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Role of tumor necrosis factor α (TNFα) in the onset of fructose-induced nonalcoholic fatty liver disease in mice☆

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Abstract

Tumor necrosis factor α (TNFα) is known to be involved in dysregulation of hepatic lipid metabolism and insulin signaling. However, whether TNFα also plays a casual role in the onset of fructose-induced nonalcoholic fatty liver disease (NAFLD) has not yet been determined. Therefore, wild-type and TNFα receptor 1 (TNFR1)−/− mice were fed with either 30% fructose solution or plain tap water. Hepatic triglycerides, markers of inflammation and ATP concentration as well as plasma ALT levels were determined. Hepatic PAI-1, SREBP-1, FAS mRNA expression was assessed by real-time RT-PCR. Furthermore, lipid peroxidation and indices of insulin resistance were determined in liver tissue and plasma. In comparison to water controls, chronic intake of 30% fructose solution caused a significant ~5-fold increase in triglyceride accumulation and neutrophil infiltration in livers of wild-type mice and a ~8-fold increase in plasma ALT levels. In TNFR1−/− mice, hepatic steatosis was attenuated and neutrophil infiltration in the liver as well as plasma ALT levels was similar to water controls. The protective effect of the TNFR1 deletion against the onset of fructose-induced steatosis was associated with increased phospho AMPK and Akt levels, decreased SREBP-1 and FAS expression in the liver and decreased RBP4 plasma levels, whereas hepatic lipid peroxidation, iNOS protein and ATP levels were similar between wild-type and TNFR1−/− mice fed fructose. Taken together, these data suggest that TNFα plays a casual role in the onset of fructose-induced liver damage as well as insulin resistance in mice through signaling cascades downstream of TNFR1.

Keywords: Nonalcoholic fatty liver disease; Fructose; TNFα; SREBP-1; Akt

1. Introduction

Worldwide, the incidence of nonalcoholic fatty liver disease (NAFLD) has increased dramatically throughout the last three decades [1]. NAFLD usually develops in the setting of obesity and insulin resistance [2] and comprises a continuum of disease ranging from simple steatosis to steatohepatitis and cirrhosis. Herein, steatosis is the earliest and most common type of NAFLD and has long been thought to be a relatively benign state of liver injury. However, in recent years it became clear that fatty livers are more vulnerable to injury from various causes [3] and can progress to steatohepatitis, increasing the probability of further liver-related morbidity and mortality [4]. Therapeutic options are still limited as mechanisms involved in the development of NAFLD are not yet fully clarified. Therefore, a better understanding of the biochemical and pathological changes that cause the early stages of NAFLD (e.g., steatosis) is desirable to improve intervention strategies.

Results of recent human studies suggest that a diet rich in carbohydrates and herein particularly fructose may be a major cause of NAFLD and may increase the odds of later stages of the disease (e.g., nonalcoholic steatohepatitis) (for an overview see Ref. [5]). The hypothesis that the intake of carbohydrates and particularly the mono- and disaccharides fructose and sucrose might play a critical role in the pathogenesis of NAFLD is also supported by a number of studies performed in animals. In these studies, it was shown that an increased consumption of fructose (e.g., up to 60% of daily calories derived from fructose) may result in increased lipid accumulation in the liver accompanied by insulin resistance, elevated plasma triglyceride levels and oxidative stress [6-10]. We recently found that chronic fructose consumption can lead to increased intestinal translocation of bacterial endotoxin, induction of lipid peroxidation as well as hepatic tumor necrosis factor α (TNFα) and, subsequently, liver steatosis in mice, whereas a similar effect was not found in animals chronically exposed to glucose [7]. In these studies, the concomitant treatment with antibiotics or the loss of the endotoxin

Abbreviations: Akt, protein kinase B; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; CCL2, chemokine (C-C motif) ligand 2; CCL19, chemokine (C-C motif) ligand 19; FAS, fatty acid synthase; iNOS, inducible nitric oxide synthase; ICAM-1, intercellular adhesion molecule 1; NAFLD, nonalcoholic fatty liver disease; PAI-1, plasminogen activator inhibitor 1; PEPCK, phosphoenolpyruvate carboxykinase; SREBP-1, sterol regulatory element-binding protein 1; TNFα, tumor necrosis factor α; TNFR1, tumor necrosis factor receptor 1.

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receptor toll-like receptor (TLR)-4 both markedly attenuated the effect of fructose on mouse liver [7,11]. Specifically, lipid peroxidation, TNFα expression and steatosis were markedly lower in livers of fructose-fed mice treated with antibiotics or TLR-4 mutant mice [7,11].

A role of the proinflammatory cytokine TNFα in the pathogenesis of NAFLD has been suggested before. Indeed, an increased expression of TNFα has been reported repeatedly to be associated with the development of NAFLD in different animal models (e.g., MCD diet, high-fructose diet, high-fat diet) [7,12,13]. Furthermore, the importance of TNFα in NAFLD has also been indicated by the findings of Li et al. [14] who reported that an anti-TNFα therapy attenuated NAFLD in ob/ob mice; also, the results obtained in ob/ob mice by Memon et al. [15] are controversial. However, whether TNFα is also causally involved in the onset of fructose-induced NAFLD has not yet been clarified. Starting from this background, the aim of the present study was to assess whether TNFα through its receptor 1 is also a critical factor in the development of hepatic steatosis in a mouse model of the onset of fructose-induced NAFLD.

2. Material and methods

2.1. Animals and treatments

Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures were approved by the local Institutional Animal Care and Use Committee (IACUC). Six-week-old C57BL/6J and TNFR1−/− mice (Tnfrsf1a$^{tm1Mak/J}$ ID: B6.129, Jackson, ME, USA) were fed either plain water or water containing 30% fructose for a period of 8 weeks (n=4–6). Body weight was assessed weekly over the 8-week feeding period. Animals were anesthetized with 80 mg ketamin/kg and 6 mg xylazin/kg body weight by intraperitoneal injection. Blood was collected just prior to sacrifice. Portions of the liver were either frozen immediately in liquid nitrogen, fixed in neutral-buffered formalin or frozen-fixed in OCT mounting media (Medite, Burgdorf, Germany).

2.2. Hepatic triglyceride determination and lipid staining

Mouse livers were homogenized in 2× phosphate buffered saline (PBS). Tissue lipids were extracted with methanol/chloroform (1:2), dried in an evaporating centrifuge and resuspended in 5% fat-free bovine serum albumin. Colorimetric assessment of triglyceride levels was carried out using a commercially available kit (Randox, Krefeld, Germany). Values were normalized to protein concentration in liver homogenate using the Bradford assay (Bio-Rad Laboratories, Munich, Germany). To determine hepatic lipid accumulation, frozen sections of the liver (10 μm) were stained with oil red O (Sigma, Steinheim, Germany) for 12 min, washed and counterstained with hematoxylin for 45 s (Sigma).

2.3. Immunohistochemical staining for 4-hydroxynonenal adducts and inducible nitric oxide synthase

Paraffin-embedded liver sections (5 μm) were cut and stained either for 4-hydroxynonenal-protein adducts or for inducible nitric oxide synthase (iNOS) using polyclonal antibodies (4-hydroxynonenal: AG Scientific, San Diego, CA, USA; iNOS: Affinity BioReagents, Golden, CO, USA) as described previously [7,11]. To detect specific binding of primary antibody, tissue sections were incubated with a peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit; DAKO, Hamburg Germany). With the use of an image acquisition and analysis system incorporated in the microscope, the extent of staining in liver sections was defined as percent of the field area within the default colour range determined by the software. To determine means, data from eight fields (200× for 4-HNE, 630× with oil immersion for iNOS) of each tissue section were used.

2.4. Immunoblots

To prepare cytosolic proteins, liver tissue was homogenized in a lysis buffer (1 mol/L HEPES, 1 mol/L Mg_2Cl, 2 mol/L KCl, 1 mol/L DTT) containing protease and phosphatase inhibitors mix (Sigma), and 60 μg protein/well was separated in a 10% SDS-polyacrylamide gel. Proteins were then transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences, Freiburg, Germany) using a semidry electroblotter. The resulting blots were then probed with antibodies against phosphorylated and total Akt or adenosine monophosphate-activated protein kinase (AMPK); Cell Signaling Technology, Irvine, CA, USA]. Bands were visualized using Super Signal Western Dura kit (Thermoscientific, Rockford, IL, USA). To ensure equal loading, all blots were stained with Ponceau red. Protein bands were analyzed densitometrically using the Flurochem Software (Alpha Innotech, San Leandro, CA, USA).

2.5. RNA Isolation and real-time RT-PCR

Total RNA was extracted from liver tissue samples using Trifast (PEQLAB, Erlangen, Germany). RNA concentrations were determined spectrophotometrically, and 1 μg total RNA was reverse transcribed using a MuLV reverse transcriptase and oligo (dT) primers followed by a DNase digestion step (Fermentas, St. Leon-Rot, Germany). Polymerase chain reaction (PCR) primers for plasminogen activator inhibitor 1 (PAI-1) (forward: 5′TCC AAG GGG CAA CGG ATA GA 3′; reverse: 5′GAC GAA GAG CCA GGC ACA CA 3′), sterol regulatory element-binding protein 1 (SREBP-1) (forward: 5′ ACC GGC TAC TGC TGG ACT GC 3′; reverse: 5′AGA GCA AGA GGG TGC CAT CG 3′), fatty acid synthase (FAS) (forward: 5′ GGG GGT GGG AGG ACA GAG AT 3′; reverse: 5′ CAC ATG GGC TGA CAG CTT GG 3′), chemokine (C-C motif) ligand 2 (CCL2) (forward: 5′ GCC AGA CGG GAG GAA GGC CA; reverse 5′ TGG ATG CTC CAG CCG GCA AC 3′), chemokine (C-C motif) ligand 19 (CCL19) (forward: 5′ GTC GGA GCC TCG GCC TCT CA; reverse 5′ CCA CCC AGG GCT GGT CTG GA 3′), intercellular adhesion molecule 1 (ICAM-1) (forward: 5′ ACT GGA TCT CAG GCC GCA AG; reverse 5′ GCC CTC CCA GCT CCA GGT AT 3′), phosphoenolpyruvate carboxykinase (PEPCK) (forward 5′ CCC TGG GAG ATG GGG AGT TC; reverse 5′ CCC ACC ATA TCC GCT TCC AA 3′) and 18S (forward: 5′ GTA ACC CGT TGA ACC CCA TT 3′; reverse 5′ CCA TCC AAT CGG TAG TAG CG 3′) were designed using the software Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Sybr Green Universal PCR Master Mix (Applied Biosystems, Damstadt, Germany) was used to prepare the PCR mix. Primers were added to a final concentration of 300 nmol/L. The amplification reactions were carried out in an iCycler system (Bio-Rad Laboratories, Munich, Germany) with an initial hold step (95°C for 2 min) and 50 cycles of a three-step PCR (95°C for 15 s, 60°C for 15 s, 72°C for 30 s). The fluorescence intensity of each sample was measured at each temperature change to monitor the amplification of the target gene. The comparative C_T method was used to determine the amount of target gene, normalized to an endogenous reference (18S) and relative to a calibrator ($2^{-\Delta\Delta Ct}$).

2.6. Clinical chemistry, pathologic evaluation and RBP-4 ELISA

Plasma alanine aminotransferase (ALT) levels were determined using a commercially available kit (Randox, Krefeld, Germany). Paraffin sections of liver (5 μm) were stained for hematoxylin and eosin to assess liver histology. With the use of a commercially available ELISA, plasma levels of retinol binding protein (RBP)-4 were determined (Alpco Diagnostics, Salem, NH, USA). Neutrophil infiltration was evaluated by staining using AS-D chloroacetate esterase (Sigma). Neutrophil numbers in liver were counted in five randomly selected fields (200×, 0.42×0.32 μm).

2.7. ATP Assay

ATP levels in the liver tissue were determined by using a commercially available ATP assay (BioAssay Systems, Hayward, CA, USA). In brief, liver tissue was homogenized in ice-cold PBS and the supernatant was diluted with PBS (1:5) to measure the luminescence after adding the reaction buffer included in the kit.

2.8. Statistical analysis

Data are expressed as means±S.E.M. (n=4–6). One-way ANOVA with Tukey's *post hoc* test was used to determine the statistical differences between the treatment groups, as appropriate. P value <.05 was considered to be significant.

3. Results

3.1. TNFR1−/− mice are protected from fructose-induced hepatic steatosis

Fig. 1 depicts representative photomicrographs of hepatic hematoxylin-and-eosin (H&E) as well as oil red O staining and quantitative analysis of hepatic triglyceride concentration in TNFR1−/− and wild-type mice fed either water or water sweetened with 30% fructose for 8 weeks. In wild-type and TNFR1−/− mice consuming water, no significant pathological changes were found and lipid staining as well as hepatic triglyceride concentration was minimal. In livers of wild-type mice, chronic fructose feeding caused a significant ~5-fold increase in hepatic triglyceride levels in comparison to water controls (Fig. 1). In contrast, in TNFR1−/− mice, hepatic triglyceride levels were markedly reduced (approx. −50%) in comparison to wild-type controls fed fructose (P<.05); however, in comparison to both water controls, hepatic triglyceride levels were still increased (approx. +609% in comparison to wild-type controls and approx. +488% in comparison to TNFR1−/− mice). Furthermore, as TNFα has been shown to be a potent inducer of chemokine secretion (e.g.,

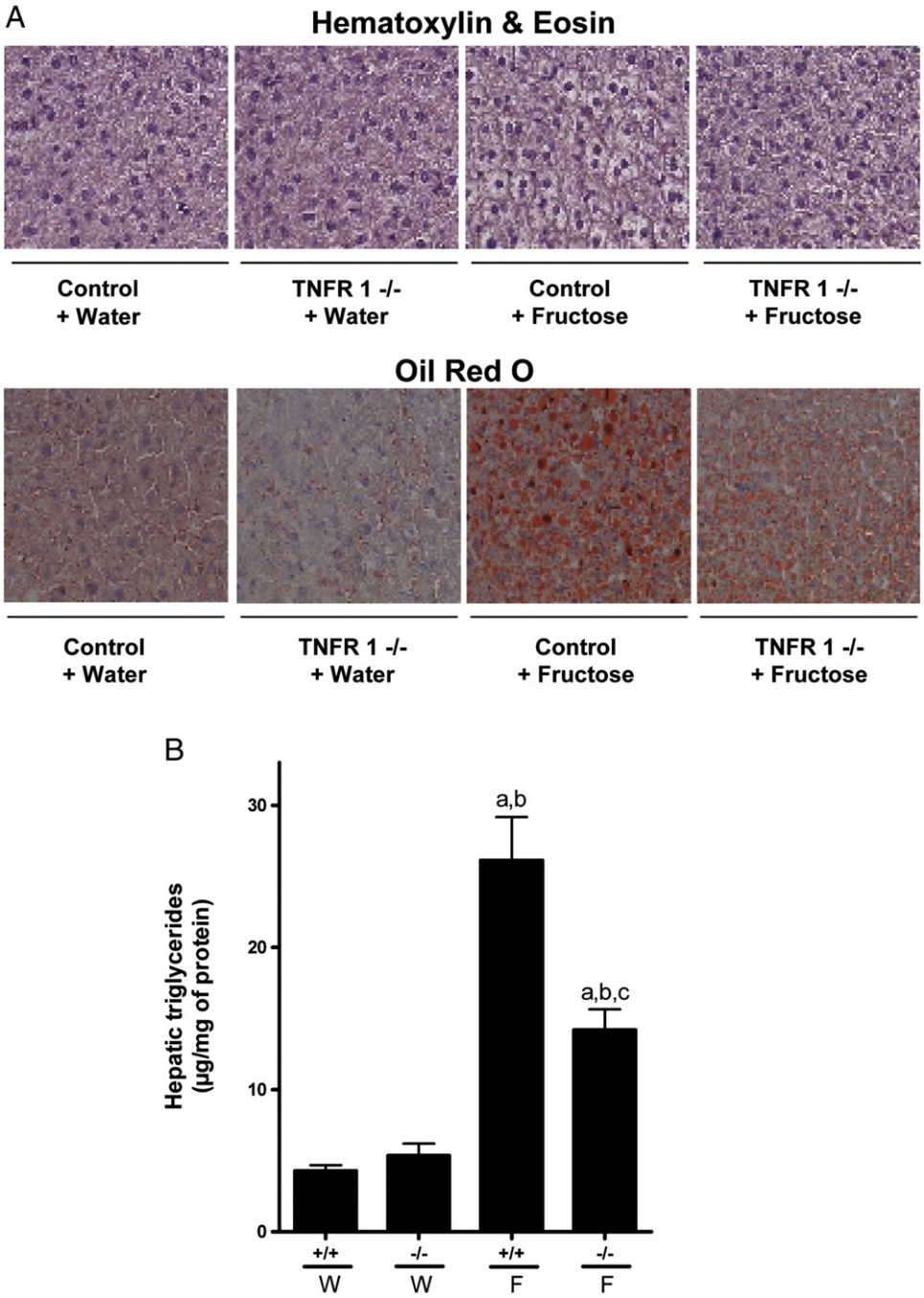

Fig. 1. Effect of TNFR1 deletion on hepatic lipid accumulation in fructose-fed mice. (A) Representative photomicrographs of hematoxylin-and-eosin (upper panel) and oil red O staining (lower panel) of liver sections (400×). (B) Quantitation of hepatic triglyceride content. Data are expressed as means±S.E.M. (n=4-6). W — water; F — 30% fructose solution. [a]P<.05 compared with wild-type mice fed with plain water. [b]P<.05 compared with TNFR1−/− mice fed with plain water. [c]P<.05 compared with wild-type fructose-fed mice.

CCL2 and CCL19) and inflammation in settings of steatosis and steatohepatitis (for a review see Ref. [16]), number of neutrophils as well as the expression of ICAM-1, CCL2 and 19 were determined (Table 1). With the exception of CCL2 mRNA expression which was markedly lower in TNFR1−/− mice fed water, no differences were found between wild-type and TNFR1−/− mice fed water. In livers of wild-type mice fed fructose solution, the number of neutrophils and expression of ICAM-1 was significantly higher than in water controls (neutrophils: approx. +5.5-fold; ICAM-1: approx. +1.8-fold). A similar effect of the fructose feeding was also found for CCL2 and CCL19 mRNA expression in livers of wild-type mice; however, as expression varied considerably between animals, the differences did not reach a level of significance (CCL2: P=.38; CCL19: P=.11, in comparison to wild-type water controls) (CCL2: P<.05; CCL19: P=.07, in comparison to TNFR 1−/− water controls). Interestingly, similar changes were not found in livers of TNFR1−/− mice fed with fructose solution. In line with these findings, plasma ALT levels were found to be significantly increased by ~6-fold in wild-type mice fed fructose, whereas plasma ALT levels of TNFR1−/− mice fed fructose did not differ from those of mice fed water (Table 2). Despite no increase in plasma ALT levels, absolute liver weight was significantly higher in TNFR1−/− mice fed fructose than in water controls and

Table 1
Effect of fructose feeding on inflammatory infiltration and expression patterns of chemokines [1]

	Control+water	TNFR1−/−+water	Control+fructose	TNFR1−/−+fructose
Neutrophil count	0.2±0.03	0.2±0.08	1.1±0.09 [a,b,d]	0.33±0.05
ICAM-1 (% of control)	100±16	126±3	182±16 [a,d]	118±18
CCL2 (% of control)	100±15	36±2	156±58 [b]	38±6
CCL19 (% of control)	100±16	99±13	207±57	82±13 [c]

[1] Values represent means±S.E.M.
[a] $P<.05$ compared with wild-type mice fed with plain water.
[b] $P<.05$ compared with TNFR1−/− mice fed with plain water.
[c] $P<.05$ compared with wild-type fructose-fed mice.
[d] $P<.05$ compared with TNFR1−/− fructose-fed mice.

higher than in wild-type mice fed fructose, whereas liver-to-body weight ratio of fructose-fed mice did not differ between strains. Furthermore, TNFR1−/− mice fed fructose solution had gained markedly more body weight (~2 g) than wild-type mice exposed to fructose throughout the feeding period.

3.2. TNFR1−/− mice are not protected against an induction of iNOS and hepatic lipid peroxidation found in fructose-fed mice

As results of our own group and those of other groups have indicated that chronic intake of fructose is also associated with an increased formation of reactive oxygen species (ROS) and lipid peroxidation [6,7,11], we determined the levels of iNOS and 4-hydroxynonenal adducts in livers of wild-type and TNFR1−/− mice fed either plain water or 30% fructose solution (see Fig. 2). Protein concentration of iNOS in livers of wild-type and TNFR1−/− mice fed water was minimal and did not differ between groups (Fig. 2A). In wild-type mice fed fructose solution, iNOS protein levels were significantly higher by ~3.2-fold in comparison to water-fed controls. Interestingly, a similar effect of the fructose feeding on iNOS protein concentration was also found in livers of TNFR1−/− mice. Levels of 4-hydroxynonenal adducts in livers of water-fed animals were minimal and did not differ between strains (Fig. 2B). In line with the findings for iNOS protein concentration, chronic intake of 30% fructose solution led to a significant ~3-fold increase in 4-hydroxynonenal adduct concentration in livers of both wild-type mice and TNFR1−/− mice in comparison to water controls.

3.3. Effect of TNFR1 deletion on ATP concentration in livers of fructose-fed mice

As it has been shown that acute intravenous exposure to fructose can lead to a depletion of ATP in the livers in humans and rodents [17-19] and that this may be associated with alteration of both apoptotic and necrotic TNFR1-mediated liver damage, we determined the ATP levels in livers of wild-type and TNFR1−/− mice chronically exposed to either 30% fructose solution or water. However, no differences were found in ATP concentration in the liver between groups (Table 2).

3.4. Effect of TNFR1 deletion on hepatic glucose metabolism and insulin signalling in liver of fructose-fed mice

As it has been shown by us and other groups that chronic intake of fructose and an induction of TNFα can alter glucose metabolism and cause insulin resistance [11,20-23], we determined the expression of the gluconeogenic enzyme PEPCK, the phosphorylation status of hepatic Akt in livers and RBP4 levels in plasma of mice exposed to either water or 30% fructose solution (Fig. 3). Expression of PEPCK in the liver was lower in TNFR1−/− mice fed water than in wild-type controls. In fructose-fed wild-type mice and TNFR1−/− mice, expression of PEPCK was lower than in water controls (wild-type: approx. −61%, $P=.06$; TNFR 1−/−: approx. −69%, $P<.05$; in comparison to wild-type water controls); however, when comparing the hepatic PEPCK expression levels of water-fed TNFR1−/− mice with those of fructose-fed TNFR1−/− animals no differences were found (Fig. 3A). As expected, in livers of wild-type mice fed fructose, phosphorylation of Akt was markedly lower than in water-fed controls (approx. −35% and approx. −77% in comparison to wild-type and TNFR1−/− mice fed plain water, respectively; $P<.05$). Interestingly, in livers of TNFR1−/− mice, a similar effect of fructose feeding was not observed; rather, similar to the findings in livers of TNFR1−/− mice fed plain water, phosphorylation of Akt was significantly higher than in livers of wild-type controls fed plain water (approx. +2-fold) (Fig. 3B). In line with these findings, plasma RBP4 levels were only found to be significantly increased in plasma of fructose-fed wild-type mice (approx. +1.6-fold in comparison to all other groups). A similar effect of fructose feeding was not found in TNFR1−/− mice (Fig. 3C).

3.5. Effect of TNFR1 deletion on hepatic AMPK phosphorylation and lipogenesis in livers of fructose-fed mice

To examine whether the protective effects of the deletion of TNFR1 in the present study was associated with alterations of the phosphorlyation status of AMPK and hepatic lipogenesis, we determined the expression levels of phospho AMPK, SREBP-1 and FAS in whole-liver homogenate (Fig. 4). In livers of water-fed wild-type, TNFR1−/− and fructose-fed wild-type animals, the levels of phospho AMPK were similar. In contrast, in livers of fructose-fed

Table 2
Effect of fructose feeding on body weight, clinical parameters of liver damage and hepatic ATP concentrations [1]

	Control+water	TNFR1−/−+water	Control+fructose	TNFR1−/−+fructose
Weight gain (g)	3.1±0.1	4.5±0.3	5.1±0.7 [a]	7.6±0.5 [a,b,c]
Liver-to-body weight ratio (%)	4.8±0.1	5.12±0.1	5.9±0.1 [a,b]	6.2±0.1 [a,b]
Absolute liver weight (g)	0.99±0.01	1.16±0.03 [a]	1.33±0.14 [a,b]	1.63±0.07 [a,b,c]
ALT (U/L)	2.7±0.8	3.4±0.9	16.1±4.1 [a,b]	5.4±0.9 [c]
ATP (μM)	0.94±0.2	1.17±0.1	1.02±0.1	1.08±0.1

[1] Values represent means±S.E.M.
[a] $P<.05$ compared with wild-type mice fed with plain water.
[b] $P<.05$ compared with TNFR1−/− mice fed with plain water.
[c] $P<.05$ compared with wild-type fructose-fed mice.

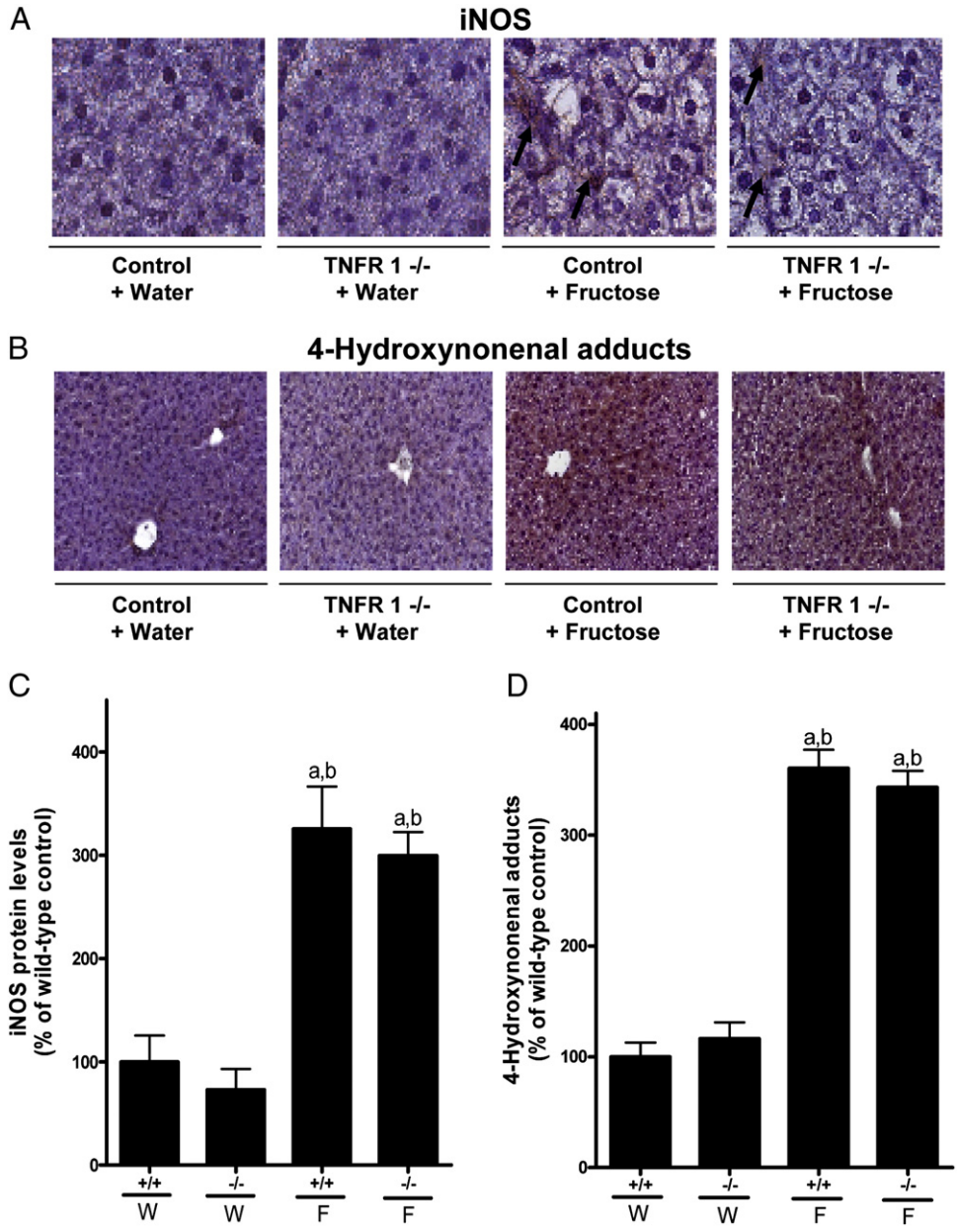

Fig. 2. Effect of knocking out TNFR1 on iNOS protein levels and hepatic lipid peroxidation in fructose-fed mice. (A) Representative photomicrographs of immunostaining of iNOS in liver sections (630×). (B) Photomicrographs of immunostaining of 4-hydroxynonenal adducts in liver sections (200×). (C and D) Densitometric analysis of the respective staining. Data are shown as means±S.E.M. (n=4–6) and are normalized to percent of wild-type control. W — Water; F — 30% fructose solution. [a]P<.05 compared with wild-type mice fed with plain water. [b]P<.05 compared with TNFR1−/− mice fed with plain water.

TNFR1−/− mice, the concentration of phosphorylated AMPK was significantly higher than in wild-type mice fed water or fructose, respectively (approx. +2-fold). In livers of water-fed controls, neither expression of SREBP-1 nor FAS differed between substrains. However, in fructose-fed wild-type mice, mRNA expression of SREBP-1 and FAS was significantly induced by ~3- and ~6-fold, respectively, in comparison to water controls. In contrast, in livers of TNFR1−/− mice, mRNA expression of SREBP-1 was at the level of controls fed plain water, whereas expression of FAS was induced by ~3.5-fold in comparison to water controls (Fig. 4).

3.6. Effect of TNFR1 deletion on hepatic PAI-1 expression in livers of fructose-fed mice

As it has been suggested that PAI-1 mRNA expression in the liver is regulated through TNFα- and insulin-dependent mechanisms and PAI-1 expression has been shown to be increased in livers of patients with NAFLD [24,25], we determined PAI-1 expression in livers of wild-type and TNFR1−/− mice exposed to plain water or 30% fructose solution (Fig. 4). PAI-1 expression did not differ and was minimal in livers of controls fed water regardless of substrain. In contrast, in livers of mice chronically fed with 30% fructose solution, mRNA expression of PAI-1 was induced significantly by ~2.2-fold. Again, a similar effect of fructose feeding was not found in livers of TNFR1−/− mice exposed to fructose. Specifically, the expression levels of PAI-1 were similar to those of mice fed plain water.

4. Discussion

An increased dietary fructose intake has been suggested to be a risk factor in the development of NAFLD in several human and animal

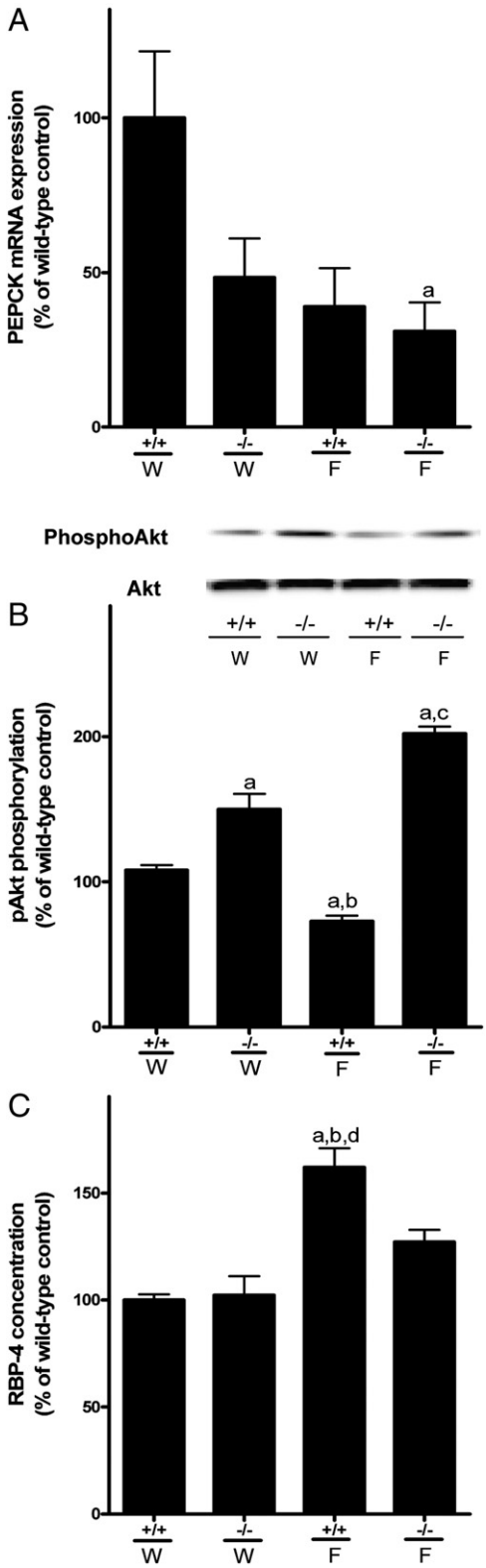

Fig. 3. Effect of TNFR1 deletion on hepatic PEPCK mRNA expression, plasma levels of RBP4 and phosphorylation status of Akt in livers of fructose-fed mice. (A) PEPCK mRNA expression. (B) Representative photographs of Western blots of phospho Akt and total Akt and quantitative analysis of blots. (C) Plasma RBP4 levels. Data are expressed as means±S.E.M. (n=4–6) and are normalized to percent of wild-type control. W — water; F — 30% fructose solution. [a]P<.05 compared with wild-type mice fed with plain water. [b]P<.05 compared with TNFR1−/− mice fed with plain water. [c]P<.05 compared with wild-type fructose-fed mice. [d]P<.05 compared with TNFR1−/− fructose-fed mice.

studies [7,11,24]. Furthermore, it has been shown in a pilot study in humans that a reduction of fructose intake may exert beneficial effects on the progression of NAFLD [26]. Our own group recently reported that the development of fructose-induced hepatic steatosis is associated with markedly increased endotoxin levels in portal plasma, an increased formation of ROS and an induction of MyD88 expression and NF B activity as well as TNFα mRNA expression in mouse liver [7,11]. We further reported that the treatment with nonresorbable antibiotics (e.g., neomycin and polymycin) as well as the loss of the endotoxin receptor TLR-4 in the liver protects animals from the onset of fructose-induced hepatic steatosis by ~50% [7,11]. In settings of acute and chronic alcohol-induced liver damage in which intestinal translocation of bacterial endotoxin has been shown to be markedly increased [27,28], the proinflammatory cytokine TNFα has been shown before to mediate the pivotal effects on the liver through its receptor 1 [29,30]. In humans with NAFLD but also in livers of obese mice (e.g., ob/ob) with nonalcoholic steatohepatitis, TNFα expression was reported to be increased, too [31,32]. In addition, a polymorphism in the promoter of TNFα has been shown to be associated with an increased susceptibility to insulin resistance and NAFLD in humans [33,34]. However, whether TNFα and its receptor 1 are also casually involved in mediating the effects of fructose on the liver has not yet been clarified. In the present study, the hypothesis that TNFα through its receptor 1 plays a casual role in the onset of fructose-induced NAFLD was tested in a mouse model. Indeed, despite similar levels of iNOS protein, 4-hydroxynonenal adducts and ATP levels in the liver and an increased liver-to-body weight ratio, plasma ALT activity remained at the level of water controls. Furthermore, hepatic fat accumulation resulting from chronic exposure to 30% fructose solution was markedly attenuated in livers of fructose-fed TNFR1−/− mice. In line with these findings, indices of inflammation (e.g., number of infiltrating neutrophils; expression of ICAM-1, CCL2 and CCL19), which were found to be elevated in livers of fructose-fed wild-type mice, were at the level of controls in livers of TNFR1−/− animals. Interestingly, the magnitude of protection against fructose-induced hepatic steatosis found in livers of TNFR1−/− mice fed fructose was similar to that reported before by our group for livers of fructose-fed TLR-4 mutant mice and fructose-fed mice treated with nonresorbable antibiotics [7,11]. In line with the here presented results, in those earlier experiments, protection was always associated with a “normalization” or “protection” against lipid peroxidation, induction of iNOS and TNFα expression in the liver, which was found to be elevated in livers of mice chronically exposed to fructose and seemed to be resulting from an increased translocation of intestinal bacterial endotoxins. Differences between our findings and those of other groups [17-19] in regard to the effects of fructose on ATP concentrations in the liver might have been resulting from differences in the experimental setups (e.g., acute intravenous exposure vs. chronic oral exposure to fructose). Taken together, these data indicate that TNFα through its receptor 1 is critically involved in mediating the onset of NAFLD. However, these data also add further weight to the hypothesis that fructose not only adds to the development of NAFLD through its insulin-independent metabolism in the liver but also through an activation of Kupffer cells associated with an induction of iNOS and increased formation of reactive oxygen species and induction of TNFα. These results do not preclude that the intake of fructose can cause hepatic steatosis through mechanisms resulting from its insulin-independent metabolism in the liver or through alterations of the bioavailability of ATP, rather our data suggest that it is likely that additional pathways (e.g., intestinal bacterial overgrowth and increased intestinal permeability) contribute to the development of fructose-induced liver damage.

Results of several human and animal studies suggest that a loss of insulin sensitivity may be a critical factor in the development of

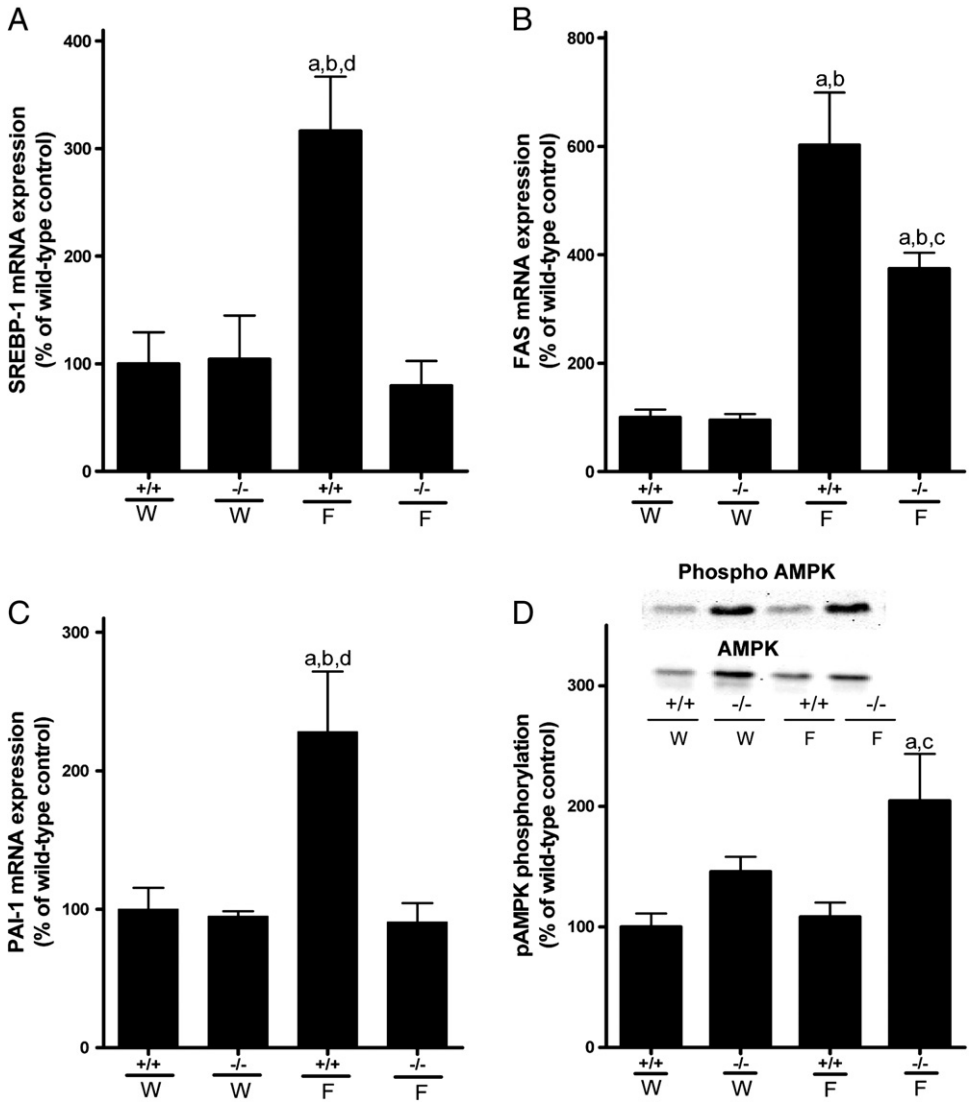

Fig. 4. Effect of TNFR1 deletion on SREBP-1, FAS and PAI-1 mRNA levels as well as phosphorylation status of AMPK in livers of fructose-fed mice. (A) SREBP-1, (B) FAS and (C) PAI-1 mRNA expression levels were normalized to 18S expression. (D) Representative photographs of Western blots of phospho AMPK and total AMPK and quantitative analysis of blots. Data are expressed as means±S.E.M. (n=4–6) and are normalized to percent of wild-type control. W — Water; F — 30% fructose solution. [a]P<.05 compared with wild-type mice fed with plain water. [b]P<.05 compared with TNFR1−/− mice fed with plain water. [c]P<.05 compared with wild-type fructose-fed mice. [d]P<.05 compared with TNFR1−/− fructose-fed mice.

NAFLD (for an overview see Ref. [35]). Recently, we reported that the phosphorylation status of Akt in the liver and plasma RBP4 levels, both being markers of insulin resistance [11], was altered in mice chronically exposed to fructose. In line with these findings, in the present study phosphorylation status of Akt in the liver as well as RBP4 plasma levels was altered in mice fed fructose solution, too. However, a similar effect of fructose feeding was not found in TNFR1−/− mice fed fructose, suggesting that TNFα and its receptor 1 may be critical in mediating insulin resistance in mice chronically exposed to fructose. In line with these findings, the expression levels of the gluconeogenic enzyme PEPCK were also lower in livers of fructose-fed wild-type mice in comparison to water-fed wild-type controls. Interestingly, when comparing the mRNA expression of hepatic PEPCK between wild-type mice and TNFR1−/− animals, expression of the latter regardless of feeding was found to be significantly lower than in wild-type controls. These data suggest that PEPCK or even gluconeogenesis may be regulated differently in wild-type mice and mice lacking the TNFR1 receptor, which might be an adaptive response of TNFR1−/− mice to the lack of the inhibitory effect of TNFα on PEPCK reported before by others performing *in vitro* studies [36]. Furthermore, it has been suggested that AMPK may be involved in the regulation of hepatic lipogenesis (e.g., through suppression of SREBP1 and subsequently FAS). It has been suggested further that an induction of TNFα may suppress the activation of AMPK in the liver, thereby contributing to the development of fatty liver. Indeed, levels of phospho AMPK were markedly higher in livers of fructose-fed TNFR1−/− mice, whereas in livers of fructose-fed wild-type mice phospho AMPK was at the level of controls. In line with these findings, expression of FAS and SREBP-1 being key regulators of lipogenesis in the liver (for a review see Ref. [37]) was only found to be markedly induced in livers of wild-type mice fed fructose. Furthermore, expression of PAI-1, which also has been suggested to be dependently regulated by insulin [30] and was shown before to be involved not only in mediating hepatic inflammation but also in exporting triglyceride [25,30,38], was significantly induced in livers of wild-type mice but not in livers of TNFR1−/− mice fed fructose. Taken together, these data suggest that TNFα through its receptor 1 may at least partly be responsible for the

insulin resistance associated with chronic fructose intake in the liver. Furthermore, these data also suggest that TNFα either directly or through indirect mechanisms (e.g., impairing insulin signalling, suppressing AMKP activation) induces lipogenesis and expression of PAI-1 in livers of animals chronically exposed to fructose. However, the exact underlying mechanisms and signalling cascades involved remain to be determined.

5. Conclusion

The results of the present study suggest that TNFα through its receptor 1 may be a critical factor in the onset of fructose-induced steatosis in mice. These data also support the hypothesis that TNFα is involved in the development of hepatic insulin resistance and may add to alterations found in lipogenesis as well as to the induction of hepatic PAI-1 expression associated with fructose-induced NAFLD. These data further suggest that fructose consumption may not only add to the development of fatty liver through its insulin-independent metabolism but also other factors such as increased activation of Kupffer cells and subsequent induction of TNFα and depending pathways may also contribute to the onset of NAFLD.

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