

# Female Mice Are More Susceptible to Nonalcoholic Fatty Liver Disease: Sex-Specific Regulation of the Hepatic AMP-Activated Protein Kinase–Plasminogen Activator Inhibitor 1 Cascade, but Not the Hepatic Endotoxin Response

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As significant differences between sexes were found in the susceptibility to alcoholic liver disease in human and animal models, it was the aim of the present study to investigate whether female mice also are more susceptible to the development of non-alcoholic fatty liver disease (NAFLD). Male and female C57BL/6J mice were fed either water or 30% fructose solution *ad libitum* for 16 wks. Liver damage was evaluated by histological scoring. Portal endotoxin levels and markers of Kupffer cell activation and insulin resistance, plasminogen activator inhibitor 1 (*PAI-1*) and phosphorylated adenosine monophosphate-activated protein kinase (*pAMPK*) were measured in the liver. Adiponectin mRNA expression was determined in adipose tissue. Hepatic steatosis was almost similar between male and female mice; however, inflammation was markedly more pronounced in livers of female mice. Portal endotoxin levels, hepatic levels of myeloid differentiation primary response gene (88) (*MyD88*) protein and of 4-hydroxynonenol protein adducts were elevated in animals with NAFLD regardless of sex. Expression of insulin receptor substrate 1 and 2 was decreased to a similar extent in livers of male and female mice with NAFLD. The less pronounced susceptibility to liver damage in male mice was associated with a superinduction of hepatic *pAMPK* in these mice whereas, in livers of female mice with NAFLD, *PAI-1* was markedly induced. Expression of adiponectin in visceral fat was significantly lower in female mice with NAFLD but unchanged in male mice compared with respective controls. In conclusion, our data suggest that the sex-specific differences in the susceptibility to NAFLD are associated with differences in the regulation of the adiponectin–*AMPK*–*PAI-1* signaling cascade.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2012.00223

## INTRODUCTION

Throughout the last decades, the prevalence of nonalcoholic fatty liver disease (NAFLD) markedly increased worldwide as the prevalence of the main risk factors (for example, overweight, obesity and insulin resistance) has reached almost epidemic proportions (1). Indeed, NAFLD, a disease comprising a contin-

uum of liver damage ranging from simple steatosis to cirrhosis, by now is accounted to be among the most frequent liver diseases in the world (2,3). Previous results of our own and other groups have shown that the development of NAFLD is associated with alterations of the intestinal barrier function, and also may be associated with an increased for-

mation of reactive oxygen species (ROS) in the liver and an induction of tumor necrosis factor  $\alpha$  (*TNF $\alpha$* ) (4–6). It was further shown in animal models of NAFLD that *TNF $\alpha$*  can alter insulin-dependent signaling cascades, subsequently leading to an induction of plasminogen activator inhibitor-1 (*PAI-1*) and alterations of the hepatic lipid export (7). However, despite intense research efforts, molecular mechanisms involved in the onset, and even more in the progression of the disease, are not yet understood fully, and universally accepted therapies or prevention strategies are still lacking. Therefore, a better understanding of the biochemical and pathological changes that cause the early stages of NAFLD (for example, steatosis)

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Submitted May 19, 2012; Accepted for publication August 28, 2012; Epub ([www.molmed.org](http://www.molmed.org)) ahead of print August 29, 2012.

is desirable to improve both prevention and therapeutic strategies.

Results of several epidemiological studies suggest that, similar to the findings for alcoholic liver disease, sex differences also exist in the susceptibility of NAFLD. However, results are so far contradictory. For instance, the results of the study of Pinidiyapathirage *et al.* (8) conducted in Sri Lanka and that of Zhang *et al.* (9) performed in China indicate a higher prevalence of NAFLD and nonalcoholic steatohepatitis (NASH) in men than in women, with men also displaying more significant metabolic impairments (for example, more features of the metabolic syndrome and/or more pathological values of parameters determined). On the other hand, the studies of Fernandes *et al.* (10) and Haentjens *et al.* (11) suggest that women and girls, particularly when being obese, are at higher risk of developing NAFLD than men. Furthermore, a recent study carried out in adolescents in Australia suggests that sex differences in adolescents with NAFLD are related to differences in adipose distribution and adipocytokines. Indeed, in that study, the male phenotype of NAFLD was associated with more adverse metabolic features and greater visceral adiposity than the female phenotype despite a lower prevalence of NAFLD in males (12).

Both genetic and dietary modifications have made it possible to produce pathological changes in rodent liver that resemble alterations found in humans with NAFLD (13). For instance, the chronic feeding of fructose produces pathological changes in the liver and also in the intestine and adipose tissue that resemble many of the early alterations (for example, steatosis and insulin resistance) that also occur in humans with NAFLD (14). By using a mouse model in which mice were exposed chronically to 30% fructose solution to induce NAFLD, the present study had two main objectives: (1) to test the hypothesis that sex-specific differences exist in regards to susceptibility to NAFLD between female and male

mice and, if so, (2) to determine underlying molecular mechanisms (for example, differences in intestinal barrier function, hepatic endotoxin response and insulin signaling, as well as adiponectin expression in visceral fat).

## MATERIALS AND METHODS

### Animals and Treatments

Six- to eight-week-old male and female C57Bl/6J mice (Janvier SAS, Le-Genest-St-Isle, France) were housed in a specified and opportunistic pathogen-free (SOPF) 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). Mice ( $n = 6$  per group) had free access either to plain tap water or water enriched with 30% fructose for 16 wks. In pilot studies, we found that, in SOPF mice, the development and progression of fructose-induced steatosis was markedly slower than in specific pathogen-free (SPF) mice (data not shown); therefore, the feeding period was expanded to 16 wks in the present study. Body weight, as well as consumption of chow and drinking solution, was assessed twice weekly throughout the 16 wks of feeding. Mice were anesthetized with 80 mg ketamine and 6 mg xylazine/kg body weight by intraperitoneal (IP) injection and blood was collected from the portal vein prior to euthanization. Portions of liver were snap frozen immediately, frozen fixed in optimal cutting temperature (OCT) mounting media (Mediate, Burgdorf, Germany) or fixed in neutral-buffered formalin.

### Clinical Chemistry and Histological Evaluation of Liver Sections

Alanine-aminotransferase (ALT) activity ( $n = 5-6$  for lack of plasma in some groups) was determined by an Olympus AT200 Autoanalyzer (Olympus Europa Holding GmbH, Hamburg, Germany) using commercially available kits (Beckman Coulter Biomedical GmbH, Krefeld,

Germany). In addition, after paraffin-embedded sections of liver (5  $\mu\text{m}$ ) were stained with hematoxylin and eosin, histology was evaluated by scoring photomicrographs captured at a 100 $\times$  magnification using a system incorporated in a microscope (Axio Vert 200M, Zeiss, Jena, Germany) by using the semiquantitative "Nonalcoholic steatohepatitis Clinical Research Network system for scoring activity and fibrosis in nonalcoholic fatty liver disease" (modified from [15]) (16). According to this system, livers were scored as follows: steatosis grade 0: <5%; 1: 5% to 33%; 2: 34% to 66%; 3: >66%; lobular inflammation 0: none; 1: <2; 2: 2-4; 3: >4; hepatocellular ballooning 0: none; 1: few ballooned cells; 2: many ballooned cells. Representative photomicrographs were captured at a 400 $\times$  magnification.

### Oil Red O Staining

For determination of hepatic lipid accumulation, frozen sections of liver (10  $\mu\text{m}$ ) were stained with oil red O (Sigma, Steinheim, Germany) for 12 min, washed, and counterstained with hematoxylin for 45 s (Sigma). Representative photomicrographs were captured at a 400 $\times$  magnification using a system incorporated in a microscope (Axio Vert 200M, software: AxioVision V4.5.0.0, Zeiss).

### RNA-isolation and Real-Time RT-PCR

Total RNA was extracted from liver and fat tissue using peqGOLD TriFast (PEQLAB, Erlangen, Germany). After spectrophotometric determination of RNA concentrations, DNA was digested using a DNase (Fermentas, St. Leon-Rot, Germany) and 1  $\mu\text{g}/\mu\text{L}$  total RNA was transcribed with MuLV reverse transcriptase and oligo dT primers. Polymerase chain reaction (PCR) primers for the detection of adiponectin, adiponectin receptor 1 (*AdipoR1*), chemokine (C-C motif) ligand 2 (*CCL2*), microsomal prostaglandin E synthase 1 (*mPGES1*), *mPGES2*, insulin receptor substrate (*IRS*)-1, *IRS*-2, insulin receptor (*InsR*), and  $\beta$ -actin were designed using

**Table 1.** Effect of chronic intake of 30% fructose solution on caloric intake in male and female mice after 16 wks.<sup>a,b</sup>

	Female		Male	
	Control	Fructose	Control	Fructose
kcal/mouse/d from solid food	72.4 ± 1.2	33.9 ± 0.8 <sup>c</sup>	91.5 ± 1.6	53.2 ± 1.9 <sup>c</sup>
kcal/mouse/d from liquid <sup>a</sup>	-	64.7 ± 2.7	-	59.6 ± 2.9
Total caloric intake (kcal/mouse/d)	72.4 ± 1.2	98.6 ± 3.8 <sup>c</sup>	91.5 ± 1.6	112.2 ± 4.5 <sup>c</sup>

<sup>a</sup>Feeding of 30% fructose solution is described in Material and Methods.

<sup>b</sup>Data are means ± SEM (n = 6).

<sup>c</sup>p < 0.001 versus male control or female control, respectively.

**Table 2.** Effect of chronic intake of 30% fructose solution on indices of liver steatosis in male and female mice after 16 wks.<sup>a,b</sup>

	Female		Male	
	Control	Fructose	Control	Fructose
Weight gain (g)	4.6 ± 0.3	5.7 ± 0.4	9.4 ± 0.7	14.3 ± 2.1 <sup>c</sup>
Liver weight (g)	1.1 ± 0.0	1.5 ± 0.0 <sup>d</sup>	1.6 ± 0.1	2.1 ± 0.0 <sup>d</sup>
Liver/body weight ratio (%)	5.0 ± 0.1	6.2 ± 0.1 <sup>d</sup>	5.0 ± 0.2	5.6 ± 0.2
ALT (U/L)	10.8 ± 2.8	18.0 ± 0.8	9.2 ± 1.3	9.8 ± 3.0
Neutrophils (number per field)	0.3 ± 0.1	0.7 ± 0.1 <sup>c</sup>	0.1 ± 0.1	0.4 ± 0.0
CCl2 mRNA expression (-fold induction)	2.0 ± 0.3	3.6 ± 0.6 <sup>c</sup>	1.5 ± 0.3	2.2 ± 0.4

<sup>a</sup>Feeding of 30% fructose solution is described in Material and Methods.

<sup>b</sup>Data are means ± SEM (n = 6 mice per group with exception of ALT (n = 5–6)).

<sup>c</sup>p < 0.05 versus male control or female control, respectively.

<sup>d</sup>p < 0.01 versus male control or female control, respectively.

Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) (for primer sequences see Supplementary Table S1). PCR mix was prepared using SybrGreen Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Amplification reactions were carried out in an iCycler (Bio-Rad Laboratories, Munich, Germany) 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, 72°C for 30 s). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. To determine the amount of target, the comparative C<sub>T</sub> method was used. The expression level was calculated as x-fold change of the gene of interest with *β-actin* as a reference gene and relative to a calibrator (2<sup>-ΔΔC<sub>T</sub></sup>). The purity of the PCR products was verified by melting curves and by running a gel electrophoresis.

#### ELISAs for TNFα and active PAI-1

Levels of hepatic protein concentration of TNFα and active PAI-1 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Alpco Diagnostics, Salem, MA, USA and Assaypro, St. Charles, MO, USA).

#### Endotoxin Assay

Plasma samples were heated at 70°C for 20 min to measure endotoxin levels. Plasma levels of endotoxin were determined using a commercially available limulus amoebocyte lysate assay with a concentration range of 0.015–1.2 EU/mL (Charles River, L'Arbaesle, France).

#### Neutrophil Staining in Liver Tissue

Neutrophil granulocytes were stained in paraffin-embedded liver sections using a commercially available naphthol AS-D chloroacetate-esterase staining kit (Sigma).

#### Immunohistochemical Staining for MyD88 Protein and 4-Hydroxynonenal Protein Adducts in the Liver

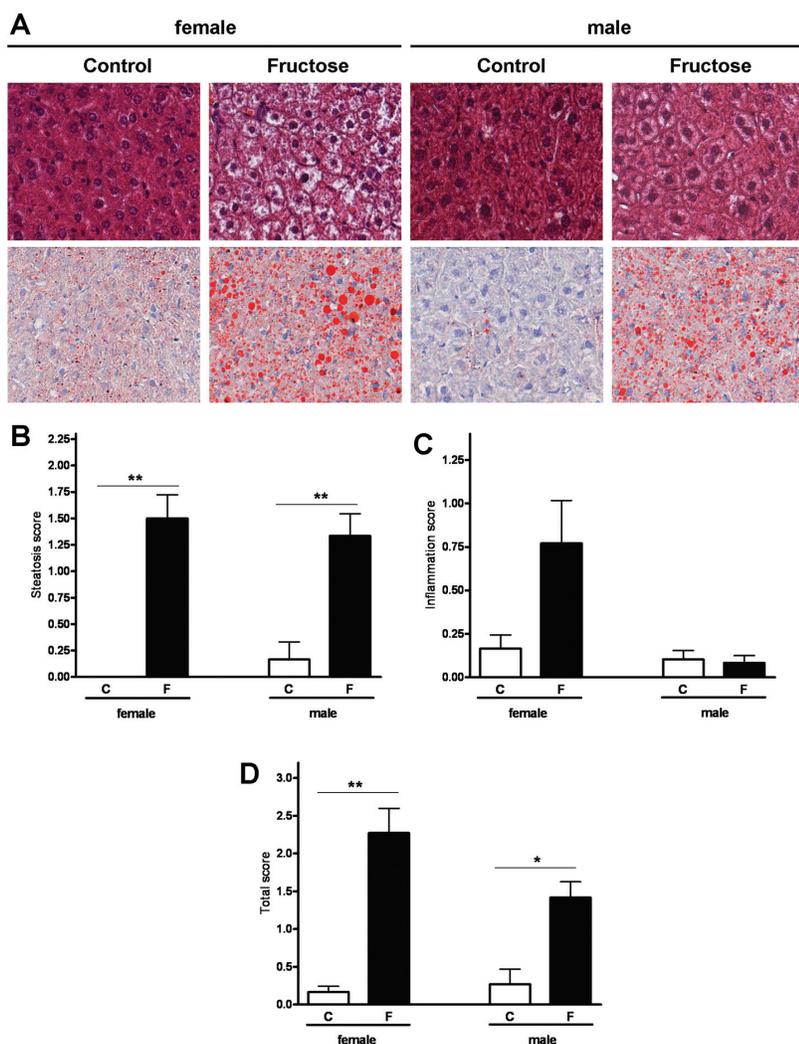
Paraffin-embedded liver sections (5 μm) were stained for myeloid differentiation primary response gene (88) (*MyD88*) protein as well as 4-hydroxynonenal (4-HNE) protein adducts using polyclonal antibodies (*MyD88*: Santa Cruz Biotechnology, Heidelberg, Germany; 4-HNE: AG Scientific, San Diego, CA, USA) as described previously (6). In brief, tissue sections were incubated with a peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit; DAKO, Hamburg, Germany) to detect specific binding of primary antibody. The extent of staining in liver sections defined as a percentage of the field area within the default color range was determined by using an image acquisition and analysis system incorporated in the microscope (Axio Vert 200M, Zeiss). Data from eight fields (200×) of each tissue section were assessed for determination of means.

#### Immunohistochemical Staining of F4/80-Positive Cells in Visceral Fat

F4/80 is a cell surface marker of macrophages and F4/80-positive cells were stained in paraffin-embedded sections (5 μm) of visceral fat tissue of mice as described previously using a monoclonal primary antibody (Abcam, Cambridge, MA, USA) (17). To determine macrophage infiltration in adipose tissue, numbers of F4/80-positive cells were counted in eight fields (630× with oil immersion, Axio Vert 200M, Zeiss) of each section to determine means as described previously by others (17–19).

#### Immunoblots

To prepare cytosolic protein lysates, liver tissue was homogenized in a lysis buffer (1 mol/L HEPES, 1 mol/L MgCl<sub>2</sub>, 2 mol/L KCl and 1 mol/L DTT) containing a protease and phosphatase inhibitors mix (Sigma) to prepare total protein lysates. Proteins were separated in 10% SDS polyacrylamide gels and transferred onto Hybond-P polyvinylidene di-



**Figure 1.** Sex differences in hepatic lipid accumulation in mice with steatosis induced by feeding a 30% fructose solution. (A) Representative photomicrographs of hematoxylin and eosin staining as well as oil red O staining of liver sections (400 $\times$ ). (B–D) Evaluation of liver histology using the semiquantitative “Nonalcoholic steatohepatitis Clinical Research Network system for scoring activity and fibrosis in nonalcoholic fatty liver disease” (modified from (15)) (16). Data are shown as means  $\pm$  SEM ( $n = 6$ ). C—water (control); F—30% fructose solution; \* $p < 0.05$  compared with respective water-fed control mice; \*\* $p < 0.01$  compared with respective water-fed control mice.

fluoride membranes (Amersham Biosciences, Freiburg, Germany) using a semidry electroblotter. The resulting blots were then probed with antibodies against phosphorylated adenosine monophosphate-activated protein kinase (*pAMPK*) and *AMPK* (Cell Signaling Technology, Danvers, MA, USA). Bands were visualized using the 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).

#### Statistical Analyses

All results are reported as means  $\pm$  standard error of mean (SEM) ( $n = 4-6$ ).

Unpaired Mann-Whitney *t* test was used for the determination of statistical significance among male and female treatment groups, respectively. A *p* value  $< 0.05$  was selected as the level of significance before the study was performed.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

## RESULTS

### Caloric Intake and Weight Gain of Male and Female Mice

In Tables 1 and 2, fructose and chow intake and body weight gain of female and male mice throughout the 16-wk feeding period are summarized. Both male and female mice exposed to the 30% fructose solution reduced their general chow intake significantly (male mice:  $\sim 42\%$  in comparison to male controls; female mice:  $\sim 53\%$  in comparison to female controls); however, when taking caloric intake derived through the consumption of the 30% fructose solution into account, which was similar between groups, total caloric intake of both male and female fructose-fed mice exceeded that of the respective controls by  $\sim 24$  kcal/mouse/d ( $p < 0.05$  for both sexes). In line with these findings, absolute body weight gain of fructose-fed mice was higher than that of controls, with this effect being more pronounced in male mice fed fructose than in female animals (male fructose-fed mice:  $+ \sim 4.9$ g in comparison to male controls,  $p < 0.05$ ; female fructose-fed mice:  $+ \sim 1.1$ g in comparison to female controls, n.s.).

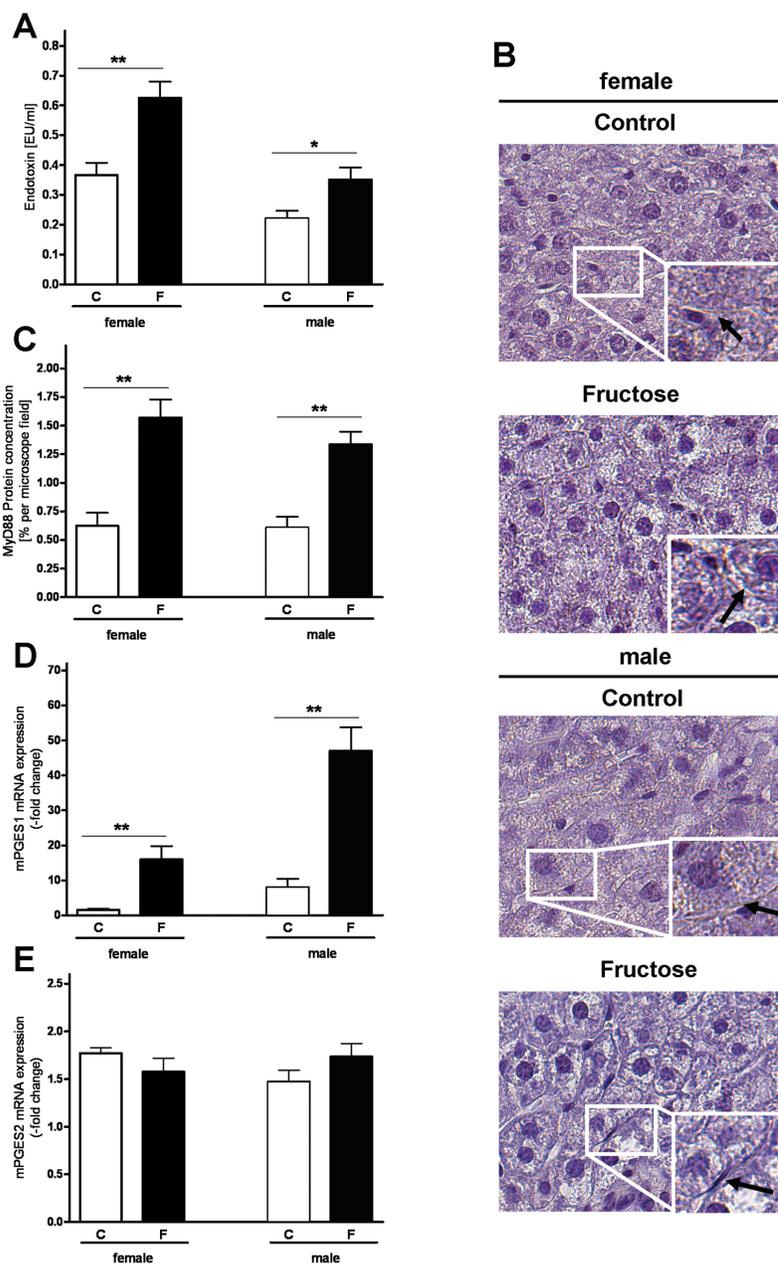
### Liver Status of Male and Female Mice

Figure 1A depicts representative photomicrographs of livers of female and male mice exposed to 30% fructose solution or tap water for 16 wks, while Figures 1B–D show pathology scores. There were no pathological changes in male or female mice exposed to tap water. As expected, chronic intake of the 30% fructose solution was associated with a marked lipid accumulation in livers, increased liver weight and liver-to-body-weight ratio in both male and female mice ( $p <$

0.05 in comparison to the respective controls (Table 2); however, despite a similar absolute fructose intake and the even higher body weight gain of male mice, focal inflammation was found significantly more often in female mice than in male mice fed the fructose solution when compared with the respective controls. Hepatocellular ballooning was not detected. In line with these findings, both expression of *CCL2* and the number of neutrophils were found to be elevated significantly only in livers of female mice fed fructose solution when compared with the respective controls (Table 2). ALT plasma levels were higher in female mice fed fructose in comparison to the respective controls (+ ~67%); however, as data varied considerable between animals, differences did not reach the level of significance ( $p = 0.1$ ). A similar effect on ALTs was not found in male mice (Table 2).

#### Portal Endotoxin Levels and Activation of the *TLR-4* Signaling Cascade as Well as Induction of *mPGES1* and *TNF $\alpha$* in the Livers of both Male and Female Mice

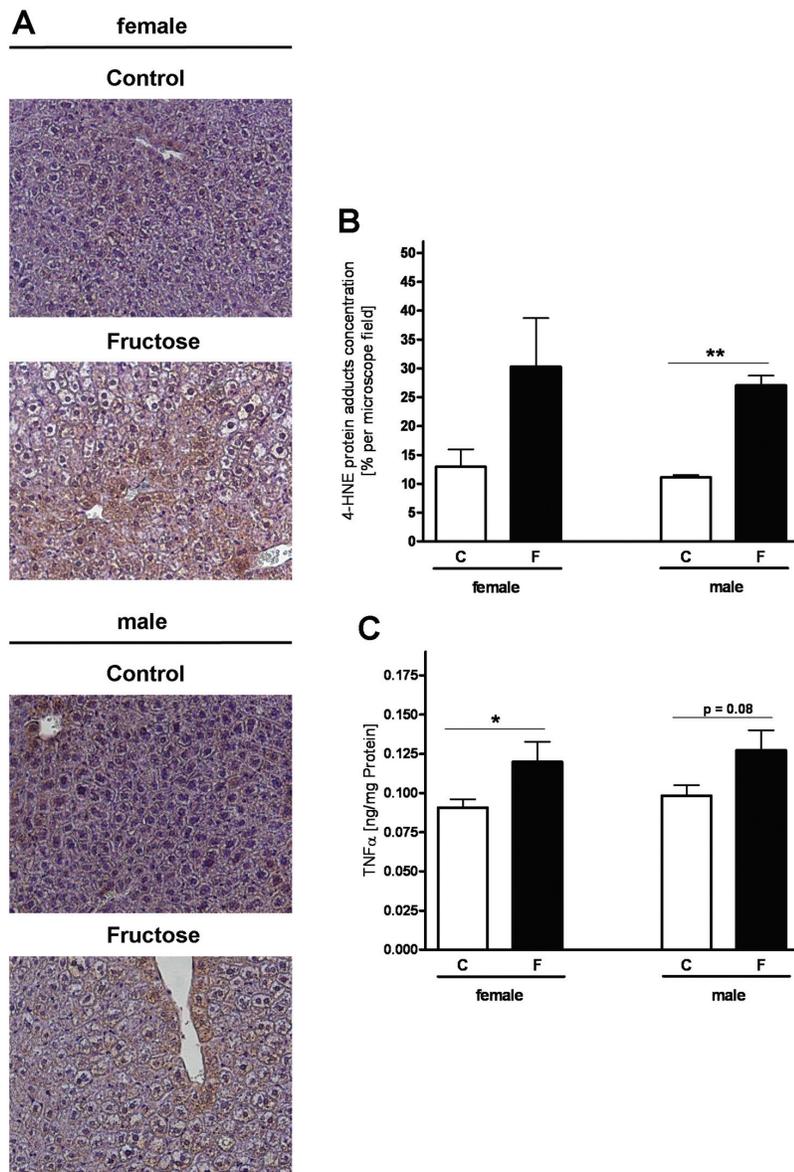
In line with our earlier findings, chronic intake of 30% fructose solution was associated with a significant increase of portal endotoxin levels in both male and female mice (+ ~1.7-fold in comparison to the respective control) (Figure 2A) (6,7). Accordingly, protein levels of the Toll-like receptor 4 (*TLR-4*) adaptor protein *MyD88* in the liver were induced in mice of both sexes exposed to fructose solution (female mice: + ~2.5-fold; male mice: + ~2.2 fold, in comparison to the respective controls,  $p < 0.05$ ) (Figure 2C). Furthermore, concentration of 4-HNE protein adducts were found to be induced in livers of both male and female mice exposed to fructose ( $p < 0.05$ ) (Figure 3B); however, as 4-HNE protein adduct concentrations varied considerably in female fructose-fed mice, differences did not reach the level of significance for female animals. Expression of *mPGES1* also was found to be induced in livers of mice that drank fructose solution; however, despite markedly lower expression levels in livers of female



**Figure 2.** Sex differences in portal endotoxin levels, protein concentration of MyD88 and *mPGES1* and  $\beta$  mRNA expression levels in livers of mice with steatosis induced by feeding a 30% fructose solution. (A) Portal endotoxin levels and (B,C) representative photomicrographs of MyD88 protein staining (630 $\times$  with oil immersion) as well as densitometric analysis of staining. Expression of (D) *mPGES1* and (E) *mPGES2* mRNA in livers of male and female mice. Expression of mRNA was determined by real time RT-PCR with  $\beta$ -actin as a reference gene in the liver. Data are shown as means  $\pm$  SEM ( $n = 6$ ) and are specified as -fold induction. C—water (control); F—30% fructose solution; \* $p < 0.05$  compared with respective water-fed control mice; \*\* $p < 0.01$  compared with respective water-fed control mice.

controls in comparison to male controls, relative changes between mice fed tap water and those fed fructose solution was

nearly identical in both sexes (Figure 2D). Protein levels of *TNF $\alpha$*  in total liver homogenate also were found to be signifi-



**Figure 3.** Sex differences in the concentration of 4-HNE protein adducts and TNF $\alpha$  in livers of mice with steatosis induced by feeding a 30% fructose solution. (A) Representative photomicrographs of hepatic 4-HNE protein adduct staining (200 $\times$ ) and (B) densitometric analysis of staining. (C) TNF $\alpha$  protein concentration in livers of mice. Data are shown as means  $\pm$  SEM ( $n = 6$ ) and are specified as -fold induction. C—water (control); F—30% fructose solution; \* $p < 0.05$  compared with respective water-fed control mice; \*\* $p < 0.01$  compared with respective water-fed control mice.

cantly higher in livers of female and male mice ingesting fructose in comparison to mice fed tap water with relative changes found between controls and fructose-fed mice not differing between sexes (male and female mice:  $\sim 1.3$ -fold, in comparison to the respective controls).

### Markers of Insulin Signaling in Livers of Male and Female Mice

To determine if the differences found in regards to liver damage between male and female mice chronically exposed to the fructose solution were associated with changes in the insulin signaling cas-

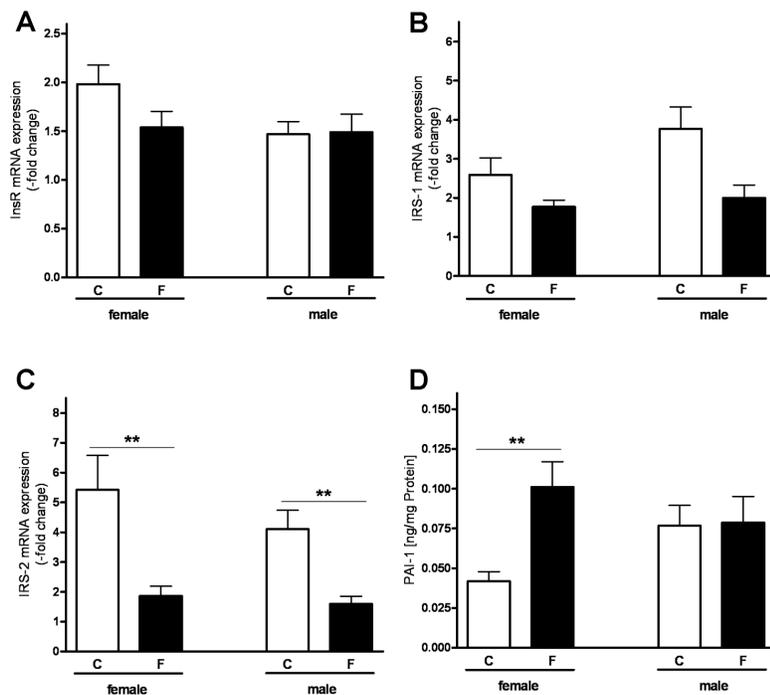
cade, we determined the mRNA expression of the insulin receptor and the *IRS-1* and *-2* in the liver. Expression of insulin receptor in the liver did not differ between groups regardless of sex and feeding (Figure 4A). Expression of *IRS-1* and *IRS-2* mRNA was markedly lower in livers of fructose-fed animals in both male and female mice in comparison to the respective controls (Figures 4B,C); however, differences only reached the levels of significance for *IRS-2*, as *IRS-1* mRNA expression in part varied considerable within groups. Furthermore, protein levels of active *PAI-1*, shown before by our group to be regulated through TNF $\alpha$  and insulin-dependent signaling cascades (4), were assessed. *PAI-1* protein levels were found to be significantly higher by  $\sim 2.5$ -fold in livers of female mice, but not in those of male mice exposed to fructose (Figure 4D).

### Markers of Inflammation and Adiponectin Expression in Visceral Adipose Tissue of Male and Female Mice

Neither expression of *mPGES1* nor the number of F4/80 positive cells in visceral fatty tissue (Figures 5A–C) differed between groups. By contrast, expression of adiponectin was significantly lower in adipose tissue of female mice chronically exposed to fructose ( $\sim 84\%$ ; Figure 5D). A similar effect of the fructose feeding was not found in adipose tissue of male mice.

### Expression of Adiponectin Receptor 1 (*AdipoR1*) and Phosphorylation of AMPK in Liver Tissue of Female and Male Mice

In line with the findings for adiponectin in visceral fatty tissue, mRNA expression of *AdipoR1* was significantly lower in livers of fructose-fed female mice than in the respective controls (Figure 6A). Similar changes were not found in male mice. Furthermore, whereas protein levels of *pAMPK* were only slightly higher in livers of female mice fed fructose ( $\sim 67\%$ ), *pAMPK* levels were approximately three-fold higher in livers of



**Figure 4.** Sex differences in hepatic insulin receptor as well as IRS-1 and -2 mRNA expression levels and concentration of active PAI-1 in mice with steatosis induced by feeding a 30% fructose solution. Expression levels of (A) insulin receptor (InsR), (B) IRS-1, and (C) IRS-2 in livers of male and female mice. (D) Hepatic PAI-1 protein concentration. Expression of mRNA was determined by real time RT-PCR with  $\beta$ -actin as a reference gene in the liver. Data are shown as means  $\pm$  SEM ( $n = 6$ ) and are specified as -fold induction. C—water (control); F—30% fructose solution. \*\* $p < 0.01$  compared with respective water-fed control mice.

male fructose-fed mice in comparison to the respective controls (Figure 6B).

## DISCUSSION

### Female Mice Are More Susceptible to NAFLD

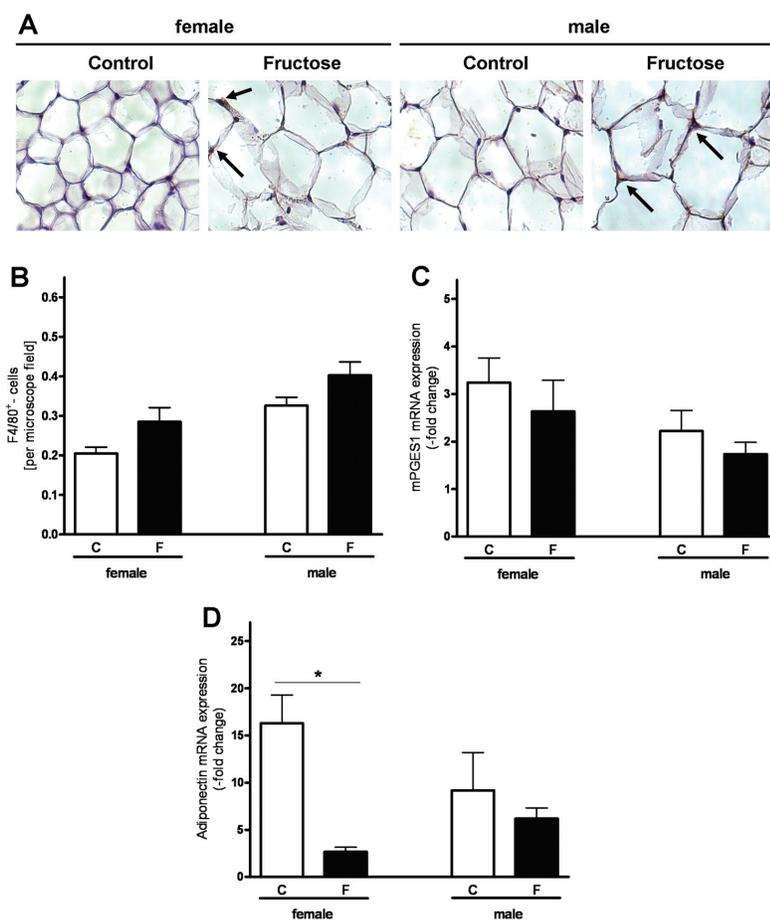
During the last decade, results of several studies suggested that gender, and particularly male gender, may be a risk factor for the development of NAFLD (20,21); however, data are still rather contradictory and the mechanisms involved are not yet clarified (22,23). Animal models resembling conditions of early phases of NAFLD in humans (for example, steatosis and steatohepatitis) have been found to be useful tools in investigating the mechanisms and pathophysiology underlying the development of NAFLD. In the present study, the hypothesis that male and female mice differ in regards of susceptibility to NAFLD was tested in

mice chronically fed a moderate fructose-enhanced diet (for example, 30% fructose in drinking solutions) for 16 wks. Despite a similar intake of fructose and a markedly higher absolute weight gain in male mice, hepatic damage was significantly more severe in female mice. Indeed, lobular inflammation was almost absent in male mice while being frequently observed in livers of female animals. In line with these findings, expression of *CCL-2* mRNA expression and also the number of neutrophils was markedly higher in livers of female mice. Interestingly, the increased weight gain of male mice fed fructose was not associated with an excessive intake of calories when compared with female mice fed fructose; rather, both male and female mice exposed to fructose increased their daily caloric intake by  $\sim 24$  kcal/mouse/d suggesting either that metabolism of fructose differs markedly between sexes or

that female mice were more physically active. However, mechanisms underlying the increased weight gain found in male mice and the lack of a weight gain in female mice, respectively, will have to be clarified in future studies. The more pronounced stage of NAFLD found in female mice in the present study is somewhat contrary to most the findings in epidemiological studies in humans (24,25); however, contrary to human studies, mice were the same age, did not differ in regards to body fat distribution (for example, android versus gynoid fat distribution in female study participants) and had a similar dietary intake. Taken together, these data suggest that female mice are more susceptible to NAFLD induced by a chronic exposure to fructose.

### Portal Endotoxin, 4-HNE Protein Adducts, *TNF $\alpha$* levels and Markers of Insulin Resistance are Similarly Altered in Livers of both Male and Female Mice with NAFLD

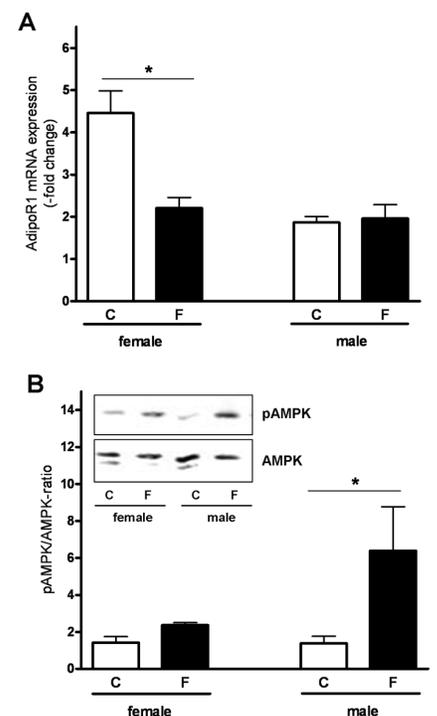
Results of human studies of our own lab and also those of other groups suggest that an increased translocation of bacterial endotoxin may be involved in the development of NAFLD (26–28). Indeed, both intestinal permeability and endotoxin levels in peripheral blood and expression of the endotoxin receptor *TLR-4* in the liver have been shown to be markedly higher in patients with NAFLD than in controls (5,27,28). Furthermore, results obtained in animal models of alcohol-induced liver disease suggest that estrogen is a modulator of intestinal barrier function as portal endotoxin levels of alcohol-exposed ovariectomized female rats were at the level of those of male rats, whereas portal endotoxin levels of sham-operated female animals were markedly higher (29). In addition, Ikejima *et al.* found that sensitivity of Kupffer cells to challenges with endotoxin after being exposed to estrogen was markedly enhanced (30). Furthermore, Enomoto *et al.* showed that the enhanced sensitivity of Kupffer cells to challenges with endotoxin found in estriol-treated rats was associated with an increased translocation



**Figure 5.** Sex differences in markers of inflammation and adiponectin expression in visceral adipose tissue in mice with steatosis induced by feeding a 30% fructose solution. (A) Representative photomicrographs of immunohistochemical staining of F4/80<sup>+</sup> cells (630 $\times$  with oil immersion) and (B) number of F4/80<sup>+</sup> cells in visceral adipose tissue of male and female mice ( $n = 4-6$  as histological material lacked). Expression levels of (C) mPGES1 ( $n = 5-6$ ) and (D) adiponectin ( $n = 6$ ) mRNA in visceral adipose tissue of mice. Expression of mRNA was determined by real time RT-PCR with  $\beta$ -actin as a reference gene in visceral fat. Data are shown as means  $\pm$  SEM ( $n = 5-6$ ) and are specified as -fold induction. C—water (control); F—30% fructose solution. \* $p < 0.05$  compared with respective water fed control mice.

of bacterial endotoxin from the gut and induction of cluster of differentiation 14 (CD14) mRNA expression (31). In the present study, basal levels of portal endotoxin were indeed slightly higher in female mice than in male animals; however, the development of NAFLD was associated with an increase of portal endotoxin levels that was similar between the two sexes (+ ~1.7-fold in both male and female mice). In line with these findings, expression of *MyD88* and *mPGES1*, being markers of an activation of *TLR-4*-dependent signaling cascades (6,32) and

also the concentration of 4-HNE protein adducts and *TNF $\alpha$*  protein in the liver also were found to be induced to a similar extent in male and female mice with NAFLD. Differences between the findings of Yin *et al.* (29) and those of the present study may have resulted from differences in the animal models used (for example, alcohol exposure versus fructose ingestion) or the species studied (for example, rats versus mice). Taken together, these data suggest that the more pronounced liver damage found in the livers of female animals in the present study was not pri-



**Figure 6.** Sex differences in expression of adiponectin receptor 1 and phosphorylation status of AMPK in liver tissue in mice with steatosis induced by feeding a 30% fructose solution. (A) mRNA expression of AdipoR1 in livers of mice as well as (B) representative Western blots of hepatic phosphorylation status of AMPK in male and female mice. Expression of mRNA was determined by real time RT-PCR with  $\beta$ -actin as a reference gene in the liver. Data are shown as means  $\pm$  SEM ( $n = 6$ ) and are specified as -fold induction. C—water (control); F—30% fructose solution. \* $p < 0.05$  compared with respective water-fed control mice.

marily a result of an enhanced translocation of bacterial endotoxin, activation of *TLR-4*-dependent signaling cascades, lipidperoxidation or release of *TNF $\alpha$* . These results do not preclude a role of endotoxin, lipidperoxidation or *TNF $\alpha$*  in the development of NAFLD in both female and male animals, but rather suggest that additional pathways may be involved in the greater susceptibility of female animals to NAFLD (see below).

Besides an enhanced translocation of bacterial endotoxin and the subsequent activation of *TLR-4*-dependent signaling

cascades in the liver, a loss of insulin sensitivity has been claimed to be a key factor in the development of NAFLD (33). A decreased expression of the insulin receptor as well as *IRS-1* and *-2* has been shown repeatedly to be associated with impairments of insulin signaling and insulin resistance (34–37). Indeed, García-Monzón C *et al.* have shown that in patients with NASH expression of *IRS-1* and *-2* was markedly lower than in controls (38). In addition, recently it was shown in female rats exposed to fructose for 2 wks that glucose tolerance was markedly impaired and that this was associated with a decreased expression of *IRS-2* mRNA in the liver (39); however, in that study, glucose metabolism was not found to be impaired in male rats exposed to fructose. Furthermore, Kong *et al.* have shown that a decreased expression of the insulin receptor also was associated with decreased insulin sensitivity in the liver (40). In the present study, the development of NAFLD was associated with a lower expression of *IRS-1* and *-2* in the liver of both female and male mice, whereas expression of the insulin receptor remained unchanged. Differences between the results of the present study and those of Kong *et al.* (40) and Vila *et al.* (39) may have resulted from differences in species and genetic strains used (for example, rats and KK-Ay mice versus C57Bl/6J mice fed fructose in the present study) as well as the duration of the fructose exposure (2 wks versus 16 wks in the present study). Taken together, these data suggest that the greater susceptibility of female animals to the development of more severe stages of NAFLD found in the present study was also not primarily a result of a more pronounced impairment of insulin signaling in the liver.

#### The More Pronounced Liver Damage in Female Mice Is Associated with Increased Levels of Active PAI-1 in the Liver and an Impaired Adiponectin Expression in Visceral Fatty Tissue

In recent years, the acute phase protein *PAI-1* has been shown to be involved in

the development of NAFLD (7). Indeed, in human studies, it was shown that hepatic expression and circulating plasma *PAI-1* levels in humans are closely related to the degree of liver steatosis (27,28,41). Furthermore, our own group recently showed that *PAI-1*<sup>-/-</sup> mice are protected from the onset of NAFLD and that this protection was associated with a markedly lower accumulation of lipids in the liver and also with a decrease in markers of inflammation (7). It also was shown recently in a mouse model that *AMPK* through small heterodimer partner (*SHP*)-dependent signaling cascades may function as a negative regulator of *PAI-1* in the liver (42). It further has been shown that adiponectin, through its receptor 1, may modulate the activity of *AMPK* and subsequently *PAI-1* (43). In the present study, the more pronounced liver damage found in female mice was associated with markedly increased levels of active *PAI-1* in the liver and a markedly lower expression of adiponectin in visceral adipose tissue as well as its receptor 1 in the liver. By contrast, in livers of male fructose-fed mice, levels of active *PAI-1* were almost at the level of controls and expression of adiponectin in visceral adipose tissue as well as of its receptor 1 in hepatic tissue were unchanged. Furthermore, in livers of male mice with NAFLD, phosphorylation of *AMPK* was markedly higher than in controls; a similar effect was not found in livers of female mice with NAFLD. Contrary to the findings of the present study, Vila *et al.* recently showed that, in livers of female rats exposed to a 10% w/v fructose drinking solution for 2 wks, phosphorylation of *AMPK* was markedly higher than in controls (39). In that study, a similar effect of the fructose feeding on *AMPK* in the liver was not found in male rats exposed to fructose. Differences between the findings of Vila *et al.* (39) and those of the present study may have resulted from the different experimental setups (for example, Vila *et al.* 2 wks, 10% w/v fructose to rats versus 16 wks, 30% w/v fructose fed to mice). However, in support of the results of the present study, Ikejima *et al.* showed previously that

mouse models lacking the physiological upregulation of adiponectin, such as the KK-A(y) mice, are more susceptible to hepatic steatosis, inflammation and fibrosis induced by a MCD-diet as compared with C57Bl/6 control mice (44). Furthermore, results obtained in rats suggest that the expression of adiponectin as well as AdipoR1 is, at least in part, regulated through estrogen receptor  $\beta$ -dependent signaling cascades in adipose tissue (45). In addition, in mice fed a high fat diet, the treatment with 17- $\beta$  estradiol was associated with increased levels of *PAI-1* mRNA expression in the liver (46). Taken together, these data suggest that the higher susceptibility of female mice to NAFLD found in the present study may, at least in part, have resulted from a decreased adiponectin release in visceral adipose tissue, subsequently leading to alterations in the adiponectin-*AMPK*-signaling cascade in the liver, which, in turn, may have led to increased levels of active *PAI-1* in the liver. However, further studies will have to elucidate molecular mechanisms underlying the protection of male mice against the loss of adiponectin and its receptor 1 as well as the superinduction of *AMPK* in livers of these mice.

#### CONCLUSION

Taken together, our data suggest that female mice are more susceptible to NAFLD than male mice. Our data further suggest that the enhanced liver damage found in female mice does not result primarily from an enhanced intestinal permeability or greater sensitivity of Kupffer cells toward endotoxin, but rather results from alterations of the adiponectin-*AMPK*-*PAI-1* signaling cascade in the liver. However, additional studies are necessary to further explore underlying mechanisms and possible resulting therapeutic strategies (for example, *PAI-1*).

#### ACKNOWLEDGMENTS

This work was supported by a grant from the German Ministry of Education and Science (BMBF) (03105084) (I Bergheim).

## DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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