH-8091 Zurich, Switzerland
[5] Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, CH-8001 Zurich, Switzerland

* Corresponding author
e-mail: andreas.geier@usz.ch

Abstract

The nuclear bile acid receptor FXR (farnesoid-X-receptor) has recently been implicated in the pathophysiology of non-alcoholic fatty liver disease because selective FXR-agonists improve glucose and lipid metabolism in rodent models of obesity. However, the regulation of FXR and other relevant nuclear receptors as well as their lipogenic target genes in fatty liver is still not revealed in detail. Livers were harvested from 14-week-old male *ob/ob* mice and wild-type controls. Serum bile acids were quantified by radioimmunoassay. mRNA and protein expression of transporters and nuclear receptors was analyzed by reverse transcriptase-polymerase chain reaction and Western blotting, whereas DNA binding to the IR-1 element was examined by electrophoretic mobility shift assay. In this study we show: (i) bile acid retention in *ob/ob* mice, (ii) a resulting FXR upregulation and binding to the IR-1 element in *ob/ob* animals and (iii) concomitant activation of the fatty acid synthase as a potential lipogenic FXR target gene *in vivo*. The present study suggests a potential role of hepatic bile acid retention and FXR activation in the induction of lipogenic target genes. Differences between intestinal and hepatic FXR could explain apparent contradictory information regarding its effects on fatty liver disease.

Keywords: bile acid transporters; cholestasis; FXR; non-alcoholic fatty liver disease; nuclear receptors; *ob/ob* mouse.

[a] Present address: Department of Internal Medicine II, Division of Nephrology and Immunology, RWTH Aachen University, D-52074 Aachen, Germany.
[b] These authors contributed equally to this work.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that affects 10–24% of the general population and comprises liver disorders ranging from steatosis and non-alcoholic steatohepatitis (NASH) to advanced fibrosis and cirrhosis (Angulo, 2002; Adams et al., 2005; Stefan et al., 2008; Erickson, 2009). The increasing global burden of obesity and related diseases highlights the necessity to define targeted future strategies of intervention based on pathophysiology.

Bile acids are physiological ligands of the nuclear bile acid receptor FXR (farnesoid-X-receptor) that can be referred to as an intracellular bile acid sensor which is the key regulator in bile acid homeostasis (Makishima et al., 1999). Recent studies in genetic mouse models of obesity and diabetes (*ob/ob* and *db/db* mice) highlight a link between FXR activation and development of the metabolic syndrome because treatment with its synthetic ligand GW4064 significantly improves insulin sensitivity (Cariou et al., 2006; Zhang et al., 2006). Hepatocellular bile acid retention is mainly caused by reduced canalicular bile salt secretion as the rate-limiting step in transport. Decreased expression of canalicular transporters including the bile-salt export pump (BSEP/Bsep; encoded by ABCB11) and the conjugate export pump (multidrug resistance-associated protein; MRP2/Mrp2, encoded by ABCC2) have been described in various form of cholestasis (reviewed in Geier et al., 2007) and also in rodent models of fatty liver disease and diabetes (Pizarro et al., 2004; Geier et al., 2005b). As an adaptive response, accumulating bile acids mediate indirect negative feedback regulation of hepatocellular bile acid uptake transporters such as the high-affinity Na^+-dependent bile-salt transporter NTCP/Ntcp (encoded by SLC10A1) by an FXR-dependent activation of the transcriptional repressor small heterodimer partner (Shp) (Geier et al., 2007). Furthermore, bile acid-activated FXR represents a negative transcriptional regulator of cholesterol 7-α-hydroxylase (CYP7A1) expression, which represents the rate-limiting enzyme in the *de novo* synthesis of bile acids from cholesterol (reviewed in Chiang, 2009).

Interestingly, general $FXR^{-/-}$ mice not only exhibit elevated serum bile acid concentrations but also develop fatty liver which is severely aggravated when fed a 1% cholesterol diet (Sinal et al., 2000; Lambert et al., 2003). Several reports confirm an inverse correlation between bile acid-dependent FXR-pathway activation and plasma triglyceride levels because bile acid-feeding in different models of hypertriglyceridemia decreased *de novo* lipogenesis through down-regulation of sterol regulatory element binding protein-1c (SREBP-1c) resulting in reduced plasma triglycerides (Watanabe et al., 2004; Bilz et al., 2006).

Despite the central role of FXR and other nuclear receptors in bile acid and lipid metabolism, their endogenous expression in fatty livers is still elusive. These nuclear receptors undergo marked changes in their expression and activity during cholestasis and inflammation (Trauner et al., 1998; Denson et al., 2000; Geier et al., 2003; Ghose et al., 2004; Geier et al., 2005b,c; Zollner et al., 2005) both of which are characteristic of NAFLD also. We hypothesize that bile acid accumulation in fatty livers leads to the activation of nuclear receptors, particularly FXR, and respective target genes under these specific conditions.

Therefore, the aim of this study was to characterize the expression of FXR and other class II nuclear receptors in fatty livers of *ob/ob* mice and to elucidate potential effects of accumulating bile acids on lipogenic FXR-target genes under these conditions.

Results

Ob/ob mice develop liver steatosis without inflammation

Leptin-deficient *ob/ob* mice spontaneously developed hepatic steatosis at 14 weeks of age. In these animals hematoxylin and eosin staining of paraffin-embedded tissue sections demonstrated a severe hepatic steatosis without signs of histopathological inflammation compared with unaffected wild-type controls (Figure 1A). The liver/body weight ratio of 14-week-old *ob/ob* mice (9.0±0.2%) was increased in comparison with wild-type controls (5.0±0.4%; n=6 each). mRNA expression levels of proinflammatory cytokines are increased in *ob/ob*-mice compared with control animals [2.26±0.26-fold for interleukin (IL)-1β and 4.05±0.32-fold for tumor necrosis factor-α (TNF-α), 3.19±0.74-fold for IL-8, respectively; n=6; $p<0.05$ each]. No change was observed for IL-12 (data not shown) (Figure 1B).

Decreased bile acid transporter expression renders fatty livers in *ob/ob* mice cholestatic

Whether *ob/ob* mice are cholestatic, as recently reported for *fa/fa* Zucker rats, has not been investigated so far (Geier et al., 2005a). Serum bile acid concentrations as measured by radioimmunoassay (RIA) are increased sevenfold in *ob/ob* mice in comparison with wild-type controls (14.9±5.4 vs. 1.9±1.0 μM; n=6; $p=0.00080$) (Figure 3).

Subsequently, hepatic bile salt and organic anion transporter expression in *ob/ob* mice has been determined at both mRNA and protein expression level using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis (Figures 2 and 3). RNA expression of basolateral uptake transporters was significantly decreased for Oatp1a1 and Oatp1a4 (organic anion transporter) compared with control mice to 2±1% and 55±6%, respectively. Similarly, protein expression was reduced to 9±1% (Oatp1a1) and 11±6% (Oatp1a4) (both $p<0.05$). Whereas Ntcp mRNA expression was largely unchanged, Ntcp protein levels were significantly decreased in *ob/ob*-mice compared with wild-

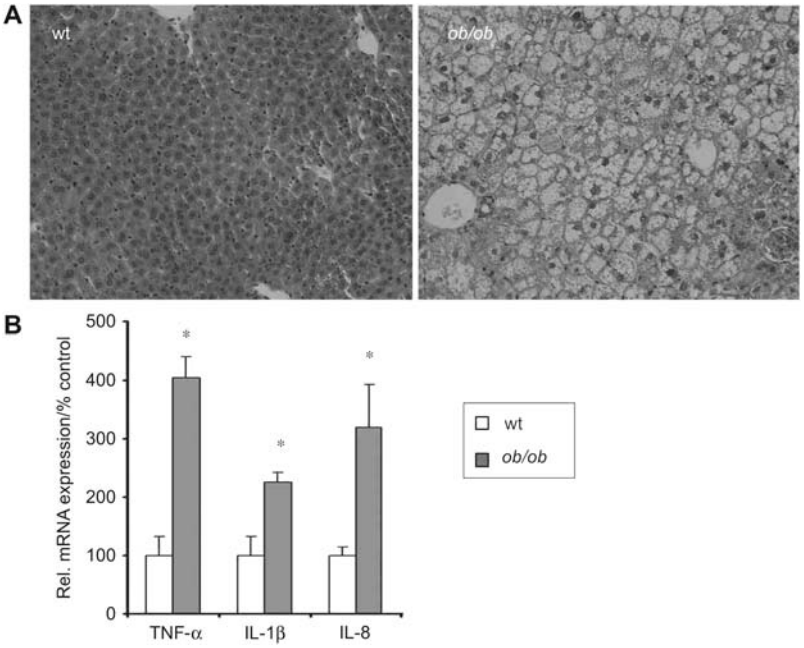

Figure 1 Liver histology and expression of inflammatory cytokines in *ob/ob* mice.
(A) Representative hematoxylin and eosin staining of liver sections from wild-type and *ob/ob* mice (200×magnification). Accumulation of hepatocellular lipid vacuoles are present in *ob/ob* livers (right) compared with wild-type livers with normal histological appearance (left). (B) Analysis of inflammatory cytokine gene expression. Quantitative RT-PCR was performed with RNA samples isolated from liver of controls and *ob/ob*-mice (n=6). The asterisk indicates $p<0.05$ as determined by Student's *t*-test.

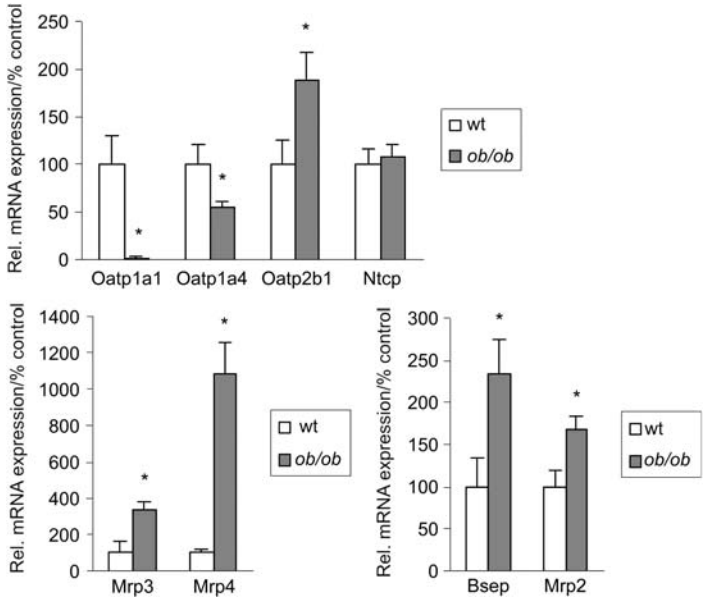

Figure 2 Hepatic transporter gene expression is altered in *ob/ob* mice.
Quantitative TaqMan RT-PCR was performed with RNA samples isolated from liver of controls and *ob/ob*-mice (n=6). The asterisk indicates $p<0.05$ as determined by Student's *t*-test.

type controls (70±9%; $p<0.05$). In contrast to other basolateral transporters, Oatp2b1 mRNA was significantly increased to 188±30% of controls. Expression of basolateral export systems was strongly upregulated at the mRNA (Mrp3 332±45%; Mrp4 1086±170%) and protein level (Mrp4 618±65%; $p<0.05$) each compared with controls.

On the contrary to other rodent models of cholestasis, but similar to *fa/fa* rats, expression of canalicular transporters was mainly decreased at the protein level. Whereas Bsep and Mrp2 mRNA expression levels were even increased in *ob/ob* mice to 234±40% and 168±16% of wild-type controls ($p<0.05$) microsomal protein levels were decreased to 59±10% for Bsep and 67±16% for Mrp2 ($p<0.05$).

Consistent with decreased transporter protein abundance and bile acid retention in cholestatic *ob/ob* mice Cyp7a1 mRNA expression is profoundly suppressed compared with non-cholestatic lean animals to 10±4% ($p=0.0001$) (Figure 4A).

FXR and other nuclear receptors are upregulated in *ob/ob* mice

In contrast to rodent models of inflammatory liver disease nuclear receptor activity is not suppressed in obese *fa/fa* rats (Geier et al., 2005a,b). To examine the influence of bile acid-retention and induction of proinflammatory cytokines on nuclear receptors in *ob/ob* mice, we analyzed both mRNA and protein expression of several nuclear receptors implicated in bile acid-signaling including FXR, pregnane X receptor (PXR) and vitamin D receptor (VDR) (Figure 4). Most prominently, FXR nuclear protein was increased to 330±70% (mRNA expression 196±21%) in *ob/ob* mice compared with controls ($p<0.05$ each). VDR and PXR expression were similarly increased at both nuclear protein (225±43% and 258±34%, respectively) and mRNA levels (309±27% and 130±26%, respectively). Likewise, liver X receptor (LXR) protein and mRNA levels increased by 146±20% and 169±19% compared with the wild-type.

Finally, Shp mRNA and protein expression was analyzed to determine whether FXR activation results in the induction of this target gene. In contrast to other rodent models of cholestasis, Shp mRNA and protein expression in *ob/ob* mice were not significantly increased and comparable with their control littermates (Figure 4A).

FXR DNA-binding activity and lipogenic target genes including fatty acid synthase are increased in *ob/ob* mice

To investigate whether the observed increase in FXR nuclear protein results in increased DNA binding activity and activation of respective target genes, we analyzed binding to the FXR-responsive element IR-1 which is present in mouse promoters of the *Bsep* (bile-salt export pump) and *Fas* (fatty acid synthase) gene (Ananthanarayanan et al., 2001; Matsukuma et al., 2006). Using electrophoretic mobility shift assays (EMSAs) DNA binding activity to the IR-1 element is doubled in nuclear extracts from obese mice (196±3% compared with lean controls; $p<0.05$) (Figure 5). Following the trend of an increased mRNA expression of Bsep (see Figure 2), FAS mRNA expression, another FXR target gene, was upregulated to 4982±1432% ($p<0.05$) (Figure 6) consistent with activated IR-1 binding.

As expected, other lipogenic genes including peroxisome proliferator-activated receptor-γ (PPAR-γ) mRNA and its target transcript Srebp-1c were upregulated in *ob/ob* mice 58-fold and seven-fold (both $p<0.05$ each), respectively (Figure 6).

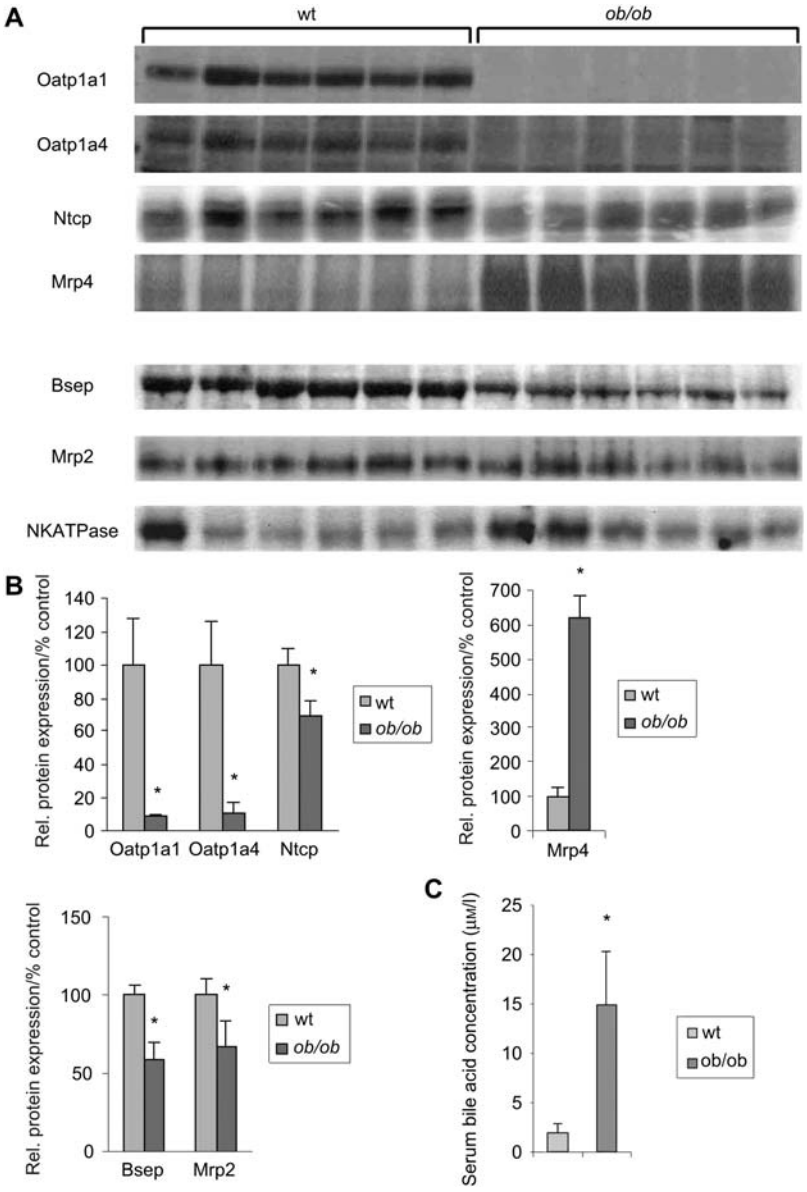

Figure 3 Western blot analysis of hepatic transporter protein expression in *ob/ob* mice and serum bile acid concentration in *ob/ob* mice. (A) Western blotting: microsomal protein of wild-type and *ob/ob* mice (n=6 each) was separated by SDS-PAGE (protein loading 50 µg/lane), blotted onto a PVDF membrane and detected by specific antibodies. (B) Densitometric analysis: quantification of relative protein expression compared with wild-type animals. The asterisk indicates $p<0.05$ as determined by Student's *t*-test. (C) Serum from wild-type and *ob/ob* animals was collected. Serum bile acids were measured by RIA. Asterisk indicates $p<0.05$ as determined by Student's *t*-test.

Discussion

Previous evidence from obese *fa/fa* Zucker rats as an established model of NAFLD, obesity and diabetes highlights the possibility that bile acid retention could play a role in the pathogenesis of the underlying metabolic disease (Pizarro et al., 2004; Geier et al., 2005b). We now used the *ob/ob* mouse model of fatty liver disease to further investigate potential bile acid-mediated effects on nuclear receptor activity and lipogenic target genes in more detail. This model resembles human fatty liver disease without histological inflammatory infiltrates and is characterized only by a subclinical extent of intrahepatic cytokine activation. The major new findings of the present study are: (i) the bile acid retention in *ob/ob* mice rendering these animals by definition cholestatic, (ii) the resulting FXR upregulation and binding to the IR-1 element in *ob/ob* animals and (iii) the concomitant activation of the fatty acid synthase as lipogenic potential FXR target gene *in vivo*.

In the current study, we describe bile acid retention and FXR activation as a potential trigger in the pathogenesis of fatty liver disease in *ob/ob* mice. This retention of bile acids is well in accordance to preliminary data from human patients with fatty liver disease which are also characterized by an increase in serum bile acid concentrations (Aranha et al., 2008; Kocabayoglu et al., 2009). Cholestasis in mice

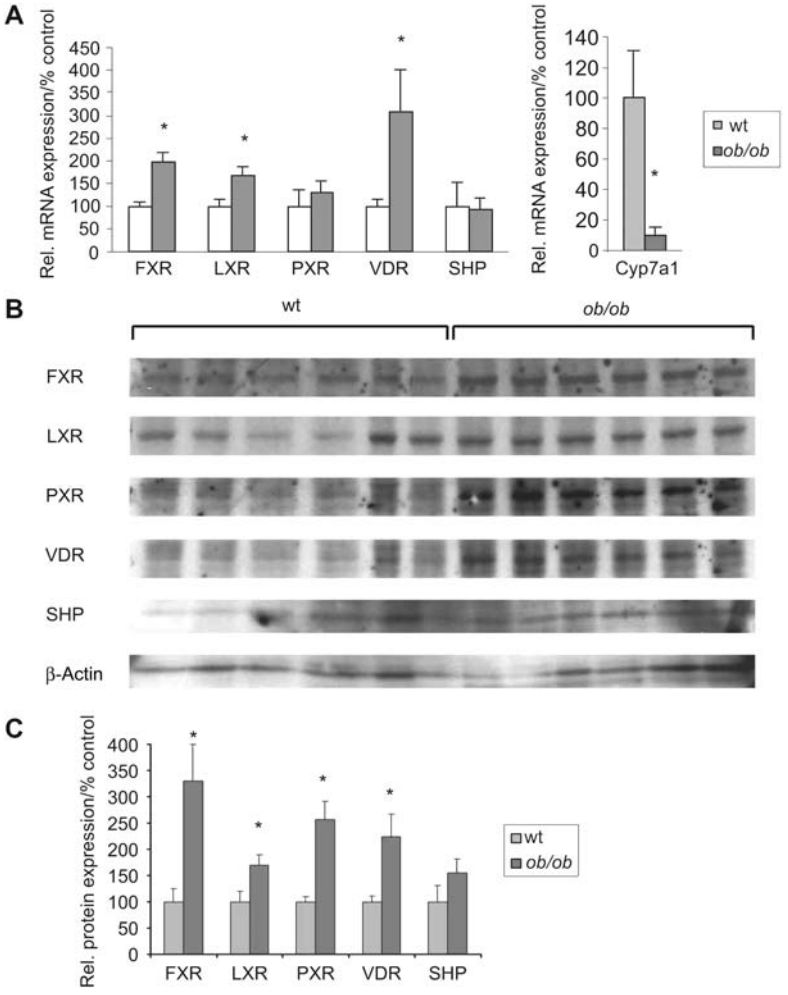

Figure 4 Nuclear receptor mRNA and protein expression in *ob/ob* mice.
(A) mRNA expression: quantitative TaqMan RT-PCR was performed with RNA samples isolated from liver of controls and *ob/ob*-mice (n=6). (B) Western blot analysis: nuclear protein and whole cell extract (SHP) were separated by SDS-PAGE (protein loading 10 μg/lane), blotted onto a PVDF membrane and detected by specific antibodies. (C) Densitometric analysis: quantification of relative protein expression shown in B compared with wild-type mice. The asterisk indicates $p<0.05$ as determined by Student's *t*-test.

with fatty livers is accompanied by a downregulation of canalicular bile acid transporter Bsep and Mrp2 proteins as the rate-limiting step in bile acid secretion. Furthermore, there seems to be a lack in the cholestatic feedback inhibition of Ntcp in *ob/ob* mice (Figures 2 and 3). Differences between maintained RNA expression and slightly decreased protein levels could be explained by post-translational modification or compartmental changes in protein location. A moderate decrease in Ntcp and Bsep protein expression is in accordance with previous observations in *fa/fa* rats (Pizarro et al., 2004; Geier et al., 2005b). Dysregulation of NTCP is even more prominent in humans where higher mRNA expression levels have been linked to disease progression in human NAFLD (Kocabayoglu et al., 2009). Of note, Cheng and coworkers observed in *ob/ob* mice a decreased Ntcp (mRNA and protein) and Bsep (mRNA) expression and an even induced Mrp2 protein (mRNA unchanged compared with lean controls) (Cheng et al., 2008) which do not parallel other findings in humans (Martin et al., 2009), rats (Pizarro et al., 2004; Geier et al., 2005b) and mice with fatty livers. Discrepancies could be explained by different breeding conditions which are particularly relevant owing to established changes in the intestinal barrier of *ob/ob* mice leading to changes in portal lipopolysaccharide levels that can contribute to hepatic inflammatory damage (Brun et al., 2007). Nevertheless, Cheng et al. report similar alterations in the expression of other basolateral bile acid transporters in *ob/ob* mice including the downregulation of Oatp1a1 and the induction of the overflow systems Mrp3 and Mrp4 in accordance with the present study (Cheng et al., 2008).

The underlying molecular mechanisms leading to transporter dysregulation might be different from (obstructive) cholestasis. Hepatic FXR expression is upregulated in *ob/ob* mice at both the mRNA and protein levels and, as expected, in the presence of bile acid retention FXR binding to the corresponding IR-1 element is activated (Figures 4 and 5). This is in line with a transcriptional activation of the FXR target gene *Bsep* via IR-1 binding but decreased Bsep protein

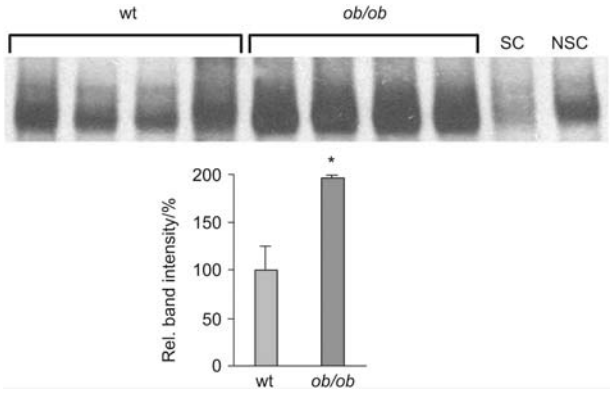

Figure 5 DNA-binding activity of FXR to the corresponding IR-1 element.
DNA-binding analysis was performed with nuclear proteins obtained from liver tissue of control and *ob/ob* mice (n=4 each). Nuclear protein was incubated with biotin-labeled oligonucleotides representing the IR-1 element. Specificity of binding was confirmed by inclusion of specific competitor (SC) oligonucleotide or an unrelated oligonucleotide at a 300-fold molar excess (NSC=non-specific competitor).

necessitates the postulation of further post-translational processing under these conditions (Figures 2, 3 and 5). However, we could not detect an upregulation of Shp as another FXR-target, as previously shown for obstructive cholestasis (Zollner et al., 2005) either on the mRNA or protein level. These results regarding an absent Shp mRNA induction have been previously reported in obese *ob/ob* and *db/db* mouse models (Zhang et al., 2006; Miao et al., 2009). Miao and coworkers could further demonstrate that Shp protein abundance is highly controlled by ubiquitin-proteasomal degradation in wild-type animals whereas *ob/ob* mice are largely protected against Shp ubiquitination.

Irrespective of ameliorating effects of FXR activation on hepatic inflammation, fibrosis during steatohepatitis and lipid

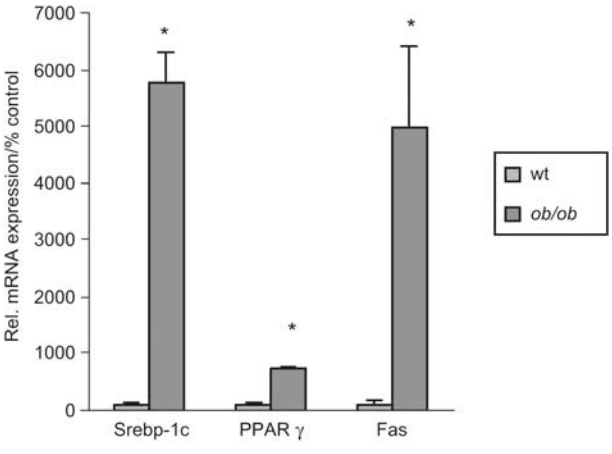

Figure 6 Hepatic mRNA expression of lipogenic genes in *ob/ob* mice.
Quantitative TaqMan RT-PCR was performed with RNA samples isolated from liver of controls and *ob/ob*-mice (n=6). Asterisk indicates $p<0.05$ as determined by Student's *t*-test.

abnormalities in the mouse model (Cipriani et al., 2009; Zhang et al., 2009) the activation of FXR could also play a role for the dysregulation of lipogenesis in the pathogenesis of fatty liver disease. Recently, a FXR-responsive element (IR-1) has been identified within the mouse *Fas* promoter which mediates a bile acid-dependent upregulation of the *Fas* gene, in addition to the established insulin-induced activation (Wang and Sul, 1998; Matsukuma et al., 2006). In the present study extremely high levels of Fas transcripts have been observed in *ob/ob* mice in the presence of increased IR-1 (FXR) binding (Figures 5 and 6). A moderately increased Fas expression in both wild-type and obese KK-A^y mice upon long-term feeding of cholic acid with a concurrent decrease of Srebp-1 support this concept in general (Watanabe et al., 2004).

Consistent with Fas activation the lipogenic transcription factor Srepb-1c and its upstream activators LXR, PPARγ and PXR are increased at the mRNA level (Figures 4 and 6). In primary mouse hepatocytes it has been shown that the activation of FXR by bile acids or synthetic agonists represses the expression of Srebp-1c and its lipogenic target genes in a Shp-dependent manner (Watanabe et al., 2004).

Under the special conditions present in the livers of *ob/ob* mice, activation of hepatic FXR could therefore affect the fatty acid homeostasis of hepatocytes, by activating FAS expression additionally to established Srebp-1c effects.

It is important to note that the role of FXR in the complex pathophysiological scenario of fatty liver disease is not conclusively clarified so far. Whereas a variety of data suggest the potentially beneficial role of bile acids in treatment of NAFLD one cannot yet unambiguously evaluate their usefulness (Orlando et al., 2007). Several interventional and knockout studies demonstrated beneficial effects of FXR activation. The general absence of FXR *in vivo* has profound consequences on systemic lipid metabolism because general $Fxr^{-/-}$ mice have increased serum triglycerides, cholesterol and free fatty acids and develop fatty livers (Sinal et al., 2000; Lambert et al., 2003; Ma et al., 2006). On one hand, a inverse correlation between the activation of FXR pathways and plasma triglyceride levels exists because bile acid-feeding in different models of hypertriglyceridemia decreased *de novo* lipogenesis through downregulation of Srebp-1c resulting in reduced plasma triglycerides (Watanabe et al., 2004; Bilz et al., 2006). On the other hand, treatment of hyperlipidemic hamsters did not significantly decrease Srebp-1c expression despite a reduction in triglyceride synthesis (Bilz et al., 2006) and hepatic Srebp-1c mRNA levels are not increased in $Fxr^{-/-}$ mice as to be expected (Zhang et al., 2004; Duran-Sandoval et al., 2005a,b; Lefebvre et al., 2009).

Studies in *db/db* and *ob/ob* mice have shown that treatment with the synthetic FXR-specific ligand GW4064 significantly improves insulin sensitivity and reduces hepatic lipid accumulation (Cariou et al., 2006; Zhang et al., 2006). Treatment with the FXR agonist GW4064 significantly represses hepatic CYP7A1 in mice with liver-specific deletion of *Fxr* (DeltaL) but not in mice with intestinal deletion of *Fxr* (DeltaIE), which opens the possibility that activation

of FXR in intestine but not in the liver could mediate hepatic effects of GW4064 (Kim et al., 2007). Keeping in mind that GW4064 is characterized by a poor pharmacokinetic profile with poor intestinal absorption into the circulation these studies do not necessarily rule out that hepatic FXR and its activation in fatty livers could play a causal role in the pathophysiology of fatty liver disease. To finally answer this question comparable studies using intestinal and liver specific FXR deletion in obese mice might be necessary.

In summary, our study opens the possibility that bile acid retention could contribute to the development of fatty liver disease. Understanding the multifaceted function of FXR in lipid homeostasis might contribute to pathophysiological and therapeutic concepts for a targeted treatment of fatty liver disease in the future.

Materials and methods

Animals

Eight-week-old male *ob/ob* mice (B6.V-*Lep*^{ob}/J) and age- and gender-matched control animals C57BL/6J were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in pathogen-free animal facilities under a standard 12-h light, 12-h dark cycle with access to regular rodent chow and autoclaved tap water *ad libitum* for 6 weeks. The mice were sacrificed, and most of the liver tissue was immediately frozen in liquid nitrogen and a small portion was immersion fixed in 4% formalin. Subsequently, paraffin-embedded sections were analyzed after hematoxylin and eosin staining for the degree of hepatic steatosis. The animals received humane care and the study protocols were approved by the local Government's Animal Care Committee.

mRNA isolation and real-time RT-PCR

Total RNA was isolated from liver by standard phenol chloroform extraction procedure using Ultraspec™ (Biotecx Lab, Houston, TX, USA) according to manufacturer's instructions. mRNA was reverse-transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). cDNA was used for RT-PCR with SYBR Green Reagent (Invitrogen, Karlsruhe, Germany) and specific primer pairs on a 7300 ABI PRISM Real-Time PCR System and with ABI PRISM 7300 SDS software (Applied Biosystems, Foster City, CA, USA). Cyp7a1 expression was analyzed using ABI TaqMan probes. Expression was normalized against 18S. All primer sequences are available from the authors upon request.

Western blotting

Nuclear and microsomal protein fractions were prepared as described previously (Gartung et al., 1997). Similar amounts of microsomal and nuclear protein (50 μg and 10 μg, respectively) were separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane and probed with the following antibodies: Oatp1a1 (Eckhardt et al., 1999), Oatp1a4 (Reichel et al., 1999), Ntcp (Stieger et al., 1994), BSEP (Gerloff et al., 1998), Mrp2 (Madon et al., 2000), Mrp4 (Rius et al., 2003), FXR (SantaCruz, CA, USA, clone Q-20, sc-1205), LXR (Abcam, Cambridge, UK, ab-28478), PXR (SantaCruz, USA, clone A-20, sc-7737), VDR (SantaCruz, USA, clone C-20, sc-1008), SHP (SantaCruz, USA, clone Q-14, sc-15283). Na/K ATPase (Abcam, Cambridge, UK, ab-7671) and β-actin (Sigma, St. Louis, MO, USA, A2066) antibodies were used as loading control for microsomal and total protein. After incubation with species-specific HRP-conjugated secondary antibody (Dako, Hamburg, Germany) immune complexes were detected using the ECL detection kit (GE Healthcare, Freiburg, Germany). Densitometric quantification of Western blots was performed using Quantity One software (Bio-Rad, Munich, Germany).

Electrophoretic mobility shift assay

Nuclear protein extracts were prepared as described previously (Geier et al., 2002). DNA binding analyses were conducted using the Lightshift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturers' protocol. The following oligonucleotides were used as probes in the analyses: IR-1, sense 5′-CTT TAG GCC ATT GAC CTA TAA-3′ and antisense 5′-TTA TAG GTC AAT GGC CTA AAG-3′ (Geier et al., 2005b). These oligonucleotides were end-labeled using the biotin 3′ end DNA labeling kit (Pierce Biotechnology). Binding reactions consisted of 1× binding buffer, 50 ng/μl poly dIdC, 20 fmol biotin-labeled DNA and 5 μg nuclear protein in a 20 μl reaction. Competition experiments included 6 pmol unlabeled oligonucleotide (300-fold molar excess). Densitometric quantification was performed using Quantity One software (Bio-Rad).

Bile acid quantification

Serum bile acids were measured by RIA using a Bile Acid RIA Kit (MP Biomedicals, Ilkirch, France) according to the manufacturer's specifications.

Statistical analysis

Statistical significance ($p<0.05$) between control animals and *ob/ob*-mice was determined by Student's *t*-test. Data represent the mean±standard deviation.

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