

Serotonin Receptor Type 3 Antagonists Improve Obesity-Associated Fatty Liver Disease in Mice

Synia Haub, Yvonne Ritze, Inga Ladel, Karolin Saum, Astrid Hubert, Astrid Spruss, Christian Trautwein, and Stephan C. Bischoff

Department of Nutritional Medicine, University of Hohenheim, Stuttgart, Germany (S.H., Y.R., I.L., K.S., A.H., A.S., S.C.B.); and Gastroenterology & Metabolic Diseases, the University Hospital RWTH Aachen, Aachen, Germany (C.T.)

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ABSTRACT

Obesity is a major cause for nonalcoholic fatty liver disease (NAFLD). Previous studies suggested that alterations in intestinal motility and permeability contribute to the development of NAFLD. Serotonin and serotonin receptor type 3 (5-HT₃R) are key factors in the regulation of intestinal motility and permeability. Therefore, we studied the effect of the 5-HT₃R antagonists tropisetron and palonosetron on the development of NAFLD in leptin-deficient obese mice. Four-week-old *ob/ob* mice and lean controls were treated for 6 weeks orally with tropisetron or palonosetron at 0.2 mg/kg per day. We determined markers of liver damage and inflammation, portal endotoxin levels, and duodenal concentrations of serotonin, serotonin-reuptake transporter (SERT), occludin, and claudin-1. Tropisetron treatment significantly reduced liver fat content (−29%), liver inflammation (−56%), and liver cell necrosis (−59%) in

ob/ob mice. The beneficial effects of tropisetron were accompanied by a decrease in plasma alanine aminotransferase and portal vein plasma endotoxin levels, an attenuation of enhanced MyD88 and tumor necrosis factor- α mRNA expression in the liver, and an increase of tight junction proteins in the duodenum. Tropisetron treatment also caused a reduction of elevated serotonin levels and an increase of SERT in the duodenum of *ob/ob* mice. Palonosetron had similar effects as tropisetron with regard to the reduction of liver fat and other parameters. Tropisetron and palonosetron are effective in attenuating NAFLD in a genetic mouse model of obesity. The effect involves the intestinal nervous system, resulting in a reduction of endotoxin influx into the liver and subsequently of liver inflammation and fat accumulation.

Introduction

Obesity is a key risk factor for the development of nonalcoholic fatty liver disease (NAFLD). NAFLD comprises a broad range of pathologic changes of the liver with the most common and early lesion being steatosis, which may progress to nonalcoholic steatohepatitis and cirrhosis (Brun et al., 1999). Despite intense research effort during the last years, mechanisms involved in the development of NAFLD are not fully understood and therapeutic options are limited. Hyper-

caloric diets that may cause alterations in intestinal motility and small intestinal bacterial overgrowth seem to be critically involved in the pathogenesis of NAFLD, possibly by inducing an impairment of the intestinal barrier and subsequent influx into the liver of bacterial products acting as triggers for steatosis and inflammation (Cope et al., 2000; Li et al., 2003; Brun et al., 2007; Bergheim et al., 2008). Human data support this concept, because elevated plasma endotoxin levels, increased tumor necrosis factor α (TNF- α) expression, and small intestinal bacterial overgrowth were found in patients with nonalcoholic steatohepatitis (Wigg et al., 2001). In support of the hypothesis that increased intestinal permeability as well as bacterial overgrowth and translocation are involved in the development of NAFLD, we and others have shown that rodents are protected from the development of NAFLD when being concomitantly treated with antibiotics (Lichtman et al., 1991; Bergheim et al., 2008). It is noteworthy that elevated endotoxin levels in the portal vein are associated with liver inflammation (Brun et al., 2007;

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ABBREVIATIONS: NAFLD, nonalcoholic fatty liver disease; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT₃R, serotonin receptor type 3; SERT, serotonin reuptake transporter; PCR, polymerase chain reaction; ALT, alanine aminotransferase; TNF- α , tumor necrosis factor α ; MyD88, myeloid differentiation factor 88; ANOVA, analysis of variance; TLR, Toll-like receptor.

Cani et al., 2008). This type of inflammation may lead to an impairment of the intestinal barrier function, consisting of an altered structure and localization of tight junctions, for example, and a subsequent development of liver damage (Bruewer et al., 2003).

The regulation of translocation of endotoxin from the intestine into the liver is unclear. It is possible that serotonin (5-hydroxytryptamine, 5-HT) and the serotonin receptor type 3 (5-HT₃R) in the intestine are involved in this process (Bakker et al., 1993; Yamada et al., 2003; Nylander and Pihl, 2006). 5-HT is one of the key signaling neurotransmitters in the gut regulating motility and inflammation (Gershon, 2004; Bischoff et al., 2009; Haub et al., 2010). In a previous study, we could show that tropisetron, a 5-HT₃R antagonist, attenuates liver damage induced by overfeeding with sugars such as glucose or fructose (Weber et al., 2009). The aim of the present study was to investigate possible underlying mechanisms and also whether the beneficial effect of tropisetron on NAFLD is restricted to a glucose-induced model of obesity or whether the effect can be generalized to different models of obesity. This drug, which is otherwise used to control radiation- and chemotherapy-induced or postoperative nausea and vomiting, might act on the intestinal, the hepatic, and the systemic level (Gan, 2005). The effects of tropisetron, which comprise not only modulatory effects on neurons but also anti-inflammatory properties, are possibly mediated not solely through 5-HT₃R (Thompson and Lummis, 2006; Mousavizadeh et al., 2009). We focused our studies on intestinal and hepatic effects of tropisetron, and we also tested the more specific 5-HT₃R antagonist palonosetron to find out whether or not the beneficial effects are rather related to the inhibition of the 5-HT₃R. The studies were performed using a well established genetic model of obesity, the *ob/ob* mouse (Anstee and Goldin, 2006), which is characterized not only by the absence of functional leptin but also by thymic atrophy and defective immune responses concerning dendritic, regulatory, and natural killer cells (Macia et al., 2006; Ilan et al., 2010).

Materials and Methods

Animals and Treatments. Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC). All procedures were approved by the local Institutional Animal Care and Use Committee (IACUC regional board, Stuttgart, Germany). Leptin-deficient *ob/ob* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). For 6 weeks, 4- to 6-week-old *ob/ob* mice and their lean littermates ($n = 3-7$ per group) had free access to water containing tropisetron (C₁₇H₂₀N₂O₂; Novartis Pharma, Basel, Switzerland) or palonosetron (C₁₉H₂₄N₂O; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (average 0.2 mg/kg b.wt.) or plain water. The average water consumption by mice was determined in earlier experiments of our group (Haub et al., 2010). Based on our finding that young adult animals consistently consumed approximately 3 ml of water per day, we adjusted the drug content in the drinking water

in order so that the animals received ~0.2 mg/kg per day of tropisetron or palonosetron. After 6 weeks, animals were anesthetized with 80 mg/kg b.wt. ketamine and 6 mg/kg b.wt. i.p. xylazine. Blood was collected in heparinized tubes containing 37.5 IU of Fragmin P Forte (Pfizer, Karlsruhe, Germany) from the portal vein just before euthanization. The plasma was separated after centrifugation at 1500g for 12 min at 15°C and was frozen at -80°C until further use. Portions of liver and duodenal tissue were either frozen immediately in liquid nitrogen for mRNA and protein analysis or fixed in neutral-buffered formalin or frozen-fixed in OCT mounting media for microscopic analysis (MEDITE GmbH, Burgdorf, Germany). We chose duodenum for tissue sampling because we found in our previous work in which we studied the effect of sugars, such as fructose on gastrointestinal barrier functions and endotoxin translocation, that sugars affect the expression of tight junctions primarily in the duodenum, the intestinal site where most sugars are absorbed (Bergheim et al., 2008; Weber et al., 2009). Therefore, we expected such changes occurring most probably in the duodenum.

RNA Isolation and Real-Time Reverse Transcription-PCR.

Total RNA was extracted from duodenum and liver tissue samples using TriFast reagent (PEQLAB, Erlangen, Germany). RNA concentrations were determined spectrophotometrically, and 1 µg of total RNA was reverse-transcribed using a Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers followed by a DNase digestion step (Fermentas, St. Leon Rot, Germany). PCR primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) (see Table 1). SYBR Green Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was used to prepare the PCR mix. The amplification reactions were carried out in an iCycler (Bio-Rad Laboratories, München, Germany) with an initial hold step (95°C for 3 min) and 40 cycles of a three-step PCR (95°C for 15 s, 60°C for 15 s, and 72°C for 30 s). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative computed tomography method was used to determine the amount of target gen, normalized to an endogenous reference (β -actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of PCR products was verified by melting curves and gel electrophoresis.

Clinical Chemistry and Pathologic Evaluation. Paraffin sections of liver (5 µm) were stained for hematoxylin and eosin to assess liver histology. Using the liver pathology score described by Nanji et al. (1989), the degree of steatosis, inflammation, and necrosis was determined as follows. Steatosis is defined as the percentage of liver tissue containing fat (absent = 0; <25% = 1+; 25-50% = 2+; 50-75% = 3+; >75% = 4+). The presence of inflammation (infiltrated cells from the immune system) and necrosis (cell lysis caused for example through inflammation) was noted with 1+ or 2+ (1 spot or less in 10× objective = 1+; 2 or more spots per 10× objective = 2+). The total liver pathology score was calculated by adding the score from each of the parameters. Plasma alanine aminotransferase (ALT) was measured using a commercially available kit following the instructions of the manufacturer (Randox, Krefeld, Germany). Neutrophil infiltration in hepatic tissue was evaluated by staining using an AS-D-chloroacetate esterase kit (Sigma-Aldrich, Steinheim, Germany) and was counted as described previously (Bergheim et al., 2008).

Oil Red O Staining. To determine hepatic lipid accumulation, frozen sections of liver (10 µm) were stained with Oil Red O (Sigma-Aldrich) for 10 min, washed, and counterstained with hematoxylin for 45 s (Sigma-Aldrich). Representative photomicrographs were cap-

TABLE 1

Primers used for real-time reverse transcription-PCR detection of MyD88, TNF- α , and β -actin

	Forward (3'-5')							Reverse (3'-5')						
MyD88	CAA	AAG	TGG	GGT	GCC	TTT	GC	AAA	TCC	ACA	GTG	CCC	CCA	GA
TNF- α	CCA	GGC	GGT	GCC	TAT	GTC	TC	CAG	CCA	CTC	CAG	CTG	CTC	CT
β -Actin	GGC	TCC	CAG	CAC	CAT	GAA		AGC	CAC	CGA	TCC	ACA	CAG	

tured at a magnification 400× using a system incorporated in a microscope (AxioVert 200M; Zeiss, Jena, Germany).

Endotoxin Assay. Portal plasma samples were heated at 73°C for 20 min. Endotoxin plasma levels were determined using a commercially available limulus amoebocyte lysate assay with a concentration range of 0.015 to 1.2 EU/ml (Charles River, L'Arbaesle, France) as described previously (Haub et al., 2010).

Measurement of the 5-HT Content of the Duodenum. Frozen duodenum was weighted, and the tissue was homogenized in 10 μ l per 1- μ g tissue of 0.05 M HCl supplemented with 0.1% ascorbic acid and centrifuged at 14,000 rpm for 5 min at 4°C. 5-HT content (in nanograms of 5-HT per milligram tissue weight) was determined in supernatants using an enzyme immunoassay kit (IBL, Hamburg, Germany).

Immunohistochemical Staining of 5-HT. For the detection of 5-HT-positive cells in the small intestine, paraformaldehyde-fixed (4%) tissue sections from the duodenum were blocked in goat serum for 1 h (4 + 0.25% Triton X-100 + phosphate-buffered saline), incubated at room temperature for 2 h with a 5-HT primary antibody (the rabbit antibody; 1:1000 phosphate-buffered saline buffer + 0.25% Triton X-100 + 1% goat serum; Alpha Diagnostics, San Antonio, TX) and Alexa Fluor 488 anti-rabbit secondary antibody for 2 h at room temperature (1:2000; Invitrogen, Darmstadt, Germany). The numbers of 5-HT-positive cells in the small intestine were counted in eight randomly selected fields per animal at a magnification of 200× using a Zeiss microscope (AxioVert 200M; Zeiss, Jena, Germany) (Bischoff et al., 2009).

Immunoblots for Detection of Intestinal and Hepatic Proteins. To prepare total tissue protein lysates, snap-frozen small intestine samples (10–30-mg tissue) were homogenized with a lysis buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS] containing a protease inhibitor mix (Roche, Mannheim, Germany). Protein lysates (30 μ g protein/well) were separated in a 10% SDS-polyacrylamide gel and transferred to Hybond-P polyvinylidene difluoride membranes. The resulting blots were then probed with antibodies against the major subunit of the 5-HT₃R (5-HT3A) (1:1500, in 5% skim milk overnight; Santa Cruz Biotechnology, Inc.), SERT (1:500 in 1% skim milk overnight; Santa Cruz Biotechnology, Inc.), occludin (1:500 in 5% skim milk overnight; Zymed, San Francisco, CA), claudin-1 (1:500, in 5% skim milk overnight; Invitrogen), and MyD88 (1:500, in 5% skim milk overnight; Santa Cruz Biotechnology, Inc.), TNF- α (1:500, in 5% skim milk overnight; Cell Signaling, Danvers, MA), respectively. After incubation blots were washed and incubated at room temperature for 45 min with Fc-specific horseradish peroxidase-conjugated secondary antibodies, the bands were visualized using SuperSignal Western Dura kit (Thermo Fisher Scientific, Waltham, MA; and Perbio Science UK Ltd., Chester, Cheshire, UK). To ensure equal loading, all blots were stained with Ponceau Red; signals were normalized to β -actin, which was detected using a commercially available antibody (1:750 in 2.5% bovine serum

albumin overnight; New England Biolabs, Frankfurt, Germany). Protein bands were identified by their molecular weights using a Full Rainbow Ladder (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and densitometrically analyzed by use of the software AlphaEaseFS as described previously (Haub et al., 2010).

Statistical Analysis. Results are reported as means \pm S.E.M. ($n = 5-7$). Results from the four treatment groups were compared by analysis of variance (ANOVA). Before performing ANOVA, data were tested for homogenous variances using the Bartlett test. ANOVA was performed only if homogeneity of variances was confirmed. Statistically significant differences between the groups were considered if ANOVA yielded $p < 0.05$. In this case, we additionally performed a Tukey's post hoc test to identify the individual differences between the means. In the figures and tables, the results from Tukey's test are indicated. No Tukey test was performed if ANOVA was not significant. In addition, an unpaired t test (two-tailed) was used to determine the statistical significance between two individual groups. The SPSS software package, release 18.0, was used.

Results

The serotonin receptor 5-HT₃R is thought to be a major receptor of serotonin produced in the gut (Gershon, 2004). Therefore, we asked the question of whether leptin-deficient mice have an altered 5-HT₃R expression in the duodenum. Indeed, we found in 10- to 12-week-old mice that duodenal protein expression of 5-HT3A was significantly increased by 2- to 3-fold in *ob/ob* mice compared with lean control mice (Fig. 1A). To determine whether the enhanced expression of the 5-HT3A might be involved in the pathogenesis of NAFLD, 4-week-old *ob/ob* mice and lean control animals were treated with tropisetron, palonosetron, or vehicle for 6 weeks. Both drugs had no significant effect on 5-HT3A expression in lean control mice; however, treatment with tropisetron, and probably palonosetron as well, reduced 5-HT3A expression in *ob/ob* mice to almost normal levels (Fig. 1).

Neither tropisetron nor palonosetron treatment affects body weight; however, tropisetron reduced liver weight and liver-to-body weight in *ob/ob* mice significantly (Table 2). However, no significant effect of palonosetron on liver weight and liver-to-body weight was seen, which might be related to the lower number of experiments performed with palonosetron compared with tropisetron (Table 2B).

Histological data revealed that in *ob/ob* mice the liver is packed with micro- and macrovesicular fat that was visibly reduced after tropisetron or palonosetron treatment (Fig. 2). If the *ob/ob* mice were treated for 6 weeks with tropisetron, steatosis was markedly reduced by ~30%, and hepatic in-

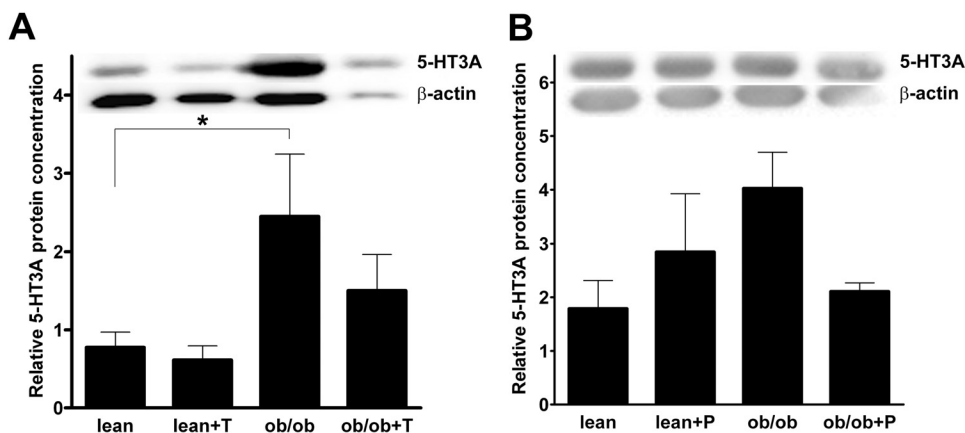


Fig. 1. Relative protein expression of the 5-HT3A in the duodenum of lean mice and *ob/ob* mice treated with tropisetron (A) or palonosetron (B), respectively. All experiments include four animal groups, genetically normal mice (lean), lean mice treated with tropisetron (lean + T) or palonosetron (lean + P), leptin-deficient mice (*ob/ob*), and *ob/ob* mice treated with tropisetron (*ob/ob* + T) or palonosetron (*ob/ob* + P). Western blot data from one representative experiment and combined data from several experiments are shown ($n = 5-7$ per group for tropisetron experiments, $n = 3-5$ per group for palonosetron experiments; means \pm S.E.M.). Statistical analysis (for details, see *Materials and Methods*): *, $p < 0.05$.

TABLE 2

Effect of 5-HT₃R antagonists on body weight, liver weight, and inflammatory parameters in lean and obese ob/ob miceMeans \pm S.E.M. are shown. Significant differences are indicated by small letters.

Parameters	lean	lean + T	lean + P	ob/ob	ob/ob + T	ob/ob + P
Tropisetron						
<i>n</i>	5–7	5–7		5–6	5–6	
Body weight, g	23.5 \pm 0.26	22.9 \pm 0.37		45.9 \pm 0.58 ^{a,b}	46.7 \pm 0.51 ^{a,b}	
Liver weight, g	1.28 \pm 0.04	1.28 \pm 0.07		3.71 \pm 0.11 ^{a,b}	3.25 \pm 0.08 ^{a,b,c}	
Liver/body weight ratio, %	5.49 \pm 0.23	5.59 \pm 0.29		8.09 \pm 0.26 ^{a,b}	6.98 \pm 0.20 ^{a,b,c}	
ALT, units/l	14.31 \pm 6.50	15.91 \pm 5.50		60.29 \pm 12.58 ^{a,b}	25.72 \pm 2.05 ^c	
Neutrophils, no. per field	0.29 \pm 0.10	0.63 \pm 0.12		2.97 \pm 0.77 ^{a,b}	1.40 \pm 0.16	
Palonosetron						
<i>n</i>	5		3–4	5		3–4
Body weight, g	21.0 \pm 0.20		22.2 \pm 0.08	56.8 \pm 1.59 ^{a,b}		59.7 \pm 0.24 ^{a,b}
Liver weight, g	1.07 \pm 0.06		1.23 \pm 0.04	3.51 \pm 0.27 ^{a,b}		3.16 \pm 0.20 ^{a,b}
Liver/body weight ratio, %	5.10 \pm 0.26		5.54 \pm 0.28	6.16 \pm 0.32 ^a		5.96 \pm 0.36 ^a
TNF- α , relative liver protein expression	0.016 \pm 0.010		0.0017 \pm 0.001	0.032 \pm 0.012		0.022 \pm 0.010

T, tropisetron; P, palonosetron.

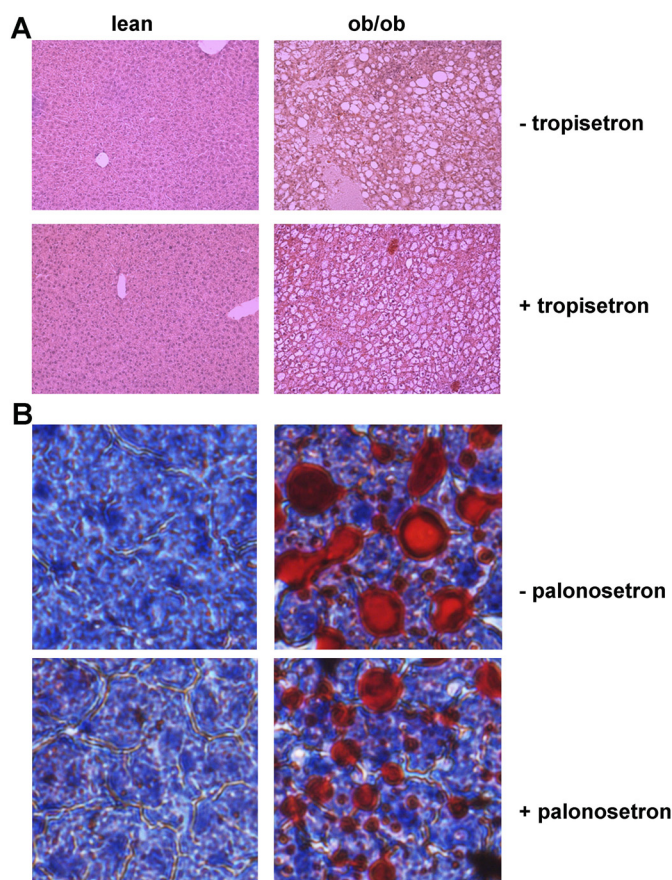
^a $p < 0.05$ compared with lean mice without treatment.^b $p < 0.05$ compared with lean mice-fed water containing drug.^c $p < 0.05$ compared with ob/ob mice without treatment.

Fig. 2. Effect of the tropisetron treatment with 5-HT₃R antagonists on fat accumulation in lean and ob/ob mice. A, effect of tropisetron treatment (representative photomicrographs of liver tissue stained with H&E, 100 \times). B, effect of palonosetron (Oil Red O staining, 400 \times).

flammation and necrosis were reduced by approximately 50 to 60% ($p < 0.05$) (Fig. 3). Consistent with these treatment-related benefits on hepatic histology, tropisetron significantly decreased plasma ALT levels and numbers of infiltrated neutrophils in the liver (Table 2A). However, neither treatment with tropisetron nor palonosetron normalized the indices of liver damage completely.

To clarify the mechanism of the beneficial effects of tropisetron and palonosetron, we studied the hepatic endotoxin/

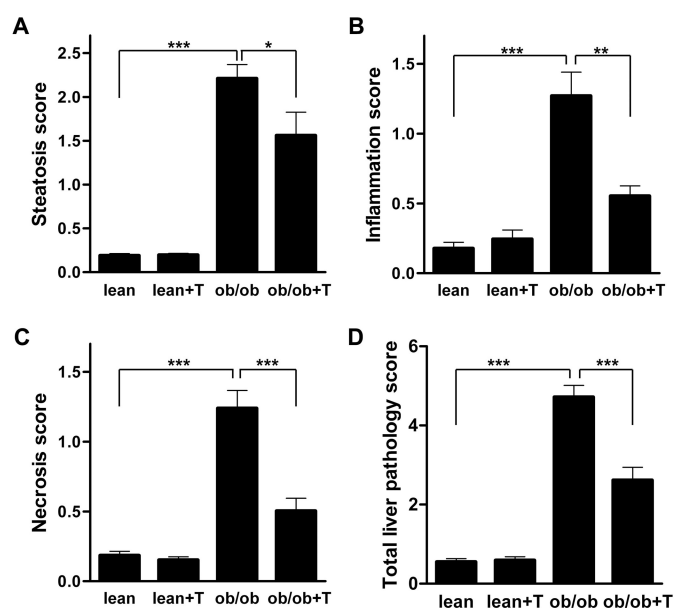


Fig. 3. Effect of tropisetron on liver pathology. Four animal groups (as indicated in Fig. 1) were examined regarding liver steatosis (A), liver inflammation (B), liver cell necrosis (C), and overall liver pathology (D) using established scores described under *Materials and Methods*. Means \pm S.E.M. are shown for all groups ($n = 5-7$ per group, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Toll-like receptor (TLR) 4 signaling cascade because lipopolysaccharide translocation from the intestine has been suggested to be a relevant trigger for the development of NAFLD (Bergheim et al., 2008). Expression of MyD88 mRNA in the liver was significantly increased by ~ 1.9 -fold in ob/ob compared with normal control mice. Treatment with tropisetron decreased MyD88 mRNA expression almost to the level of lean controls (Fig. 4A). The protective effects of tropisetron on hepatic MyD88 mRNA expression were associated with an almost complete normalization of hepatic TNF- α mRNA expression, which was induced by ~ 11 -fold in ob/ob control mice ($p < 0.001$) (Fig. 4C). The mRNA data on MyD88 and TNF- α could be confirmed on the protein level, although the drug-induced reductions did not reach statistical significance, probably because of the large variation of protein levels between the animals (Fig. 4, B and D). In tendency, the

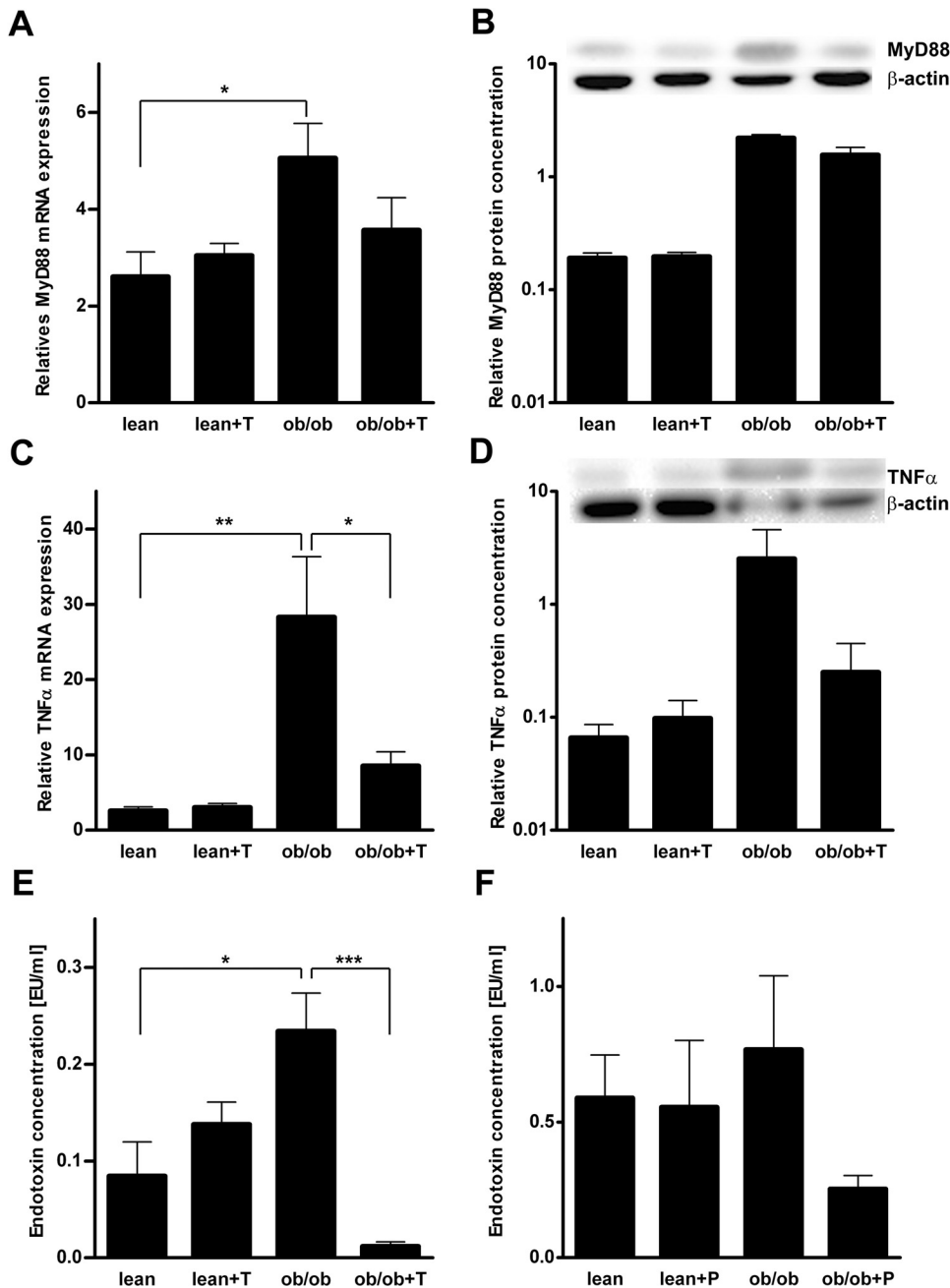


Fig. 4. Markers of liver inflammation and endotoxin translocation. A–D, expression of MyD88 mRNA (A) and protein (B) and of TNF- α mRNA (C) and protein (D) in the liver with or without tropisetron treatment. E and F, endotoxin levels in portal vein plasma after treatment with tropisetron (E) or palonosetron (F). All parameters were measured 6 weeks after treatment with drugs or vehicle in lean or *ob/ob* mice. Means \pm S.E.M. are shown. Group sizes and statistical analysis as described in Fig. 1. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

TNF- α data could be confirmed in another set of experiments in which mice were treated with palonosetron (Table 2).

To further delineate the mechanisms underlying the hepatoprotective effect of tropisetron and palonosetron in *ob/ob* mice, we determined the level of endotoxin in the portal blood plasma. We found that elevated endotoxin levels observed in *ob/ob* mice (up to 2.7-fold in comparison to lean controls for tropisetron; see Fig. 4E) were normalized or became even lower than normal in *ob/ob* mice treated with tropisetron or palonosetron (Fig. 4, E and F). These data strongly suggest that tropisetron, as well as palonosetron treatment, alters intestinal permeability for microbiota-derived endotoxin. To answer the question of whether these results might be related to an altered tight junction protein expression, we determined protein concentrations of occludin and claudin-1 in the duodenum. Indeed, the protein concentration of occludin

in the duodenum of *ob/ob* control mice was tendentially decreased by approximately 30% in *ob/ob* mice compared with normal littermates (Fig. 5A); however, this finding was not consistent (Fig. 5, B and C). More importantly, in *ob/ob* mice, but not in normal mice, treatment with tropisetron or palonosetron caused a significant enhancement by ~4- to 6-fold of the protein levels of occludin in the duodenum (Fig. 5, A and C). Moreover, claudin-1 protein concentrations in the duodenum were also increased in *ob/ob* mice treated with tropisetron (Fig. 5B).

The effect of tropisetron on the intestinal serotonergic system was further assessed by measuring the number of 5-HT-positive cells, total 5-HT content, and protein levels of SERT in the duodenum (Fig. 6). The number of 5-HT-positive cells remained unchanged in *ob/ob* mice compared with lean mice, independent of whether or not animals were treated with

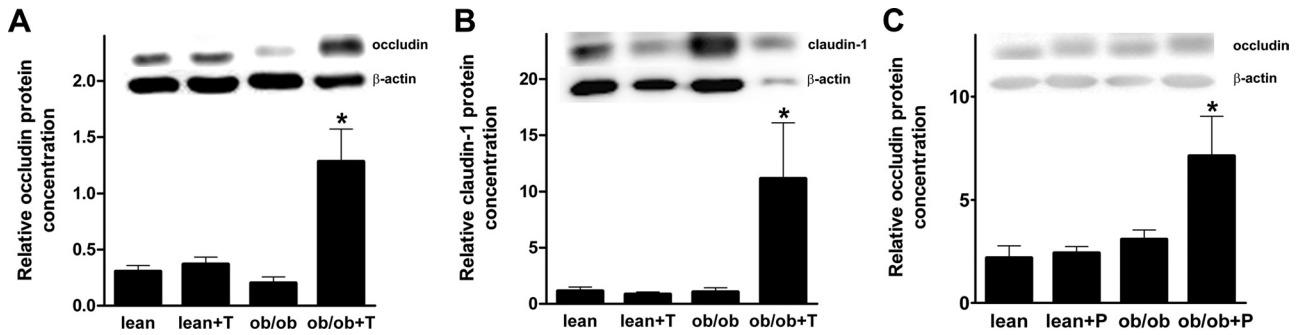


Fig. 5. Effect of tropisetron and palonosetron treatment on tight junction protein expression in the duodenum. Representative Western blots of occludin (A and C) and claudin-1 (B) with or without treatment with tropisetron (A and B) or palonosetron (C), respectively, are shown (animal groups and group sizes as in Fig. 1). *, $p < 0.05$.

tropisetron. However, total content of 5-HT in the duodenum, which was markedly increased in *ob/ob* mice, suggesting a higher level of 5-HT production and/or release per enterochromaffin cell in these animals compared with lean mice, was virtually normalized after treatment with tropisetron. A possible explanation for this finding could be that the protein concentration of SERT, which was similar in lean and *ob/ob* mice, was elevated in *ob/ob* animals treated with tropisetron (Fig. 6).

Discussion

The present study shows that treatment with the 5-HT₃R antagonists tropisetron and palonosetron improves liver pathology in a genetic model of obesity-associated NAFLD. The beneficial effects of the treatment were associated with a marked decrease of the intestinal translocation of bacterial endotoxin—most probably because of the stabilizing effect of tropisetron and palonosetron on the intestinal barrier integrity, as suggested by the induction of occludin concentration in *ob/ob* mice.

Our findings extend our previous findings using tropisetron in a mouse model of glucose-induced obesity (Weber et al., 2009) and suggest that 5-HT₃R antagonism attenuates NAFLD independent of detailed mechanisms leading to NAFLD. Moreover, the relevance of our results is emphasized considering the findings of Brun et al. (2007), who showed that a loss of the tight junction proteins occludin and ZO-1 is associated with the development of NAFLD in *ob/ob* mice. However, in our experiments, we saw only a slight decrease of occludin, but not claudin-1, in *ob/ob* mice.

As a consequence of a decreased endotoxin influx caused by tropisetron, the TLR4-dependent signaling in the liver, as determined by MyD88 and TNF- α mRNA expression, is attenuated. The fact that palonosetron attenuated TNF- α , but not MyD88, may be either because of unspecific binding of tropisetron to other receptors than 5-HT₃R, or because of the limited number of experiments we could perform with palonosetron. For example, tropisetron is known to act as a partial agonist of the α 7-acetylcholine receptor through which TLR4-dependent signaling might be modulated or other anti-inflammatory effects might be triggered (Abdrakhmanova et al., 2010; Kohnomi et al., 2010). Nevertheless, our findings might be of clinical importance because several previous studies from various groups clearly showed that an impairment of the intestinal barrier, which results in enhanced translocation of bacterial lipopolysaccharides, is a

major mechanism leading to fatty liver disease (Arteel, 2003; Bode and Bode, 2003; Bergheim et al., 2008). In turn, any dietetic or pharmaceutical approach that supports stabilization of the intestinal barrier could improve fatty liver disease. The pronounced decrease of endotoxin levels in the portal vein caused by tropisetron and palonosetron is probably a result of the strong induction of occludin and, after tropisetron treatment, claudin-1 protein concentration in the intestine as well. The enhanced expression of occludin can be interpreted as a kind of protective mechanism by which these drugs attenuate fatty liver disease. Alternatively, tropisetron and palonosetron might modulate not only expression but also function of tight junction proteins and other barrier-regulating molecules in the intestine.

Our finding that SERT protein concentrations are also affected by tropisetron points to more than one mechanism by which tropisetron and also probably palonosetron attenuate liver damage in *ob/ob* mice. SERT up-regulation could compensate for increased 5-HT secretion occurring in inflammatory conditions (Bischoff et al., 2009), whereas occludin and claudin-1 up-regulation could counteract the gut barrier impairment associated with enhanced 5-HT₃R expression and signaling. Indeed, experimental data in the rat showed that expression of tight junction proteins in the duodenum is down-regulated by 5-HT, possibly through 5-HT₃R, and can be normalized by administration of tropisetron (Yamada et al., 2003). 5-HT receptors other than 5-HT₃R might be involved because, in mammary gland epithelial cells, the 5-HT₇R located on the basal side of the cells is involved in tight junction regulation (Stull et al., 2007). The mechanisms of how leptin deficiency and/or the deficient immune system of *ob/ob* mice lead to an up-regulation of the 5-HT₃A in the intestine are unknown. In either way, *ob/ob* mice could be either more sensitive to 5-HT signaling or exposed to an increased extracellular 5-HT concentration because of reduced SERT protein content or both. Our previous finding supports the assumption that SERT protein concentration is reduced in a mouse model of fructose-induced NAFLD (Haub et al., 2010), which was associated with an increased intestinal permeability. The present study, together with our previous studies, shows that tropisetron can improve NAFLD in different experimental models of obesity. The effect is probably 5-HT₃R-dependent because similar effects were obtained with the more specific 5-HT₃R antagonist, palonosetron. Independent of whether the cause of obesity was overfeeding (Weber et al., 2009) or genetic alterations in the leptin and immune system (the present study), the animals

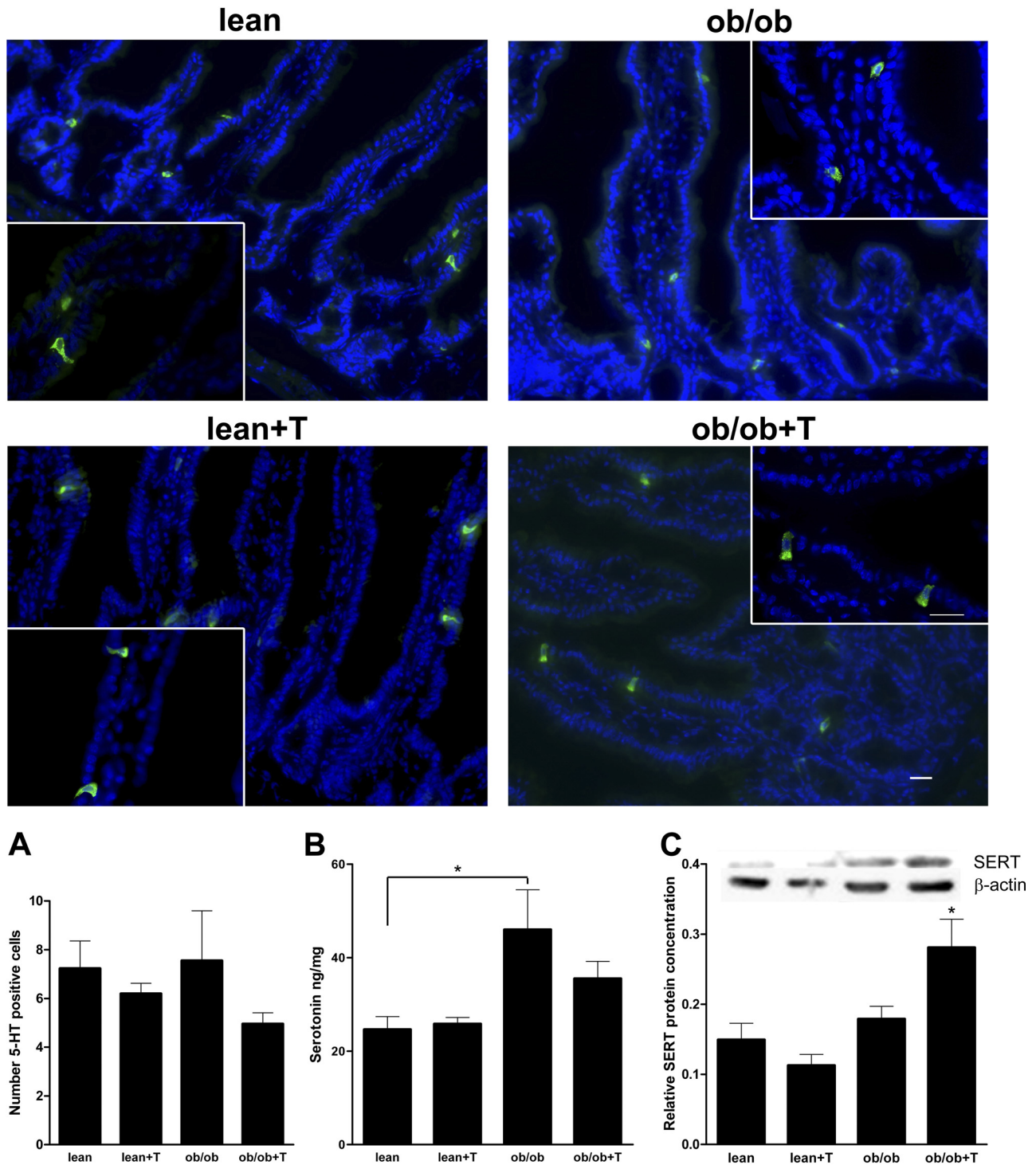


Fig. 6. Effect of tropisetron treatment on the intestinal serotonergic system. Top, the numbers of 5-HT-positive cells are shown by representative immunostainings of duodenal tissue derived from obese mice (*ob/ob*) and their lean littermates (*lean*) with or without treatment with tropisetron (T). White bars indicate 20 μ m. The immunofluorescent data are summarized in A. The 5-HT content in the duodenal tissue assessed by enzyme-linked immunosorbent assay is shown in B. A representative Western blot of SERT and β -actin as well as the combined data of the blots are shown in C. In all panels, means \pm S.E.M. from five to seven independent experiments with different animals are shown. Statistical analysis as in Fig. 1 (*, $p < 0.05$).

showed signs of enhanced 5-HT signaling and impaired intestinal barrier function resulting in enhanced influx of endotoxin and subsequent induction of inflammation and fat deposition in the liver. Fat deposition results in increased liver weight and liver-to-body ratio in *ob/ob* mice that was reduced after tropisetron but not after palonosetron treatment. If this discrepancy results from the limited numbers of

experiments or, as speculated in the literature (Hookman and Barkin, 2003), from a reduction of fat redistribution from liver to adipose tissue depots possibly regulated via the 5-HT₃R is unclear at present.

It is clear that our present data suggest that the spectrum of 5-HT functions can be extended to regulation of epithelial tight junctions and mucosal permeability in addition to the

well established intestinal functions, such as regulation of peristalsis, secretion, and sensibility (Costedio et al., 2007; Spiller 2007; Margolis and Gershon, 2009). Because tropisetron is not a fully specific 5-HT₃R, we cannot confirm that the effects we observed are solely mediated by the 5-HT₃R expressed in the intestine. For example, tropisetron also blocks 5-HT₄R-mediating intestinal motility, but only at high doses that are hardly reached in our study (Talley, 1992). Murine studies with tropisetron and other 5-HT₃R as well as 5HT₄R antagonists revealed that motility is disturbed in the duodenum after treatment with all of these drugs, with the exception of tropisetron, suggesting that its effect on the 5-HT₄R is of minor extent (Nylander and Pihl, 2006). Another particular feature of tropisetron is its anti-inflammatory effect that has been shown in experimental colitis in the rat, for example (Mousavizadeh et al., 2009). It is not clear from the literature to which extent this effect is dependent on 5-HT₃R blockage or on other mechanism such as modulation of the α 7-acetylcholine receptor pathway that also mediates anti-inflammatory effects (Abdrakhmanova et al., 2010; Kohnomi et al., 2010). Alternatively, the calcineurin pathway in human T-cells might play a role in the anti-inflammatory effects of tropisetron. Vega Lde et al. (2005) reported that tropisetron inhibits T-cell activation by targeting the calcineurin pathway, resulting in decreased interleukin-2 synthesis. These findings provide some evidence for 5-HT₃R-independent anti-inflammatory activity of tropisetron; however, these in vitro studies need to be confirmed in vivo. Finally, we cannot rule out central effects being involved in the liver-protecting effects, because tropisetron also acts centrally in humans, for example, by reducing chemotherapy-induced emesis (Gan, 2005). On the other hand, we provide some evidence for the notion that at least parts of the tropisetron effects are indeed 5-HT₃R-dependent by showing that similar effects can be obtained with the more specific 5-HT₃R antagonist palonosetron.

Taken together, our findings clearly indicate that 5-HT is an important modulator of intestinal integrity in experimental obesity resulting in loss of tight junctions, translocation of bacterial endotoxin, and development of NAFLD. Furthermore, we show that the 5-HT₃R antagonist tropisetron, through 5-HT₃R blockage and possibly other mechanisms, can attenuate pathological events such as fat deposition in the liver, leading to the development of NAFLD. The latter mechanism is similar to that described previously for alcohol-induced liver disease (Arteel, 2003; Bode and Bode, 2003). Therefore, it is tempting to speculate that 5-HT₃R blockage could also improve liver damage in alcohol-induced fatty liver disease. At present, it is unclear what induces 5-HT release in obesity and how the immune system and other mechanisms apart from 5-HT are involved in the pathophysiology of obesity-associated fatty liver disease. However, tropisetron and palonosetron seem to be new therapeutic options that deserve further investigation.

Authorship Contributions

Participated in research design: Bischoff and Trautwein.

Conducted experiments: Haub, Ritze, Ladel, Saum, and Hubert.

Performed data analysis: Haub and Ritze.

Wrote or contributed to the writing of the manuscript: Bischoff, Trautwein, Haub, and Ritze.

Other: Bischoff for acquired funding for the research.

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Address correspondence to: Dr. Stephan C. Bischoff, Department of Nutritional Medicine (180), University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany. E-mail: bischoff.stephan@uni-hohenheim.de
