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Metformin protects against the development of fructose-induced steatosis in mice: role of the intestinal barrier function

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To test the hypothesis that metformin protects against fructose-induced steatosis, and if so, to elucidate underlying mechanisms, C57BL/6J mice were either fed 30% fructose solution or plain water for 8 weeks. Some of the animals were concomitantly treated with metformin (300 mg/kg body weight/day) in the drinking solution. While chronic consumption of 30% fructose solution caused a significant increase in hepatic triglyceride accumulation and plasma alanine-aminotransferase levels, this effect of fructose was markedly attenuated in fructose-fed mice concomitantly treatment with metformin. The protective effects of the metformin treatment on the onset of fructose-induced non-alcoholic fatty liver disease (NAFLD) were associated with a protection against the loss of the tight junction proteins occludin and zonula occludens 1 in the duodenum of fructose-fed mice and the increased translocation of bacterial endotoxin found in mice only fed with fructose. In line with these findings, in metformin-treated fructose-fed animals, hepatic expression of genes of the toll-like receptor-4-dependent signalling cascade as well as the plasminogen-activator inhibitor/cMet-regulated lipid export were almost at the level of controls. Taken together, these data suggest that metformin not only protects the liver from the onset of fructose-induced NAFLD through mechanisms involving its direct effects on hepatic insulin signalling but rather through altering intestinal permeability and subsequently the endotoxin-dependent activation of hepatic Kupffer cells.

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Recently, it has become more and more obvious that hepatic steatosis, which for a long time had been thought of as a relatively benign state of liver injury, actually increases the vulnerability of the liver to injuries from various causes.[1] The development of non-alcoholic fatty liver disease (NAFLD) is frequently associated with obesity and insulin resistance and ranges from simple steatosis to steatohepatitis and cirrhosis.[2] Despite intense research efforts throughout the last decade, mechanisms involved in the development of NAFLD are still not fully understood and therapeutic as well as preventive strategies are still rather limited.

Results of several epidemiological studies performed in different countries suggest that besides a general over-nutrition a diet rich in carbohydrates and/or a dietary pattern leaning toward a high intake of fructose (eg, as high-fructose corn syrup) may be a critical factor in the development of NAFLD.[3,4] In line with the results of these human studies, it was shown in several animal experiments that a diet rich in fructose (eg, up to 60% of the daily caloric intake) may lead to the development of NAFLD in rodents but may also result in the development of insulin resistance, dyslipidemia, and oxidative stress.[5–8] We recently found that the development of fructose-induced steatosis was associated with an increased translocation of bacterial endotoxin and activation of toll-like receptor (TLR)-4-dependent signalling cascades in the liver.[7,9] Interestingly, similar alterations were not found in mice chronically exposed to glucose; however, chronic intake of glucose resulted in a markedly higher weight gain of mice in comparison with fructose.[7]

Metformin (dimethylbiguanide) is a drug used in the treatment of type 2 diabetes for >50 years. During more recent years, it was shown that metformin may have additional beneficial effects besides lowering blood glucose levels (for overview, see Duvnjak *et al*[10]). For example, results of

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several clinical studies have shown that metformin may improve liver status of patients with NAFLD.[11–13] In line with these findings, it was reported that metformin also improves liver damage in ob/ob mice through mechanisms involving an interference with tumor necrosis factor (TNF)α-dependent signalling cascades.[14] Furthermore, results of animal studies also have shown that metformin may exert protective effects on the development of alcohol- and endotoxin-induced liver diseases through inhibiting the induction of the plasminogen-activator inhibitor (PAI)-1.[15] Here, using a mouse model of fructose-induced liver steatosis, the hypothesis that metformin protects against fructose-induced liver damage was tested, and potential mechanisms involved in this effect were determined.

MATERIALS AND METHODS

Animals and Treatments

Six- to eight-week-old C57BL/J6 mice (Janvier SAS, Le-Genest-St-Isle, France) were housed in a pathogen-free barrier facility accredited by the AAALAC. All procedures were approved by the local IACUC. Mice ($n = 4$–6 per group) had free access either to plain tap water or to water containing 30% fructose solution ± 300 mg metformin/kg body weight/day added to the drinking solution for 8 weeks. Body weight as well as consumption of chow and drinking solution was assessed twice weekly over the 8-week feeding period. Animals were anesthetized with 80 mg ketamine and 6 mg xylazine/kg body weight by i.p. injection and blood was collected from the portal vein. While portions of the liver and duodenum were frozen immediately others were fixed in neutral-buffered formalin, or frozen-fixed in OCT mounting media (Medite, Burgdorf, Germany). Mesenteric lymph nodes were taken from upper part of the small intestine and fixed in neutral-buffered formalin, as well.

Triglyceride Determination in Liver

Liver samples were homogenized in ice-cold 2 × phosphate-buffered saline. Tissue lipids were extracted with methanol/chloroform,[1,2] dried, and resuspended in 5% fat-free bovine serum albumin. Triglyceride levels were determined using a commercially available kit (Randox, Krefeld, Germany). Values were normalized to protein concentration measured by Bradford assay in the liver homogenate used for the triglyceride isolation (Bio-Rad Laboratories, Munich, Germany).

Oil Red O Staining

Frozen sections of liver (10 μm) were stained with oil red O (Sigma, Steinheim, Germany) for 12 min, washed, and counterstained with hematoxylin for 45 s (Sigma) to determine hepatic lipid accumulation. Representative photomicrographs were captured at a × 100 magnification using a system incorporated in a microscope (Axio Vert 200M; Zeiss, Jena, Germany).

Neutrophils Staining in Livers and Staining of Bacteria in the Small Intestine as well as in MLNs of Mice

Paraffin-embedded liver sections (5 μm) were stained for neutrophils using a commercially available Naphthol AS-D Chloroacetate Estearse kit (Sigma). Neutrophils were counted in eight microscopic fields (× 200) of each liver section to determine means. Moreover, paraffin-embedded tissue sections of the small intestine and mesenteric lymph nodes (5 μm) were stained with toluidine blue solution for 60 min. Representative pictures were taken at a × 400 magnification.

RNA Isolation and Real-Time RT-PCR

After extraction of total RNA from liver and duodenum samples using peqGOLD TriFast (PEQLAB, Erlangen, Germany), RNA concentrations were determined spectrophotometrically. In all, 1 μg total RNA was reverse transcribed after a DNase digestion (Fermentas, St Leon-Rot, Germany) using a MuLV reverse transcriptase and oligo dT primers. PCR primers for myeloid differentiation factor 88 (MyD88), inducible nitric oxide synthase (iNOS), interferon regulatory factor (IRF)-3 and -7, TNFα, PAI-1, apolipoprotein B (ApoB), interferon γ (IFNγ), interleukin 1β (IL-1β), matrix metalloproteinase (MMP)-9,-13, and β-actin as well as 18S (for primer sequences, see Table 1) were designed using Primer3 software (Whitehead Institute for Biomedical Research). Sybr Green Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was used to prepare the PCR mix. Amplification reactions were carried out in an iCycler (Bio-Rad Laboratories) with an initial hold step (95°C for 3 min) and 50 cycles of a three-step PCR (95°C for 15 s, 60°C for 15 s, and 72°C for 30 s). To monitor amplification of the target gene, the fluorescence intensity of each sample was measured at each temperature change. The comparative C_T-method was used to determine the amount of target, normalized to an endogenous reference (β-actin in the liver and 18S in the duodenum) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of the PCR products was verified by melting curves and gel electrophoresis.

Clinical Chemistry and ELISA for RBP4, TIMP-1, Leptin, and Adiponectin

Alanine-aminotransferase (ALT) activity was determined using a commercially available kit (Randox). Furthermore, plasma levels of retinol binding protein (RBP)-4 (Alpco Diagnostics, Salem, USA), leptin (Abcam, Cambridge, UK), as well as adiponectin (Alpco Diagnostics), and protein concentration of tissue inhibitor of MMP (TIMP)-1 (RayBiotech, Norcross, USA) in duodenum of mice were determined using commercially available ELISA kits.

Endotoxin Assay

To determine plasma endotoxin levels, samples were heated at 70°C for 20 min. Endotoxin plasma levels were measured using a commercially available limulus amebocyte lysate assay with a concentration range of 0.015–1.2 EU/ml (Charles River, L'Arbaesle, France).

Table 1 Primers used for real-time RT-PCR detection of ApoB, PAI-1, TNFα, iNOS, MyD88, IRF3, IRF7, IFNγ, IL-1β, MMP-9, -13, β-actin, and 18S

	Forward (5′–3′)	Reverse (5′–3′)
ApoB	TCA CCA TTT GCC	CAG GTC AAC ATC
	CTC AAC CT	GGC AAT CA
PAI-1	TCC AAG GGG CAA	GAC GAA GAG CCA
	CGG ATA GA	GGC ACA CA
TNFα	CCA GGC GGT GCC	CAG CCA CTC CAG
	TAT GTC TC	CTG CTC CT
iNOS	CAG TG GGC TGT ASA	CAT TGG AAG TGA
	AAC CTT	AGC GTT TCG
MyD88	CAA AAG TGG GGT	AAA TCC ACA GTG
	GCC TTT GC	CCC CCA GA
IRF3	AAC CGG AAA GAA	GCA CCC AGA TGT
	GTC TTG CG	ACG AAG TCC
IRF7	ACA GGG CGT TTT	TCC AAG CTC CCG
	ATC TTG CG	GCT AAG
IFNγ	CAC TGC AGC TCT	CAA GCG GCT GAC
	GAA TGT TTC TTA TT	TGA ACT CA
IL-1β	TGG CTG TGG AGA	GTC CGA CAG CAC
	AGC TGT GG	GAG GCT TT
MMP-9	CTG GAC TCC GCC	AGA CAC GCC CCT
	TTT GAG GA	TGC TGA AC
MMP-13	GTC CCT GCC CCT	TCG GAG CCT GTC
	TCC CTA TG	AAC TGT GG
β-Actin	GGC TCC TAG CAC	AGC CAC CGA TCC
	CAT GAA	ACA CAG A
18S	GTA ACC CGT TGA	CCA TCC AAT CGG
	ACC CCA TT	TAG TAG CG

Immunohistochemical Staining for 4-Hydroxynonenal Protein Adducts in Liver and Occludin as well as Zonula Occludens 1 Protein in Duodenum

Paraffin-embedded liver sections (5 μm) were stained for 4-hydroxynonenal (4-HNE) protein adducts using a polyclonal antibody (AG Scientific, San Diego, USA) as described previously.[7] In addition, paraffin-embedded sections (5 μm) of duodenum were pre-incubated with protease (2 mg/ml) (Sigma) and stained with polyclonal primary antibodies for occludin as well as zonula occludens 1 (ZO-1) protein (Invitrogen, Camarillo, USA). In brief, to detect specific binding of primary antibody, tissue sections were incubated with a peroxidise-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit; DAKO, Hamburg, Germany). The extent of staining in sections of liver and duodenum defined as percent of the field area within the default color range was determined using an image acquisition and analysis system incorporated in the microscope. Data from eight fields (× 200) of each liver tissue section and five fields (× 400) of each duodenum tissue section were assessed to determine means.

Western Blot Analysis

For determination of the hepatic phosphorylation status of cMet as well as duodenal occludin protein concentration, liver and duodenum tissue were homogenized, respectively, in RIPA lysis buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (v/v), 0.1% SDS (w/v)) containing a protease and phosphatase inhibitors mix (Sigma). Proteins were separated in an 8% SDS-polyacrylamide gel for measurement of phospho-cMet and cMet, and a 10% gel for occludin and β-actin. Proteins were transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences, Freiburg, Germany). Blots were then probed with antibodies against phospho-cMet and total cMet (both from Cell Signaling Technology, Danvers, USA) or occludin (Invitrogen), and β-actin (Cell Signaling Technology), respectively. Bands were visualized using Super Signal Western Dura kit (Thermo Scientific, Rockford, USA). To ensure equal loading, all blots were stained with Ponceau red. Protein bands were densitometrically analyzed using the Flurochem Software (Alpha Innotech, San Leandro, USA).

Statistical Analyses

All results are reported as means ± standard error of mean (s.e.m.). One-way ANOVA with Tukey's *post hoc* test was used for the determination of statistical significance between treatment groups. A *P*-value of <0.05 was selected as the level of significance before the study was performed.

RESULTS

Effect of Metformin Treatment on Fructose-Induced Hepatic Steatosis, Markers of Hepatic Inflammation and Markers of Insulin Resistance

In line with previous studies of our own group and those of others,[3,4,7,9,16] chronic intake of fructose resulted in a marked accumulation of triglycerides in the liver (~3-fold in comparison with controls, $P<0.05$; Figure 1). In contrast, in fructose-fed mice concomitantly treated with metformin triglyceride levels were only increased by ~50% in comparison with the respective controls ($P<0.05$). In line with these findings, plasma ALT levels of fructose-fed mice were significantly higher than those of all other groups (Table 2). Furthermore, number of neutrophils in the liver was significantly higher in fructose-fed mice than in controls whereas in fructose-fed mice concomitantly treated with metformin number of neutrophils was at the level of controls (Table 2). Expression of IFNγ mRNA was also markedly higher in livers of fructose-fed mice than in all other groups; however, as data varied considerably differences did not reach level of significance (Table 2). Expression of IL-1β mRNA was higher in livers of both fructose-fed groups

Table 2 Effect of metformin treatment on caloric intake, body weight and indices of the onset of fructose-induced steatosis

	Control	Control+metformin	Fructose	Fructose+metformin
N	4–6	4–6	4–6	4–6
Total caloric intake (kcal/g BW/week)	3.4 ± 0.1	3.3 ± 0.1	3.9 ± 0.1[a,b]	4.0 ± 0.1[a,b]
Caloric intake from fructose (kcal/g BW/week)			1.9 ± 0.1	1.8 ± 0.0
Weight gain (g)	4.8 ± 0.4	4.8 ± 0.3	5.6 ± 0.2	6.0 ± 0.4
Liver weight (g)	1.2 ± 0.0	1.2 ± 0.1	1.4 ± 0.0[a,b]	1.4 ± 0.0[a,b]
Liver-to-body weight ratio (%)	5.9 ± 0.1	5.4 ± 0.1	6.0 ± 0.1[a,b]	6.2 ± 0.1[a,b]
ALT (U/L)	2.8 ± 0.9	3.7 ± 2.2	16.2 ± 4.2[a,b,d]	4.3 ± 1.4
Neutrophils (number per microscope field)	0.4 ± 0.1	0.2 ± 0.1	1.3 ± 0.1[a,b,d]	0.5 ± 0.1
IL-1β mRNA expression (−fold induction)	2.3 ± 0.2	2.6 ± 0.5	4.4 ± 0.6	5.6 ± 1.0[a,b]
IFNγ mRNA expression (−fold induction)	3.4 ± 0.9	4.5 ± 1.3	7.6 ± 1.7	5.8 ± 0.6
RBP4 (μg/ml)	28.8 ± 0.7	30.2 ± 2.4	45.6 ± 2.4[a,b,d]	36.4 ± 2.7
Adiponectin (μg/ml)	14.2 ± 0.8	13.3 ± 0.2	12.4 ± 1.0	13.3 ± 1.5
Leptin (pg/ml)	109.2 ± 22.4	122.8 ± 13.4	1276.0 ± 382.6[a,b]	602.8 ± 173.7

Feeding of 30% fructose and metformin solution is described in 'Materials and methods'. Data are mean values ± s.e.m. ($n = 4$–6).
[a]$P < 0.05$ compared with mice fed water.
[b]$P < 0.05$ compared with mice fed water+300 mg metformin/kg body weight/day.
[d]$P < 0.05$ compared with mice fed 30% fructose solution+300 mg metformin/kg body weight/day.

(fructose + ~92%, NS in comparison with controls; fructose + metformin + ~113%, $P < 0.05$ in comparison with respective controls) (Table 2). A protective effect of the metformin treatment was also not found when comparing absolute liver weight and liver-to-body weight ratios of groups with both being significantly higher in mice fed with fructose regardless of additional treatments (Table 1). Despite markedly higher total caloric intake in both fructose-fed groups, absolute weight gain did not differ between groups as weight gain varied considerably between mice. Fructose intake per gram body weight was similar between the two fructose-fed groups (Table 1). To determine if the treatment with metformin protected mice from fructose-induced insulin resistance plasma leptin, adiponectin, and RBP-4 levels were determined (Table 2). Indeed, in line with earlier reports of our own group and those of other groups,[16–18] plasma RBP4 and leptin levels were significantly increased in fructose-fed mice in comparison with the respective controls. A similar effect of the fructose feeding was not found in fructose-fed mice treated with metformin. In contrast, plasma concentration of adiponectin varied considerable within groups (−15%, NS).

Effect of Metformin on Fructose-Induced PAI-1 Expression and Triglyceride Export in the Liver

As the protective effect of metformin on alcoholic liver disease has been shown to be dependent at least in part on its inhibitory effects on PAI-1 expression and subsequently the induction of the cMet-dependent lipid export in the liver,[15] we determined the expression of PAI-1 and ApoB as well as the phosphorylation status of cMet in the liver. Expression of PAI-1 mRNA was significantly induced by ~3.7-fold in livers of fructose-fed mice in comparison with water-fed controls (Figure 2a). In livers of fructose-fed mice concomitantly treated with metformin, this effect on PAI-1 mRNA expression was markedly attenuated. In line with these findings, hepatic phosphorylation status of cMet that did not differ between the two control groups and fructose-fed mice was significantly increased by ~2.3-fold in livers of fructose-fed mice treated with metformin (Figure 2b). Expression of ApoB in the liver also did not differ between the two control groups and fructose-fed mice; however, in livers of fructose-fed mice concomitantly treated with metformin ApoB mRNA expression was induced significantly by ~2.7-fold (Figure 2c).

Effect of Metformin Treatment on Fructose-Induced Expression of TNFα

As it has been shown by us and other groups before that the expression of PAI-1 can be regulated by the proinflammatory cytokine TNFα,[4,16,19] we determined mRNA expression of TNFα in whole liver homogenate. In line with our findings for PAI-1 expression, TNFα mRNA expression was significantly induced by ~3.7-fold in livers of fructose-fed mice; however, in livers of fructose-fed mice concomitantly treated with metformin TNFα expression was almost at the level of controls (Figure 2d).

Effect of Metformin Treatment on Fructose-Induced Induction of TLR-4-Dependent Signalling Pathways in the Liver

Results of earlier studies of our group suggest that fructose may induce TNFα expression in the liver through an

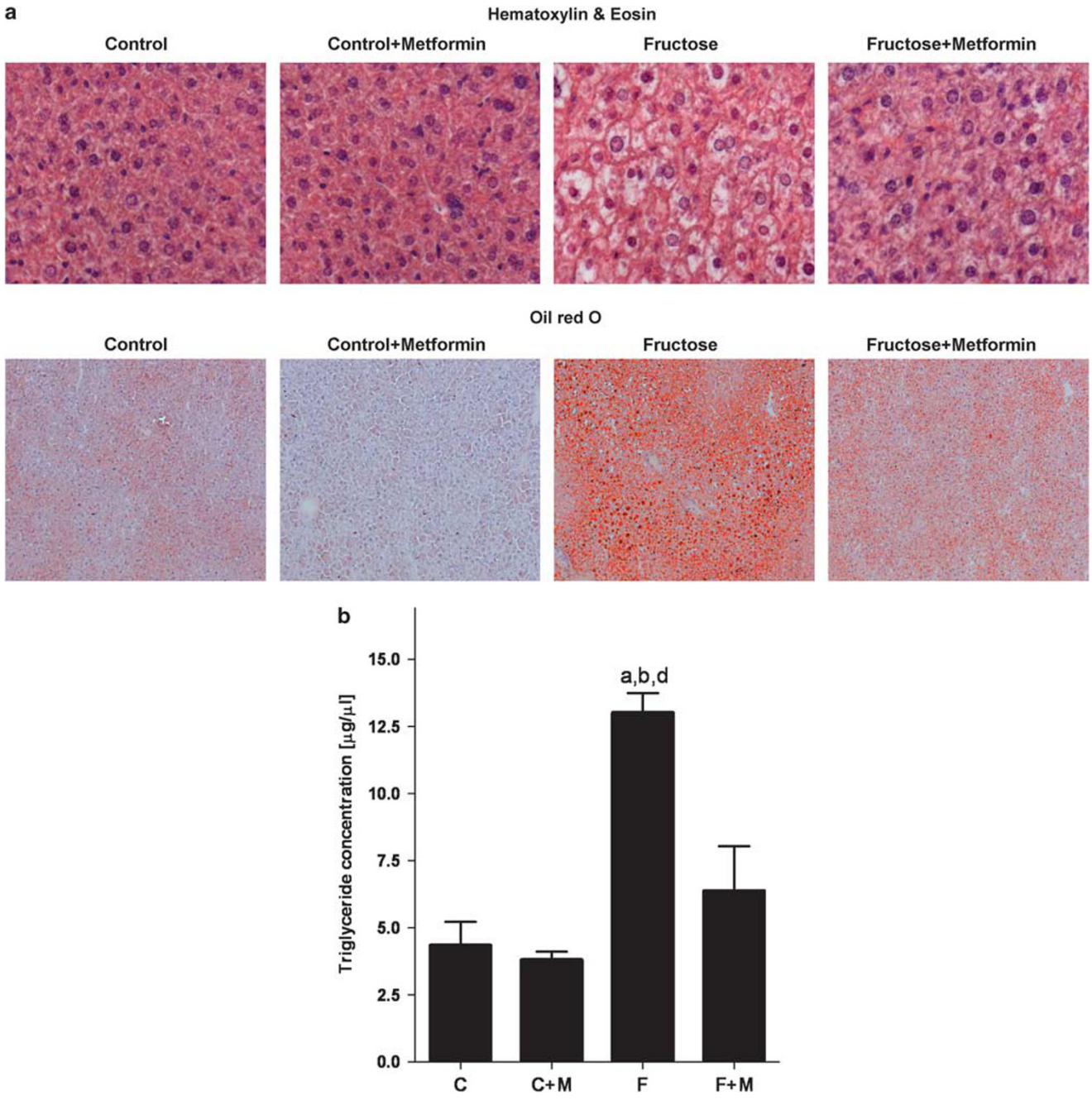

Figure 1 Effect of chronic consumption of metformin on the hepatic steatosis induced by 30% fructose solution. (**a**) Representative photomicrographs of hematoxylin and eosin staining (× 400) as well as oil red O staining of liver sections (× 100) and (**b**) quantification of the hepatic triglyceride accumulation. Data are shown as mean values ± s.e.m ($n = 4$–6). C: water; C + M: water with 300 mg metformin/kg body weight/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body/day. $^{a}P < 0.05$ compared with water-fed control mice; $^{b}P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; $^{d}P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

increased translocation of endotoxin and subsequently an activation of TLR-4-dependent signalling pathways.[7,9] To determine if the treatment with metformin may have protected livers from the onset of fructose-induced hepatic steatosis and the induction of TNFα through interfering with the TLR-4-dependent signalling cascade, we determined levels of 4-HNE protein adducts, iNOS expression, and expression of the TLR-4 adapter proteins MyD88 as well as IRF-3 and IRF-7. In line with the findings for TNFα expression levels in the liver, concentration of 4-HNE protein adducts (Figure 3a and b) and expression of iNOS mRNA (Figure 3c) were only found to be significantly elevated in

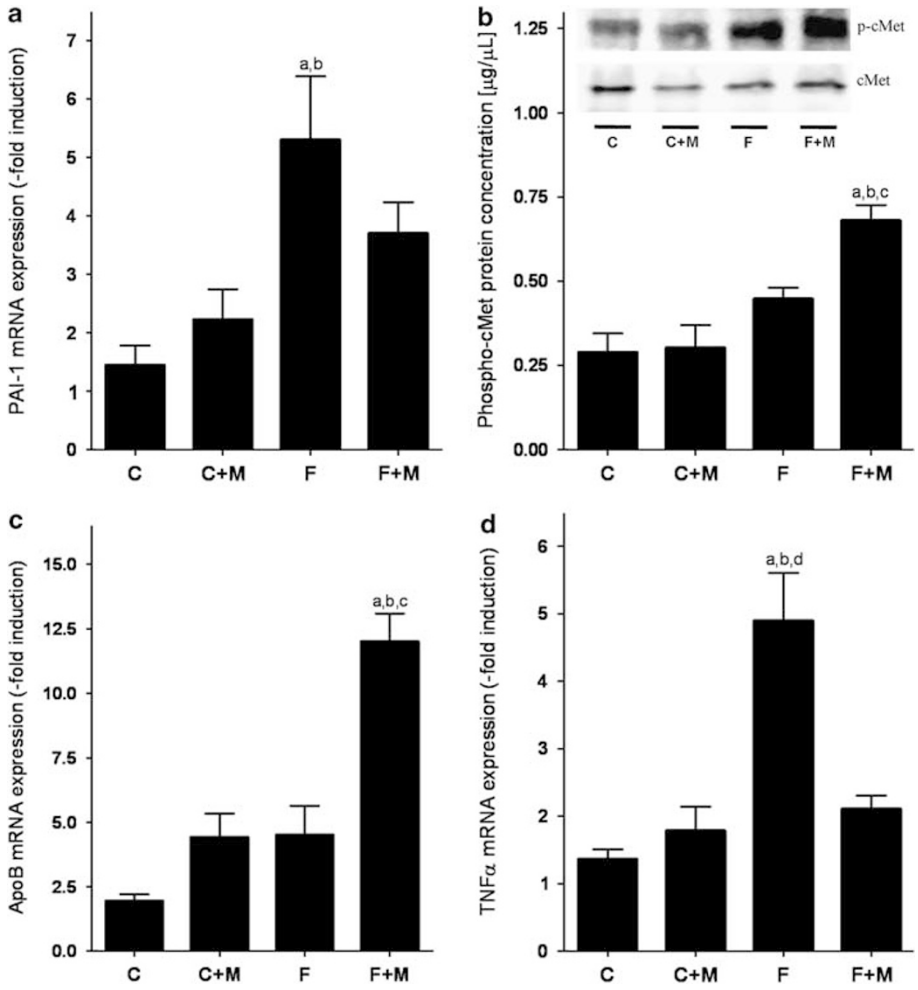

Figure 2 Effect of chronic consumption of metformin on hepatic PAI-1 expression, phosphorylation status of cMet, and ApoB as well as TNFα expression in fructose-induced steatosis. (**a**) PAI-1 mRNA expression and (**b**) representative pictures of phospho-cMet and total cMet western blots as well as quantitative analysis of blots. (**c**) ApoB and (**d**) TNFα mRNA expression. mRNA expression was determined by real-time RT-PCR and was normalized to β-actin expression in the liver. Data are shown as mean values ± s.e.m. ($n = 4$–6) and are specified as –fold induction. C: water; C + M: water with 300 mg metformin/kg body/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body weight/day. $^{a}P < 0.05$ compared with water-fed control mice; $^{b}P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; $^{c}P < 0.05$ compared with mice fed with 30% fructose solution; $^{d}P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

livers of mice fed with fructose whereas in fructose-fed mice concomitantly treated with metformin, both levels of 4-HNE protein adducts and iNOS mRNA expression remained almost at the level of controls. Furthermore, expression of the TLR-4-adaptor proteins MyD88 (Figure 4a) and IRF-3 and IRF-7 (Figure 4b and c) was significantly induced in livers of mice fed with fructose; however, in livers of fructose-fed mice concomitantly treated with metformin expression of all three adapter proteins was almost at the level of the respective controls.

Effect of Metformin Treatment on Fructose-Induced Translocation of Intestinal Bacteria and Bacterial Endotoxins as well as the Loss of the Tight Junction Protein Occludin and ZO-1 in the Duodenum

As our results suggested that the protective effect of metformin on the onset of fructose-induced steatosis may be associated with a protection against the endotoxin-dependent activation of TLR-4-dependent signalling cascades in the liver, we determined endotoxin levels in portal plasma. Levels of endotoxin in portal plasma of water controls and fructose-fed mice treated with metformin did not differ (Figure 5a). In contrast, in portal plasma of mice fed fructose plasma endotoxin levels were significantly higher by ~3.6-fold in comparison with controls. Whereas no bacteria were found in mesenteric lymphatic tissue using toluidine blue staining number of bacteria in intestinal mucosa was markedly higher in fructose-fed mice in comparison with all other groups (Supplementary Figure 1). However, number of bacteria found in the intestinal mucosa was also rather low. The increased translocation of bacterial endotoxin into the portal plasma of mice fed fructose was further associated with a marked reduction of the tight junction proteins occludin and ZO-1 in the duodenum of these

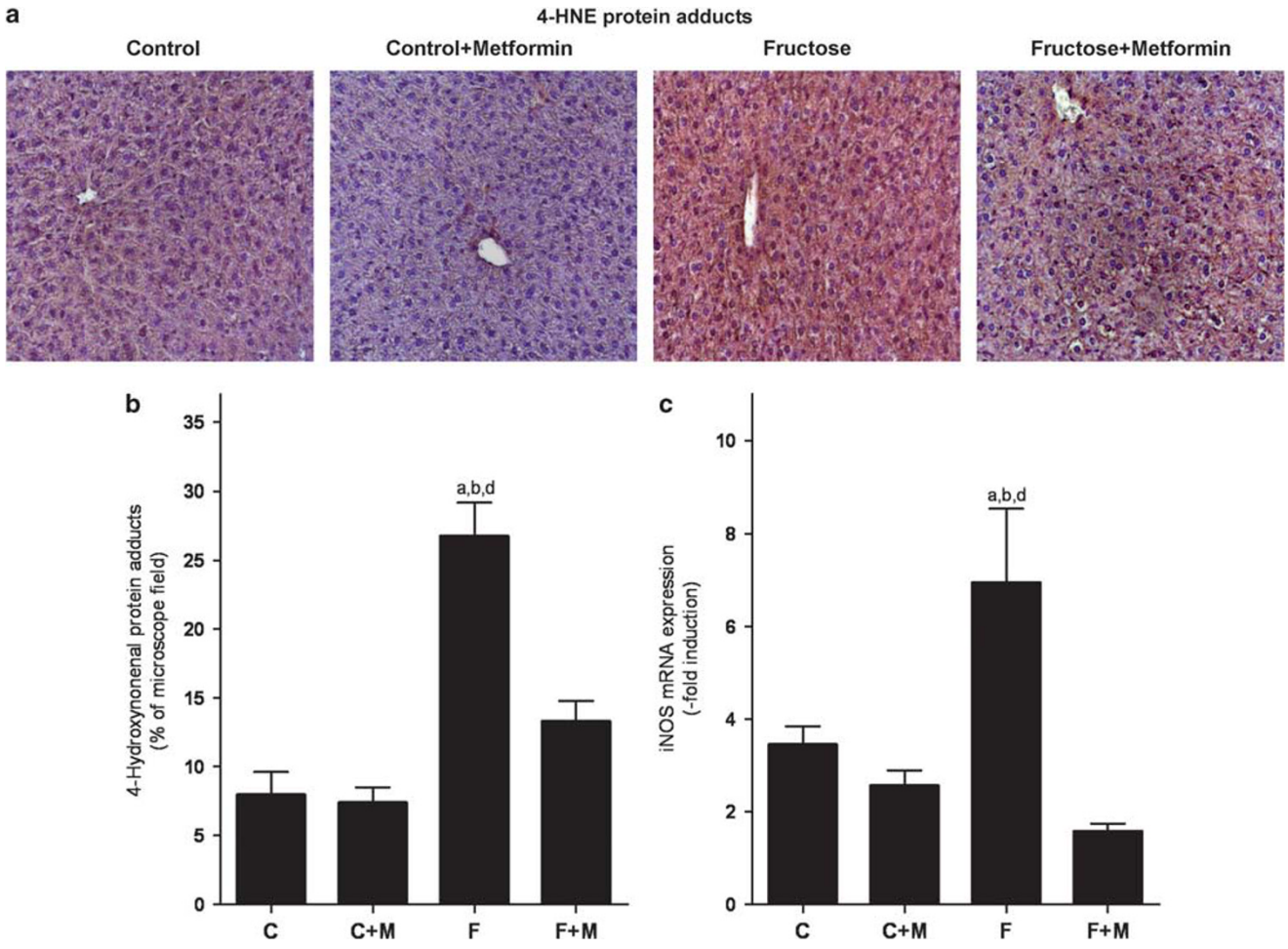

Figure 3 Effect of chronic consumption of metformin on markers of hepatic lipid peroxidation and iNOS expression in fructose-induced steatosis. (**a**) Representative photomicrographs of immunohistochemical staining of 4-HNE protein adducts (× 200) in liver and (**b**) densitometric analysis of staining. (**c**) Expression of iNOS mRNA in livers of mice. Data are shown as mean values ± s.e.m. ($n = 4$–6) and are specified as –fold induction. C: water; C + M: water with 300 mg metformin/kg body weight/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body/day. $^{a}P < 0.05$ compared with water-fed control mice; $^{b}P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; $^{d}P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

mice (Figures 5b and 6). A similar effect of fructose feeding was not found in mice concomitantly treated with metformin.

Effect of Metformin Treatment on TIMP-1 Protein and on MMP-9 and MMP-13 Expression in the Duodenum of Fructose-Fed Mice

As earlier results of our group suggest that the effect of fructose on occludin protein levels in the duodenum is not dependent upon an alteration of occludin mRNA expression[20] and as results of recent studies suggest that occludin protein levels are also regulated through MMPs,[21,22] we determined mRNA expression of MMP-9 and MMP-13 as well as protein levels of TIMP-1 in the duodenum. Expressions of MMP-9 (Figure 7a) and MMP-13 (Figure 7b) were considerably induced in the duodenum of fructose-fed groups; however, a similar effect of the fructose feeding was not detected in metformin-treated mice. Protein levels of TIMP-1 were significantly lower in the duodenum of fructose-fed mice (Figure 7c), whereas in the duodenum of fructose-fed mice concomitantly treated with metformin, protein levels of TIMP-1 did not differ from those of controls. Furthermore, the ratio of MMP-9 mRNA expression to TIMP-1 protein levels (Figure 7d) as well as MMP-13 mRNA expression to TIMP-1 protein levels (Figure 7e) was significantly elevated in the duodenum of fructose-fed mice. A similar effect of fructose treatment was not found in the duodenum of metformin-treated mice (Figure 7d and e).

DISCUSSION

Results of several human and animal studies suggest that fructose intake may be a critical factor in the development of NAFLD (for review, also see Spruss and Bergheim[23]); however, underlying mechanisms of the pivotal effects of fructose on the liver have not yet been fully understood. Results of

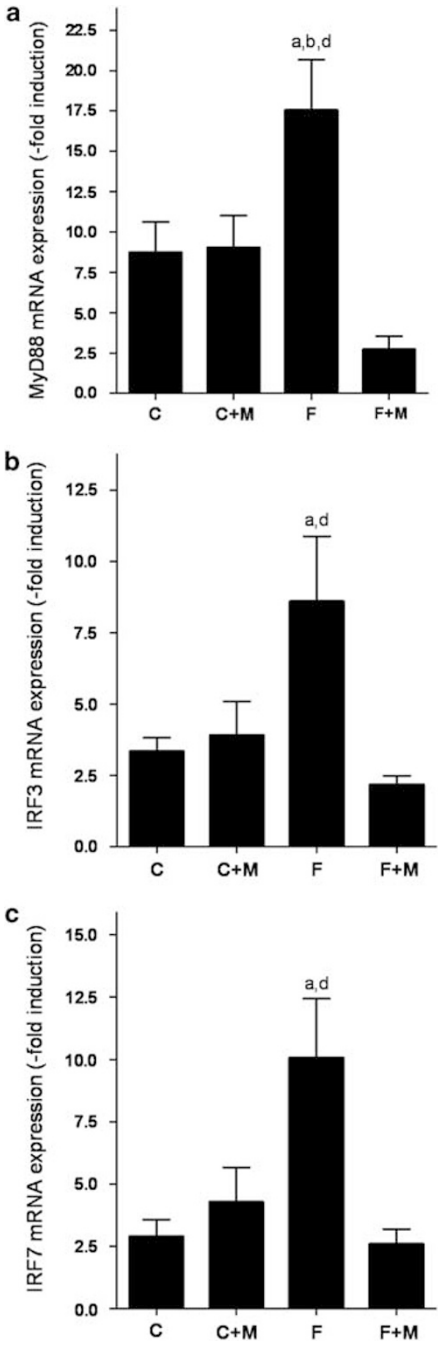

Figure 4 Effect of chronic consumption of metformin on hepatic MyD88, IRF-3, and IRF-7 expression in fructose-induced steatosis. (**a**) MyD88, (**b**) IRF-3, and (**c**) IRF-7 determined by real-time RT-PCR. Data are expressed as mean values ± s.e.m. ($n = 4$–6) and are specified as –fold induction. C: water; C + M: water with 300 mg metformin/kg body weight/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body weight/day. $^{a}P < 0.05$ compared with water-fed control mice; $^{b}P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; $^{d}P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

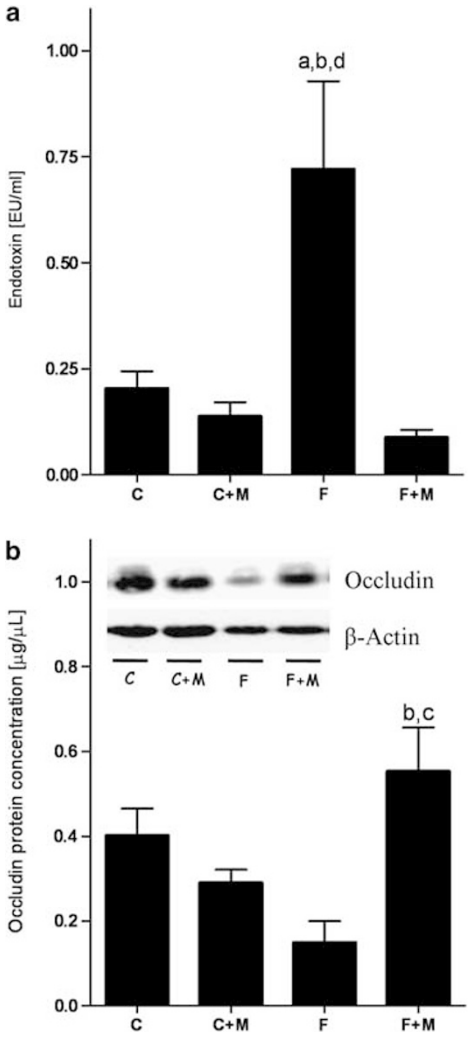

Figure 5 Effect of chronic consumption of metformin on portal endotoxin levels and occludin protein concentration in duodenum in fructose-induced steatosis. (**a**) Endotoxin levels in portal plasma. (**b**) Representative occludin and β-actin western blots and quantitative analysis of blots. Data are shown as mean values ± s.e.m. ($n = 4$–6). C: water; C + M: water with 300 mg metformin/kg body weight/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body weight/day. $^{a}P < 0.05$ compared with water-fed control mice; $^{b}P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; $^{c}P < 0.05$ compared with mice fed with 30% fructose solution; $^{d}P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

our own group obtained in animal feeding experiments suggest that fructose may cause hepatic steatosis not only through its insulin-independent metabolism but also through altering intestinal barrier function.[4,20] Indeed, we showed that chronic intake of fructose, but not glucose, may lead to a decrease in protein levels of the tight junction protein occludin and an increased translocation of bacterial endotoxins subsequently leading to a TLR-4-dependent activation of hepatic Kupffer cells and an increased release of the proinflammatory cytokine TNFα.[7,9] A sterilization of the gut with non-resorbable antibiotics or the loss of the endotoxin receptor TLR-4 was found to be associated with a marked protection on animals from the onset of

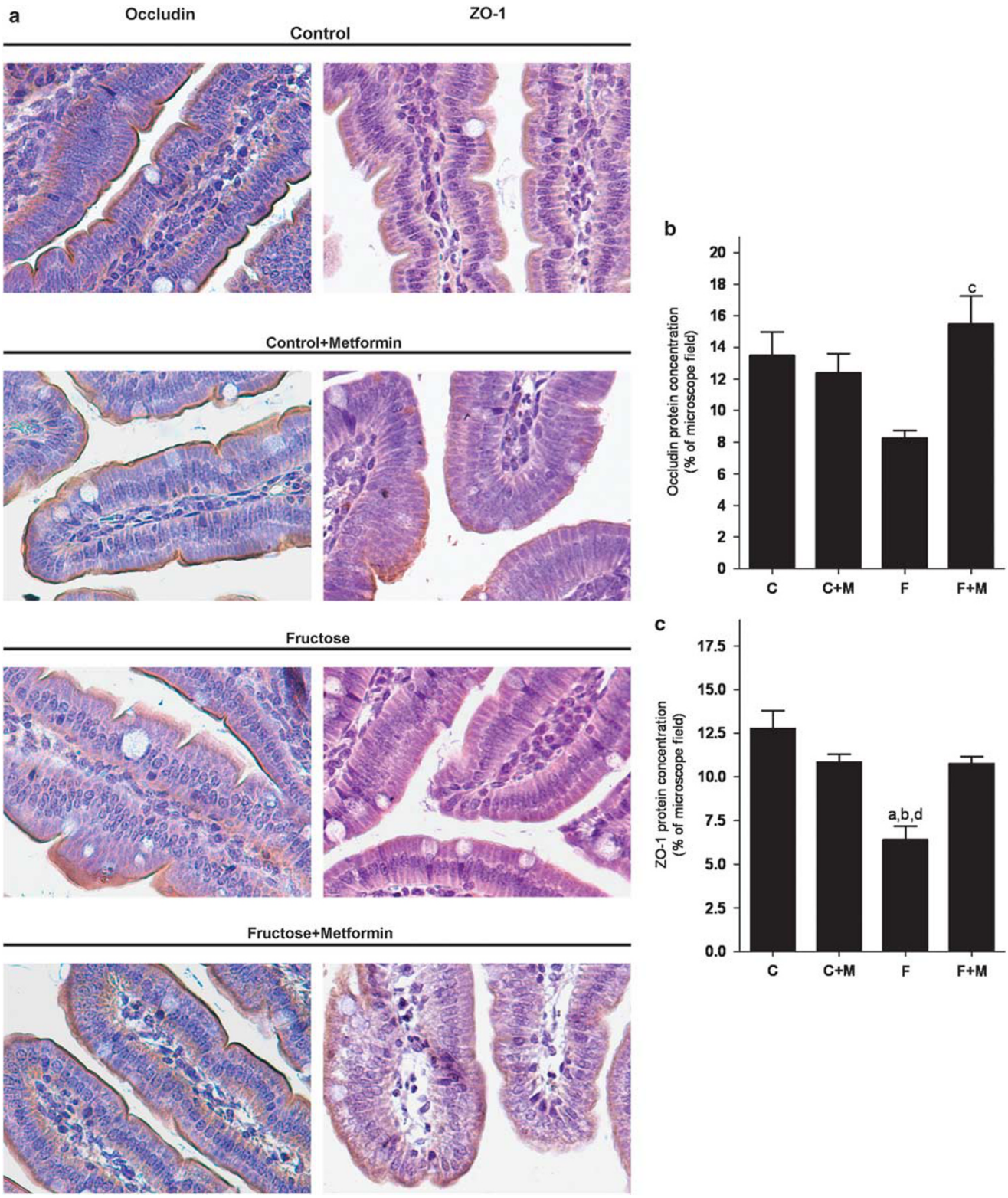

Figure 6 Effect of chronic consumption of metformin on occludin and ZO-1 protein concentration in duodenum in fructose-induced steatosis. (**a**) Representative photomicrographs of immunohistochemical staining of occludin and ZO-1 protein in sections of duodenum ($\times$ 400). Densitometric analysis of (**b**) occludin and (**c**) ZO-1 protein staining. Data are shown as mean values $\pm$ s.e.m. ($n = 4$–6). C: water; C + M: water with 300 mg metformin/kg body weight/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body weight/day. $^{a}P < 0.05$ compared with water-fed control mice; $^{b}P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; $^{c}P < 0.05$ compared with mice fed with 30% fructose solution; $^{d}P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

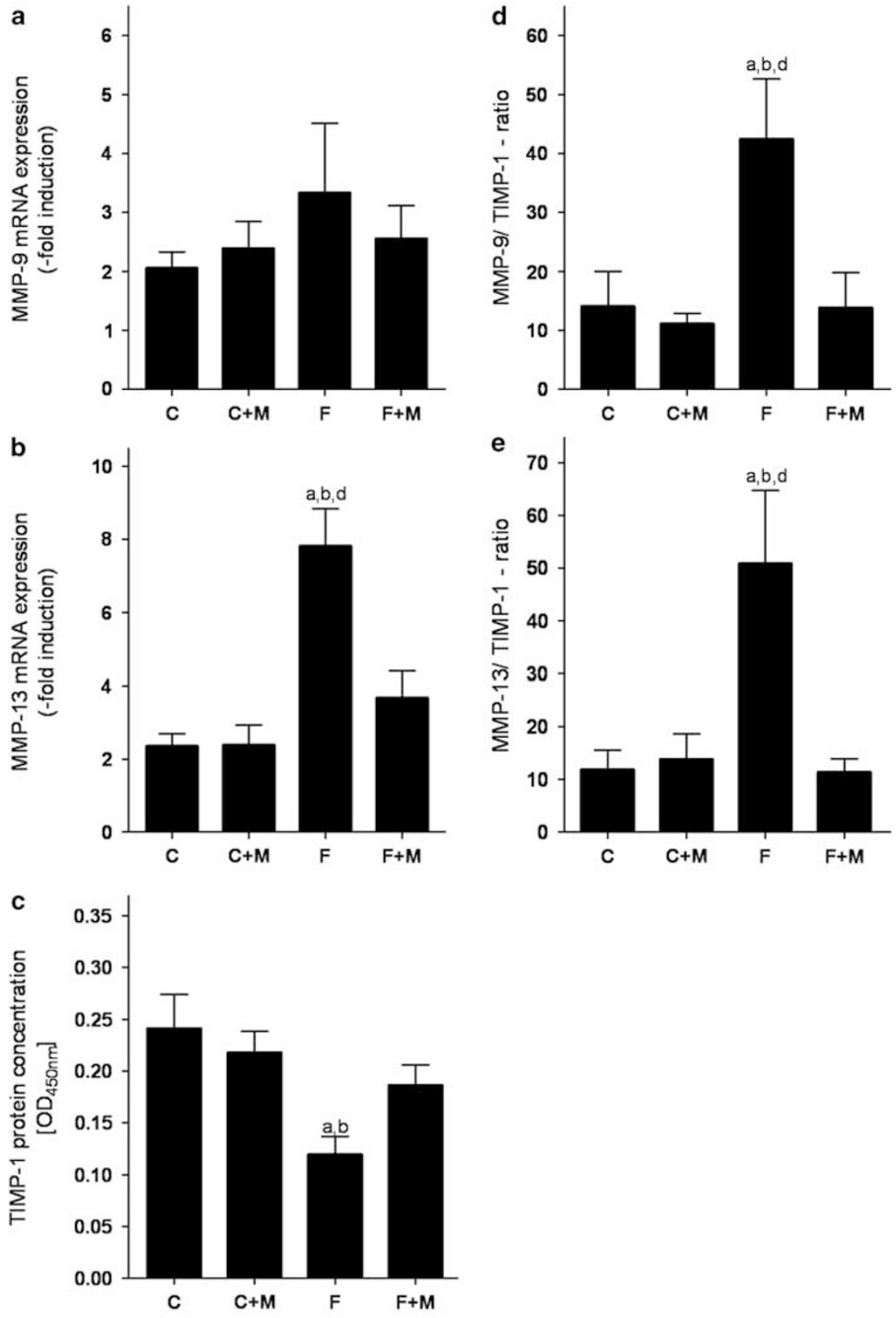

Figure 7 Effect of chronic consumption of metformin on expression of MMP-9 and MMP-13 as well as TIMP-1 protein concentration in duodenum in fructose-induced steatosis. Expression of (**a**) MMP-9 and (**b**) MMP-13. (**c**) TIMP-1 protein concentration as well as (**d**) MMP-9/TIMP-1 ratio and (**e**) MMP-13/TIMP-1 ratio. Data are shown as mean values ± s.e.m. ($n = 4$–6) and mRNA expression is specified as –fold induction. C: water; C + M: water with 300 mg metformin/kg body weight/day; F: 30% fructose solution; F + M: 30% fructose solution with 300 mg metformin/kg body weight/day. [a]$P < 0.05$ compared with water-fed control mice; [b]$P < 0.05$ compared with mice concomitantly treated with water and 300 mg metformin/kg body weight/day; [d]$P < 0.05$ compared with mice concomitantly treated with 30% fructose solution and 300 mg metformin/kg body weight/day.

fructose-induced NAFLD.[7,9] The oral antidiabetic drug metformin has been shown to have a beneficial effect not only on the development but also on the progression of NAFLD in humans and animals (eg, ob/ob mice) as well as on the development of alcohol-induced liver damage.[13,15,24] In these studies, it was shown that the protective effects of metformin on the liver were mainly attributed to an improvement of insulin signalling the liver. However, whether metformin also has a protective effect on the development of fructose-induced liver steatosis and if so what the underlying mechanisms are was not clarified to our knowledge. Here, by feeding mice chronically with 30% fructose solution and

concomitantly treating them with metformin, we tested the hypothesis that metformin has a protective effect on the development of fructose-induced liver steatosis and determined the mechanisms involved. As to be expected metformin treatment markedly improved some markers of insulin resistance (like RBP4 and leptin in plasma) in fructose-fed mice; however, plasma levels of active adiponectin were only slightly affected by the treatments as levels varied considerable between groups. Metformin had no effect on weight gain of mice, which was slightly but not significantly higher in fructose-fed mice. These findings are in contrast to those of Chen *et al*[25] who reported that in rats metformin treatment protected animals from fructose-induced weight gain. However, differences between the study of Chen and the present study may have resulted from differences in animals (rats *vs* mice) and feeding paradigms (fructose in chow *vs* fructose in drinking water). Furthermore, metformin treatment also not only protected fructose-fed mice from the development of steatosis but also attenuated the induction of some markers of inflammation like number of neutrophils and IFNγ expression. In contrast, mRNA expression of IL-1β was markedly higher in livers of animals of both fructose-fed groups. These findings are in line with earlier findings of our group, where we showed that metformin attenuated the LPS-induced upregulation of IFNγ and number of neutrophils in the liver of rats but had no effect on IL-1β protein levels.[26] However, molecular mechanisms in the lack of responsiveness of IL-1β metformin *in vivo* in the liver remain to be determined. Interestingly, the protective effects found were also associated with a decreased expression of PAI-1 in the liver and an upregulation of the hepatocyte growth factor (HGF)/cMet-dependent lipid export. These findings are in line with earlier studies in which it was shown that the protective effects of metformin on the development of alcoholic liver disease and those found in PAI-1 mice fed with fructose on steatosis are associated with an induction of the HGF/cMet-regulated lipid export.[15,27] Furthermore, PAI-1 levels repeatedly have been reported to be markedly lower in patients with diabetes that are treated with metformin.[28,29] In addition, results of *in-vitro* studies and studies in TNF receptor 1 (TNFR1)−/− mice suggest that PAI-1 is induced through TNFα receptor 1-dependent signalling cascades in the liver.[15,30] It further has been shown in *in-vitro* studies that metformin can block the TNFα-dependent induction of PAI-1 through yet not fully understood mechanisms.[15] However, in the present study, TNFα mRNA expression was only found to be induced in livers of fructose-fed mice, whereas in livers of fructose-fed mice concomitantly treated with metformin, TNFα mRNA expression was almost at the level of controls. Taken together, these data suggest that the protective effects of metformin on the development of fructose-induced hepatic steatosis found in the present study were related to a suppression of the fructose-dependent induction of PAI-1 expression and an induction of the HGF-c-Met-dependent hepatic lipid export. However, as the expression of TNFα mRNA in the liver was also found to be affected by the treatment with metformin these data also suggest that the protective effects of metformin on the development of hepatic steatosis did not primarily result from an interference of metformin with the TNFα-dependent induction of PAI-1 but that other mechanisms may be involved.

Results of several studies suggest that chronic intake of fructose can lead to an increased formation of reactive oxygen species in the liver and subsequently to the induction of TNFα.[5–8] Indeed, we recently reported that iNOS−/− mice are protected from the onset of fructose-induced NAFLD and that the protective effects were associated with a protection of animals against the activation of nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NFκB) and the induction of TNFα mRNA expression.[18] In the present study, concentration of 4-HNE protein adducts in the liver and expression of iNOS were markedly lower in livers of mice treated with metformin while being fed fructose in comparison with those not receiving the antidiabetic drug suggesting that metformin interfered with the induction of iNOS in the liver. One possible way to induce iNOS in the liver is through bacterial endotoxin/TLR4-dependent signalling pathways.[9,20] Chronic intake of fructose has been shown before by our own group to be associated with a decrease of the tight junction protein occludin in the upper parts of the small intestine as well as an increased translocation of bacterial endotoxins from the intestine leading to an induction of TLR4-dependent signalling cascades in the liver (eg, via an induction of MyD88 and IRF-3 as well as IRF-7).[7,9,20] Indeed, in the present study not only the expression of MyD88, IRF-3, and IRF-7 in the liver but also endotoxin levels in portal plasma and number of bacteria in intestinal mucosa and mesenterial tissue were found to be markedly lower in fructose-fed mice treated with metformin when compared with mice only fed with fructose. Taken together, these data suggest that metformin treatment protected mice from fructose-induced steatosis at least in part through suppressing the fructose-induced increase in translocation of bacterial endotoxins. These data by no means preclude that metformin may have also affected insulin signalling in the liver but rather suggest that other pathways not involving insulin signalling in the liver may have contributed to the protective effects of metformin.

Results of recent studies of our group and those of others suggest that a loss of tight junction proteins in the upper parts of the small intestine may be involved in the development of NAFLD in several animal models of NAFLD but also in humans.[4,7,9,31] Indeed, Miele *et al*[32] recently reported that in patients with NASH the increase in intestinal permeability was associated with lower levels of protein levels of ZO-1. In genetically obese mice (eg, ob/ob mice), the development of NAFLD was associated with a loss of the tight junction proteins ZO-1 and occludin and increased levels of the endotoxin receptor CD14 in the liver.[33] In the present study, we found that metformin treatment markedly protected mice

from the fructose-induced loss of the tight junction proteins occludin and ZO-1. The protective effects of metformin against the loss of occludin and ZO-1 were associated with a protection of mice against the induction of MMPs mRNA expression and the loss of TIMP-1 found in the small intestine of mice fed with fructose. Indeed, it has been suggested before by the results of *in-vitro* and *in-vivo* studies of others that MMPs are involved in the degradation of occludin.[21,22] Furthermore, it has been shown that MMPs and TIMPs levels are altered in situations of insulin resistance.[34,35] However, mechanisms involved in these alterations and the protective effects of metformin herein remain to be determined. Taken together, these data suggest that metformin at least in part protected the liver from the onset of fructose-induced steatosis through maintaining intestinal barrier function.

CONCLUSION

In summary, the present study adds further support to the hypothesis that the damaging effects of chronic fructose consumption may not solely be a result of an increased *de-novo* lipogenesis in the liver but at least in part also of an increased translocation of bacterial endotoxins and subsequently an induction of TLR-4-dependent signalling pathways. The results of the present study further suggest that fructose affects tight junction proteins through altering MMPs and TIMP-1 concentration in the intestine. Our data also suggest that the oral antidiabetic drug metformin may protect mice from the onset of fructose-induced liver steatosis through attenuating the effects of fructose on MMPs and TIMP-1. However, underlying mechanisms of the effect of fructose and metformin on MMPs and TIMPs as well as if similar mechanisms are also applicable in the human situation remain to be determined.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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