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ORIGINAL CONTRIBUTION

Moderate alcohol consumption diminishes the development of non-alcoholic fatty liver disease (NAFLD) in *ob/ob* mice

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Abstract
Purpose Using *ob/ob* mice as a model of non-alcoholic fatty liver disease (NAFLD), we investigated the effect of moderate alcohol intake on the development of NAFLD and molecular mechanisms involved.
Methods *Ob/ob* mice were fed water or ethanol solution (2.5 g/kg body weight/day) for 6 weeks, and markers of liver injury, insulin signalling and adiponectin in visceral adipose tissue were determined.
Results Whereas bodyweight and the degree of liver steatosis did not differ among *ob/ob* mouse groups, those consuming ethanol had markedly less macrovesicular hepatic fat accumulation, inflammatory alterations and significantly lower transaminase levels. Despite similarly elevated protein levels of tumour necrosis factor α, protein concentrations of plasminogen activator inhibitor 1 were significantly lower in livers of *ob/ob* mice consuming ethanol in comparison with controls. The hepato-protective property of moderate alcohol ingestion in *ob/ob* mice was associated with an induction of the sirtuin-1/adiponectin-signalling cascade in visceral fat tissue and an activation of Akt in the liver. Similar effects of moderate alcohol exposure were also found in vitro in 3T3-L1 and AML-12 cells.
Conclusion These data suggest that moderate alcohol intake may diminish the development of NAFLD through sirtuin-1/-adiponectin-dependent signalling cascades.

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Keywords Adiponectin · Ethanol · PAI-1 · SIRT1 · Visceral fat

Abbreviations

ACOX1	Acyl-coenzyme A oxidase
ADH	Alcohol dehydrogenase
Akt	Protein kinase B
AML-12	Alpha mouse liver 12 cells
AMPK	Adenosine monophosphate-activated protein kinase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BAX	BCL2-associated X protein
b.w.	Body weight
CCL2	Chemokine (c–c motif) ligand 2
ELISA	Enzyme-linked immunosorbent assay
FAS	Fatty acid synthase
FOXO1	Forkhead box O1
GK	Glucokinase
GLUT4	Glucose transporter type 4
H&E	Haematoxylin and eosin
IRS-1/2	Insulin receptor substrate 1/2
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
PAI-1	Plasminogen activator inhibitor 1
PEPCK	Phosphoenolpyruvate carboxykinase
PPARγ	Peroxisome proliferator-activated receptor gamma
RT-PCR	Reverse transcriptase polymerase chain reaction
SIRT1	Sirtuin-1
SREBP-1c	Sterol regulatory element-binding protein 1c
TNFα	Tumour necrosis factor alpha

Introduction

By now, non-alcoholic fatty liver disease (NAFLD), a disease comprising a continuum of diseases ranging from simple steatosis to hepatic cirrhosis, is one of the most common liver diseases worldwide [1, 2]. NAFLD is commonly associated with metabolic co-morbidities like obesity, type 2 diabetes and the metabolic syndrome [3]. Indeed, NAFLD has repeatedly been considered as the hepatic manifestation of the metabolic syndrome [2, 3]. Results of studies performed in recent years suggest that not only genetic predisposition, alterations of the intestinal barrier function, a sedentary lifestyle and over-nutrition, but also certain dietary patterns (e.g. a diet rich in fat and sugar or iron/animal-derived protein) may be critical in the development of NAFLD (for overview see [4]). However, despite intense research effort, molecular mechanisms involved in the disease onset but even more so its progression and herein especially the role of the composition of diet have not yet been clarified.

Heavy alcohol consumption (e.g. an intake >50 g/day) is unequivocally associated with the development of liver steatosis [5]. In contrast, a recent population-based study suggests that moderate alcohol consumption (<20 g on 1–3 days/week) may even decrease the odds to develop NAFLD [6, 7]. These data are in line with the findings of others who reported that moderate drinkers (<20 g/day) had a lower risk of being diagnosed with non-alcoholic steatohepatitis (NASH) but also fibrosis than lifetime non-drinkers [8]. Results of human studies suggest that the beneficial effect of moderate alcohol intake may be associated with increased levels of adiponectin [9]; however, molecular mechanisms involved in the beneficial effects of moderate intake of alcoholic drinks have not yet been fully understood. Results obtained in animal experiments are inconsistent. Indeed, using *ob/ob* mice as a model of NAFLD, a protective effect of chronic alcohol consumption (≤21 g/kg body weight (b.w.)/day) was shown [10]. In contrast, results of other studies suggest that moderate to elevated consumption of plain ethanol may actually add to the progression of NAFLD [11, 12] and that this could even be dose dependent [13, 14]. Furthermore, protective effects found in human studies might not have resulted from the intake of ethanol itself but rather from other factors associated with the intake of alcohol (e.g. intake of resveratrol in red wine or hops ingredients in beer or even changes in lifestyle [15–18]).

Starting from this background, the primary aim of the present study was to determine the effects of moderate chronic intake of ethanol (2.5 g/kg b.w./day) on the development of NAFLD in *ob/ob* mice. Because moderate alcohol intake diminished the development of NAFLD, a secondary objective of the study was to identify potential mechanisms involved in the beneficial effects of the moderate intake of alcohol.

Materials and methods

Animals and treatments

Mice were housed in a specific pathogen-free barrier facility accredited by the 'Association for Assessment and Accreditation of Laboratory Animal Care International'. All procedures were approved by the local 'Institutional Animal Care and Use Committee'. Six-week-old male C57BL/6J mice used as naïve controls ($n = 10$), and *ob/ob* mice ($n = 12$) were obtained from Janvier (Janvier Labs, France). Animals had free access to chow during the entire experiment. Furthermore, mice had either free access to plain tap water or water enriched with 2.5 g/kg b.w./day ethanol for 6 weeks. Ethanol intake was assessed daily and adjusted to liquid intake. Body weight was assessed twice weekly. This dose of ethanol did not cause any behavioural changes in mice or any signs of drunkenness. Blood was collected from the portal vein just prior to killing, and portions of liver and adipose tissue were snap-frozen immediately, frozen-fixed in Tissue-Tek® O.C.T. mounting media (Sakura, Germany) or fixed in neutral-buffered formalin.

Cell culture

3T3-L1 cells, a model for adipocytes (DSMZ, Germany), were cultured at 37 °C in a humidified, 5 % carbon dioxide atmosphere. At 80 % confluence, differentiation of 3T3-L1 cells was started by exposing cells to DMEM medium containing 10 % foetal bovine serum, 0.5 mM 3-Isobutylmethylxanthin, 2.5 µM dexamethasone, 850 nM insulin as well as 1 % penicillin and streptomycin (all PAN-Biotech, Germany). On day 9, cells were treated with 0.2 vol% ethanol for 6 h in the presence or absence of 1 mM 4-methylpyrazole in an alcohol vapour chamber as described previously by others [19].

Alpha mouse liver 12 (AML-12) cells obtained from American type culture collection (ATCC, USA) were grown in DMEM/F12 media (PAN-Biotech, Germany) supplemented with 10 % foetal bovine serum, 40 ng/mL dexamethasone, 0.005 mg/mL insulin, 5 ng/mL selenium and 0.005 mg/mL transferrin as well as 1 % penicillin and streptomycin (PAN-Biotech, Germany). At 70 % confluence, cells were serum starved for 18 h in starvation media supplemented with bovine serum albumin (0.01 %), insulin, selenium and transferrin as well as penicillin and streptomycin. Cells were then challenged with 10 ng/mL tumour necrosis factor (TNF) α (Sigma–Aldrich, Germany) ±2 µg/ml adiponectin (Bio Vendor, Germany) for 6 h. Cells were

Table 1 Primer sequences used for real-time RT-PCR

	Forward (5′–3′)	Reverse (5′–3′)
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ACOX1	GGGAATTTGGCATCGCAGAC	CATCTCCGTCTGGGCGTAGG
Adiponectin	GAAGACCTGCATCTCCTTTCTCTCC	TCATGCCGAAGATGACGTTACTACA
BAX	CGAGCTGATCAGAACCATCA	CTCAGCCCATCTTCTTCCAG
CCL2	GCCAGACGGGAGGAAGGCCA	TGGATGCTCCAGCCGGCAAC
FAS	TCT GGG CCA ACC TCA TTG GT	GAA GCT GGG GGT CCA TTG TG
FOXO1	CTCCCGGTACTTCTCTGCTG	GTGGTCGAGTTGGACTGGTT
GK	ACT TTC CAG GCC ACA AAC A	TCC CAG AAC TGT AAG CCA CTC
GLUT4	GAT TCT GCT GCC CTT CTG TC	ATT GGA CGC TCT CTC TCC AA
IRS-1	GCTCTAGTGCTTCCGTGTCC	GTTGCCACCCCTAGACAAAA
IRS-2	GAAGCGGCTAAGTCTCATGG	GACGGTGGTGGTAGAGGAAA
PEPCK	CCC TGG GAG ATG GGG AGT TC	CCC ACC ATA TCC GCT TCC AA
Perilipin 2	GTGGGTGGAGTGGAAGAGAA	TGGCATGTAGTCTGGAGCTG
Perilipin 3	CAAGCTGCTATGGAGGAACC	GGTCTTGACGCCTTTCTCAG
PPARγ	AAC GTG AAG CCC ATC GAG GA	CTG CAC GTG CTC TGT GAC GA
SIRT1	GACGCTGTGGCAGATTGTTA	GGAATCCCACAGGAGACAGA
SREBP-1c	AAC GGC TAC TGC TGG ACT GC	AGA GCA AGA GGG TGC CAT CG

rinsed twice with ice-cold phosphate-buffered saline and used for RNA isolation.

Hepatic lipid accumulation, histological evaluation and clinical chemistry

To determine hepatic lipid accumulation, triglyceride levels were measured, and Oil Red O lipid staining was performed as previously detailed [20]. Representative pictures of Oil Red O staining were taken using a camera integrated in a microscope (DM4000 B LED, Leica, Germany) at 200× magnification. Paraffin-embedded livers were cut into 5-μm sections, stained with haematoxylin and eosin (H&E, Sigma, Germany), and histology was evaluated using the NAFLD activity scoring system (NAS) according to Kleiner et al. [21]. Neutrophil granulocytes were stained using a commercially available naphthol AS-D chloro-acetate-esterase staining kit (Sigma–Aldrich, Germany). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as well as lipid profile (triglycerides, total cholesterol, HDL and LDL cholesterol and free fatty acids) were determined in heparinized plasma in a routine laboratory at the University Clinic of Jena (Beckman Coulter® Biomedical GmbH, Germany).

Enzyme-linked immunosorbent assays (ELISA) for TNFα and plasminogen activator inhibitor (PAI)-1

Protein concentration of TNFα and PAI-1 in whole liver lysates, extracted with a buffer containing 50 mM TRIS, 150 mM NaCl, 2 mM KCl, 0.5 % Triton-X 100 (Roth, Germany) and protease inhibitors (Sigma–Aldrich, Germany) were determined using commercially available ELISA kits according to the instructions of the manufacturers (TNFα: Alpco Diagnostics, USA; PAI-1: AssayPro, USA).

RNA isolation and real-time RT-PCR

Total RNA was extracted from liver and fat tissue as well as cells using peqGOLD TriFast™ (PEQLAB, Germany), and cDNA was synthetized as previously described [22]. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used for the detection of adiponectin, acyl-coenzyme A oxidase (ACOX1), BCL2-associated X protein (BAX), chemokine (c–c motif) ligand 2 (CCL2), fatty acid synthase (FAS), forkhead box O1 (FOXO1), glucokinase (GK), glucose transporter type 4 (GLUT4), insulin receptor substrate (IRS)-1 and IRS-2, phosphoenolpyruvate carboxykinase (PEPCK), perilipin 2 and 3, peroxisome proliferator-activated receptor gamma (PPARγ), sirtuin-1 (SIRT1), sterol regulatory element-binding protein 1c (SREBP-1c) and 18S (for primer sequences see Table 1) as described previously [22]. To determine the amount of target genes, the comparative C_T method was used and normalized to an endogenous reference (18S) relative to a calibrator ($2^{-\Delta\Delta Ct}$).

Caspase-9 activity

For this study, caspase-9 activity was measured using a luminescent Caspase-Glo® 9 assay (Promega, Germany).

Immunoblots

Cytosolic protein lysates were prepared by homogenizing liver tissue in a lysis buffer (1 mol/L HEPES, 1 mol/L

$MgCl_2$, 2 mol/L KCl, 1 mol/L DTT all Roth, Germany) containing a protease and phosphatase inhibitor mix (Sigma–Aldrich, Germany). Proteins (60 µg protein/well) were separated in 10 % SDS-polyacrylamide gels and transferred onto Hybond™-P polyvinylidene difluoride membranes (Amersham Biosciences, Germany). Blots were probed with antibodies against phospho-Akt (a protein kinase B) or total Akt (Cell Signalling Technology, USA). To ensure equal loading of blots, all blots were stained with Ponceau Red (Sigma–Aldrich, Germany). Bands were visualized using a Super Signal Western Dura kit (Thermo Scientific, USA), and blots were analysed using software integrated in the Chemi Doc™ MP System (Bio-Rad, Germany).

Statistical analysis

All results are reported as mean ± standard error of mean (SEM) ($n = 4$–6). Statistically significant differences between *ob/ob* groups were determined using Mann–Whitney *U* test included in the GraphPad Prism 6 software (GraphPad Prism Inc., USA). A p value <0.05 was considered to be significant. Grubbs test included in GraphPad Prism 6 software was used to identify outliers.

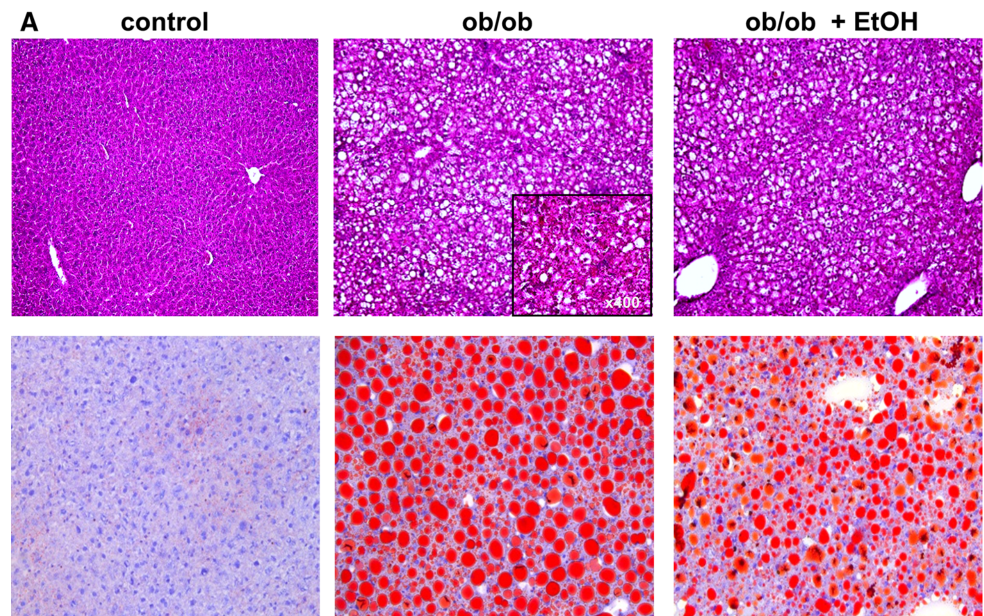
Results

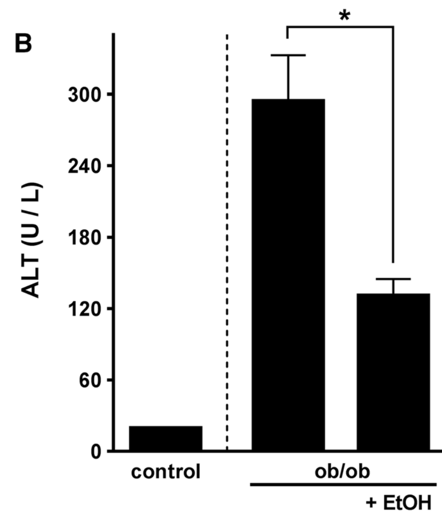
Effect of moderate alcohol consumption on liver status of *ob/ob* mice

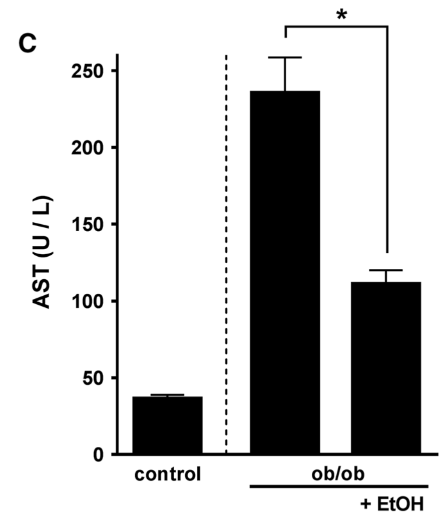
As no differences in regards to markers of liver damage, plasma lipid profile and selected other parameters (e.g. expression of insulin receptor substrates and PAI-1) between naïve wild-type mice and wild-type mice fed moderate amounts of ethanol (data not shown) were found, data from wild-type mice fed plain water are shown to represent both control groups. As expected, *ob/ob* mice fed plain water developed massive macrovesicular steatosis associated with beginning inflammatory alterations (see Fig. 1a and NAS score in Table 2). Despite a significantly higher overall caloric intake (+~6 kcal/mouse/week, $p > 0.05$) resulting from the alcohol added to the drinking water, body weight was similar between ob/ob groups. Furthermore, while liver-to-body weight ratio and number of fatty hepatocytes did not differ between groups, *ob/ob* mice chronically consuming moderate amounts of ethanol displayed only microvesicular fat accumulation in most of the hepatocytes along with markedly lower hepatic triglyceride levels (−17 %, $p = 0.056$) and less signs of inflammation when compared to *ob/ob* mice only consuming drinking water (see Table 2). In line with these findings, plasma levels of ALT and AST were significantly lower in *ob/ob* mice drinking moderate amounts of alcohol in comparison with *ob/ob* mice fed with tap water. However, both plasma AST and ALT levels were still higher than those of naïve wild-type mice (see Fig. 1b, c). Not only plasma levels of triglycerides, total cholesterol, HDL as well as LDL cholesterol but also free fatty acids were similar between *ob/ob* mouse groups (see Table 3). However, total cholesterol but also HDL and LDL cholesterol plasma levels were markedly higher than in naïve controls. In line with these findings, HDL/LDL ratios were similar between both *ob/ob* mouse groups. Furthermore, mRNA expression of perilipin 2 and 3 shown before to be involved in the formation, maintenance and involution of lipid droplets [23] was also significantly induced in livers of mice drinking plain water only (Fig. 1d, e). In contrast, in livers of mice consuming moderate amounts of ethanol, expression of perilipin 2 and 3 mRNA were at the level of naïve controls. Number of neutrophils and mRNA expression of CCL2 but also markers of apoptosis such as BAX mRNA expression and activity of caspase-9 were all markedly induced in livers of *ob/ob* mice fed with tab water. In livers of *ob/ob* mice consuming moderate amounts of alcohol, levels of these markers were almost at the level of controls; however, as some of these data varied considerably within the groups, results did not reach the level of significance for most of these parameters (Table 2).

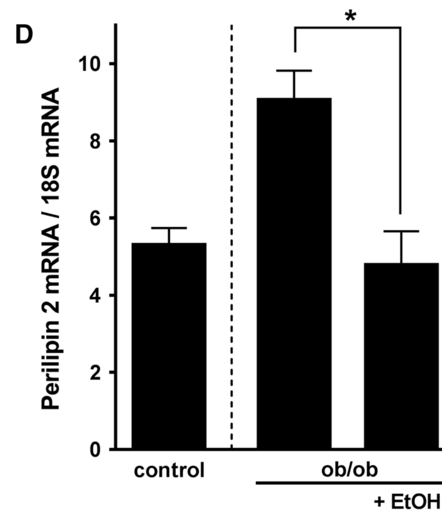
Effect of moderate alcohol consumption on protein levels of TNFα, PAI-1, markers of insulin signalling cascade as well as carbohydrate metabolism and lipogenesis in the liver

Neither protein levels of TNFα, nor expression levels of IRS-1 and 2 mRNA differed between livers of *ob/ob* mice fed plain water and those consuming moderate amounts of ethanol (Fig. 2a–c). However, TNFα protein and IRS-2 mRNA expression in liver tissue were markedly different in *ob/ob* mice regardless of additional treatment in comparison with naïve control mice. As data varied considerably, IRS-1 mRNA expression did not differ between groups. In contrast, protein levels of phosphorylated Akt were markedly higher in livers of mice drinking moderate amounts of alcohol in comparison with naïve controls and *ob/ob* mice fed plain water (Fig. 2e, f); however, as levels of phosphorylated Akt varied considerable within groups, differences did not reach the level of significance between ob/ob groups. Expression of PEPCK, GK and GLUT4 mRNA in the liver, respectively, was similar between *ob/ob* groups. However, expression of GLUT4 was higher in livers of *ob/ob* mice regardless of additional treatments when compared to naïve control mice. In contrast, mRNA expression of FAS ($p < 0.05$) and to a lesser extend SREBP-1c ($p = 0.07$) were both lower in livers of *ob/ob* mice fed moderate amounts of ethanol in comparison with *ob/ob* mice









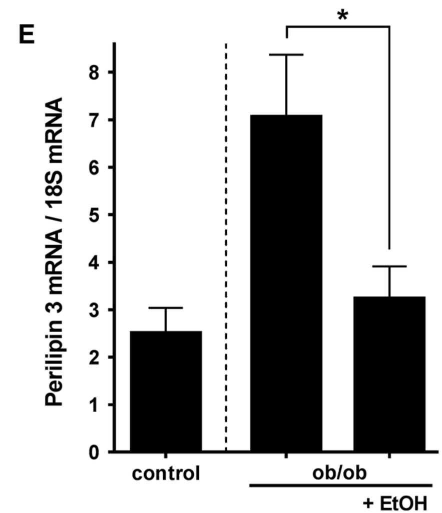


Fig. 1 Effect of moderate alcohol consumption on indices of liver damage and hepatic perilipin mRNA expression in *ob*/*ob* and control mice. **a** Representative photomicrographs of haematoxylin and eosin staining (×100 and ×400) and Oil Red O staining (×200) of liver sections. **b**, **c** Plasma ALT and AST levels **d**, **e** Perilipin 2 and 3 mRNA expression normalized to 18S mRNA in the liver. Data are shown as mean ± SEM ($n = 4$–6). *$p < 0.05$ in comparison with *ob*/*ob* mice treated with ethanol

Table 2 Effect of ethanol feeding on body weight and indices of liver damage in lean and *ob*/*ob* mice

	Lean mice	*ob*/*ob* mice	
	Control	Control	Ethanol
Body weight (g)	27.7 ± 0.6	49.1 ± 0.8	50.1 ± 1
Liver weight (g)	1.5 ± 0.1	4.0 ± 0.1	4.3 ± 0.2
Liver-to-body weight ratio (%)	5.6 ± 0.1	8.2 ± 0.2	8.5 ± 0.3
Alcohol intake (g/kg b.w./d)	–	–	2.5 ± 0.05
Food consumption (g/mouse)	22.7 ± 0.3	36.4 ± 0.3	36.7 ± 2.5
Caloric intake (kcal/mouse/week)	77.4 ± 1.1	124.5 ± 1	130.4 ± 8.4
NAS	0.02 ± 0.02	3.7 ± 0.3*	2.5 ± 0.2
Steatosis	0.0 ± 0.0	2.8 ± 0.2	2.4 ± 0.25
Inflammation	0.02 ± 0.02	0.9 ± 0.2*	0.1 ± 0.05
Triglycerides (µg/mg protein)	3.6 ± 0.6	227 ± 12.5	188 ± 7.6
Neutrophil count (per microscopic field)	0.25 ± 0.09	0.33 ± 0.07*	0.075 ± 0.05
CCL2/18S mRNA	5.4 ± 0.7	10.3 ± 2.3*	2.9 ± 0.9
BAX/18S mRNA	2.9 ± 0.4	3.3 ± 0.6	1.9 ± 0.5
Caspase-9 activity (fold induction)	3.1 ± 0.3	6.0 ± 1.05	4.0 ± 0.4

Values represent mean ± SEM (n = 4–6). * $p < 0.05$ in comparison with *ob*/*ob* mice treated with ethanol

NAS NAFLD activity score

Table 3 Effect of moderate ethanol feeding on plasma lipid profile

	Lean mice	*ob*/*ob* mice	
	Control	Control	Ethanol
Triglycerides (mmol/L)	0.8 ± 0.1	1.05 ± 0.2	1 ± 0.1
Total cholesterol (mmol/L)	2.1 ± 0.02	4.1 ± 0.2	3.7 ± 0.1
HDL cholesterol (mmol/L)	0.09 ± 0.01	0.2 ± 0.02	0.15 ± 0.01
LDL cholesterol (mmol/L)	1 ± 0.02	1.6 ± 0.06	1.5 ± 0.04
LDL/HDL	12.1 ± 1.5	7.8 ± 0.7	10 ± 1.1
Free fatty acids (mmol/L)	0.27 ± 0.04	0.34 ± 0.05	0.27 ± 0.02

Values represent mean ± SEM (n = 4–6). Lipid profile has been determined in heparinized plasma

fed plain water. Hepatic mRNA expression of ACOX-1 and PPARγ did not differ between *ob*/*ob* mouse groups; however, with the exception of SREBP-1c, expressions of genes involved in lipogenesis were markedly higher in livers of ob/ob mice than in livers of naïve lean control mice (Table 4). Furthermore, the elevated protein levels of phosphorylated Akt were associated with a marked protection against the induction of PAI-1 protein levels found in livers of *ob*/*ob* mice fed plain water (Fig. 2d).

Effect of moderate alcohol consumption on the SIRT1-/adiponectin-signalling cascade in visceral adipose tissue

Adiponectin expression in visceral adipose tissue was significantly lower in *ob*/*ob* mice fed plain water when compared to those chronically drinking moderate amounts of alcohol (Fig. 3a). Furthermore, expression of SIRT1, shown to be a key regulator of adiponectin expression in adipocytes [24], was significantly induced only in visceral adipose tissue of *ob*/*ob* mice drinking moderate amounts of alcohol (~threefold) when compared between groups (Fig. 3b).

Effect of moderate alcohol exposure on 3T3-L1 cells challenged with 4-methylpyrazole

To further delineate whether the positive effects found in *ob*/*ob* mice exposed to moderate alcohol levels may have resulted from a direct effect of ethanol on adipose tissue, differentiated 3T3-L1 cells were challenged with 0.2 vol% of ethanol in an alcohol vapour chamber. Adiponectin mRNA expression was significantly increased in differentiated 3T3-L1 cells treated with 0.2 vol% of ethanol in comparison with naïve cells (Fig. 4a). Furthermore, expressions of SIRT1 and FOXO1 mRNA were also significantly higher in 3T3-L1 cells exposed to 0.2 vol% of ethanol for 6 h in comparison with naïve cells (Fig. 4b, c). These effects of ethanol were almost completely blunted when cells were concomitantly exposed to 4-methylpyrazole, an inhibitor of alcohol dehydrogenase (ADH) [25].

Effect of adiponectin treatment on PAI-1 expression in AML-12 cells challenged with TNFα

To further address the role of adiponectin in regulating PAI-1 expression in hepatocytes, AML-12 cells, a model of mouse hepatocytes [26], were challenged with TNFα. AML-12 cells challenged with TNFα for 6 h expressed significantly higher (~+3.7-fold) levels of PAI-1 mRNA. This effect of TNFα was completely blunted in cells concomitantly treated with adiponectin when exposed to TNFα (Fig. 4c, d).

Discussion

Results of several epidemiological studies suggest that the effect on general health and herein also the liver may substantially differ between moderate (e.g. <20 g/d for men and <10 g/d for women) and elevated alcohol intake (e.g. >50 g/d for men and >40 g/d for women) (for overview also see [27–29]). Furthermore, in recent years results of epidemiological studies suggest that moderate alcohol

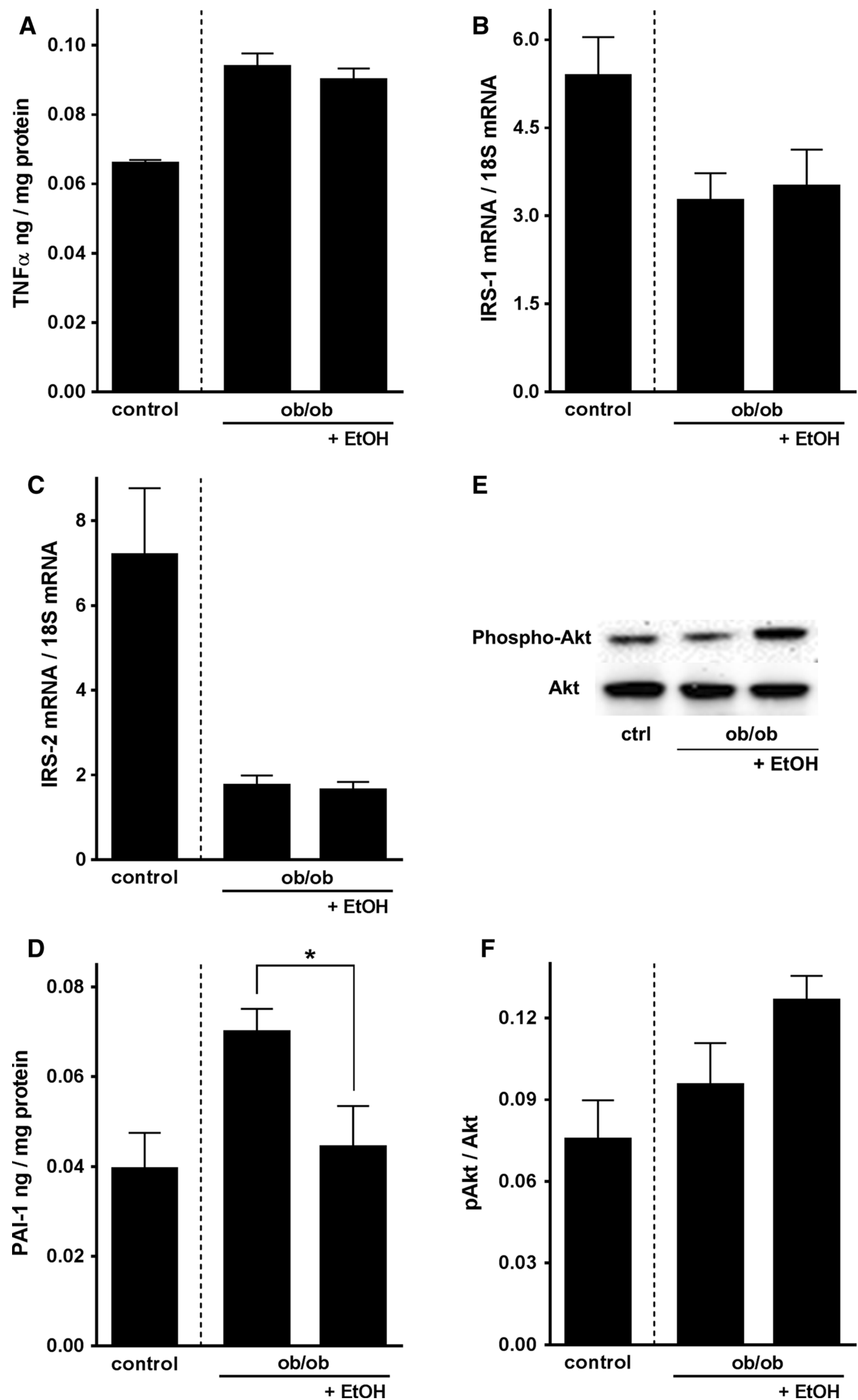


Fig. 2 Effect of moderate alcohol consumption on TNFα protein, markers of insulin signalling, PAI-1 protein levels and phosphorylation status of Akt protein in livers of *ob*/*ob* and control mice. **a** TNFα protein levels and **b, c** IRS-1 and 2 mRNA expression normalized to 18S mRNA. **d** Liver protein levels of PAI-1 and **e** representative blots of phospho-Akt and total Akt as well as densitometric analysis of the blots. Data are shown as mean ± SEM ($n = 4$–6). *$p < 0.05$ in comparison with *ob*/*ob* mice treated with ethanol

consumption may actually possess beneficial effects on the development and also the progression of liver damage in patients with NAFLD. Indeed, in a large biopsy-proven NAFLD population study, daily consumption of <20 g alcohol was associated with lower odds of NASH as well as fibrosis [6]. In line with these findings, Moriya et al. [7] reported in a cross-sectional study that drinking <20 g on 1–3 days/week is associated with a lower prevalence of fatty liver. However, from the results of these epidemiological studies, it cannot be ruled out that compounds taken in along with alcoholic beverage (e.g. compounds derived from hops or grapes or lifestyle modifications associated

Table 4 Effect of ethanol feeding on relative mRNA expression of markers of lipogenesis and carbohydrate metabolism in the liver

mRNA/18S	Lean mice	*ob*/*ob* mice	
	Control	Control	Ethanol
FAS	2.1 ± 0.6	8.4 ± 0.9*	5.9 ± 1
PPARγ	1.9 ± 0.5	5 ± 0.4	3.8 ± 0.5
ACOX1	1.8 ± 0.4	3.7 ± 0.7	3.9 ± 1
SREBP-1c	1.9 ± 0.6	1.5 ± 0.02	1.3 ± 0.1
GLUT 4	7 ± 2.8	12.9 ± 3.6	14.2 ± 3.1
GK	4.3 ± 1.4	5 ± 0.9	3.7 ± 0.9
PEPCK	8 ± 3.3	10 ± 2.6	7 ± 2

Values represent mean ± SEM ($n = 4$–6). * $p < 0.05$ in comparison with *ob*/*ob* mice treated with ethanol

with moderate alcohol intake) may contribute to the beneficial effects of alcohol consumption rather than the ethanol itself. Indeed, it has been shown that chronic intake of elevated amounts of beer and wine in comparison with spirits may have less harmful effects on the liver [15, 30]. Results of animal studies assessing the effect of plain ethanol on the liver in settings of NAFLD are inconsistent as doses and models used varied considerably between studies [10, 11, 14]. In the present study, the hypothesis that moderate alcohol consumption (e.g. 2.5 g/kg b.w. given for 6 weeks) protects against the development of NAFLD was tested in a genetic model of NAFLD (*ob*/*ob* mice). Indeed, concomitant intake of this comparably low dose of alcohol diminished the development of NAFLD as shown by markedly lower transaminases, less pronounced steatosis and inflammation and lower indices of apoptosis in livers of *ob*/*ob* mice fed ethanol. However, moderate intake of ethanol had no effects on plasma levels of free fatty acids, triglycerides, total as well as LDL and HDL cholesterol in ob/ob mice. These data are somewhat contrary to the findings of others reporting that moderate alcohol intake, e.g. resulting from beer or wine consumption, is associated with higher plasma HDL cholesterol levels in humans [31]. Differences between these studies and ours might have resulted from differences in the type of alcoholic beverages consumed (plain ethanol vs. beer or wine) but also differences in species (mice vs. humans).

An increased release of TNFα, mainly from Kupffer cells, and alterations of insulin signalling have been proposed to be key factors in the development of NAFLD (for overview see [32, 33]). Furthermore, several studies have shown that chronic intake of alcohol is associated with an induction of TNFα and impaired insulin signalling in the liver as reviewed by Cohen et al. and Purohit et al. [34, 35]. Indeed, decreased expressions of IRS-1 and IRS-2 have repeatedly been shown to be associated with impairments of insulin signalling and insulin resistance [36–39]. Furthermore, results of studies in humans suggest, that moderate alcohol consumption is associated with improved insulin sensitivity in non-diabetic humans [18, 40]. Here, neither TNFα nor IRS-1 or 2 levels were altered between livers of *ob*/*ob* mice only fed drinking water and those drinking moderate amounts of ethanol. In contrast, serine phosphorylation of Akt which was repeatedly shown to be a mediator of the cellular effects of insulin and to be negatively regulated by TNFα [41, 42] was markedly higher in livers of *ob*/*ob* mice exposed to moderate amounts of alcohol. However, mRNA expression of markers of carbohydrate metabolism, shown before to be regulated at least in part through Akt-dependent signalling pathways (for overview see [43, 44]), did neither differ between *ob*/*ob* groups nor lean control mice. In contrast, expressions of markers

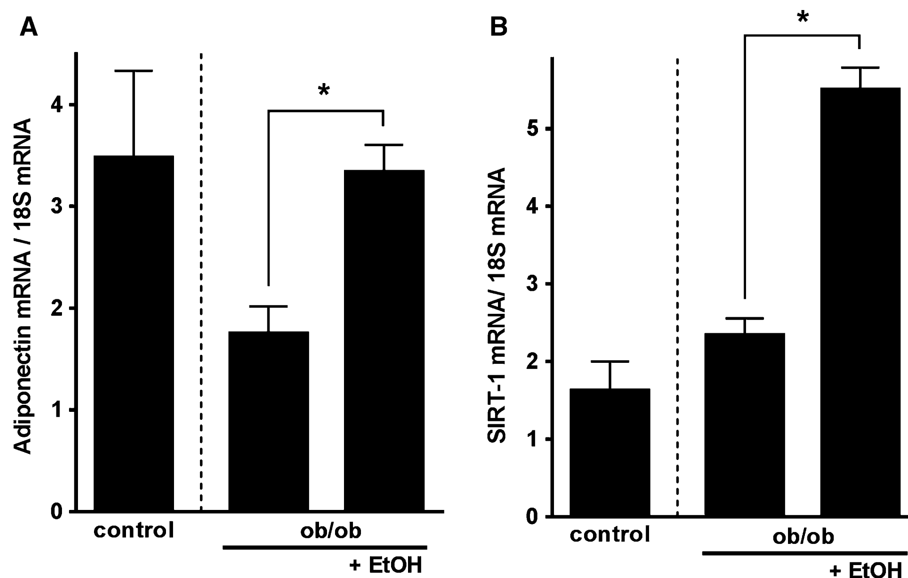


Fig. 3 Effect of moderate alcohol intake on adiponectin and SIRT1 mRNA expression in visceral adipose tissue in mice. **a** Adiponectin and **b** SIRT1 mRNA expression in adipose tissue in mice normalized to 18S mRNA ($n = 4$–6). Data are shown as mean ± SEM. *$p < 0.05$ in comparison with *ob*/*ob* mice treated with ethanol

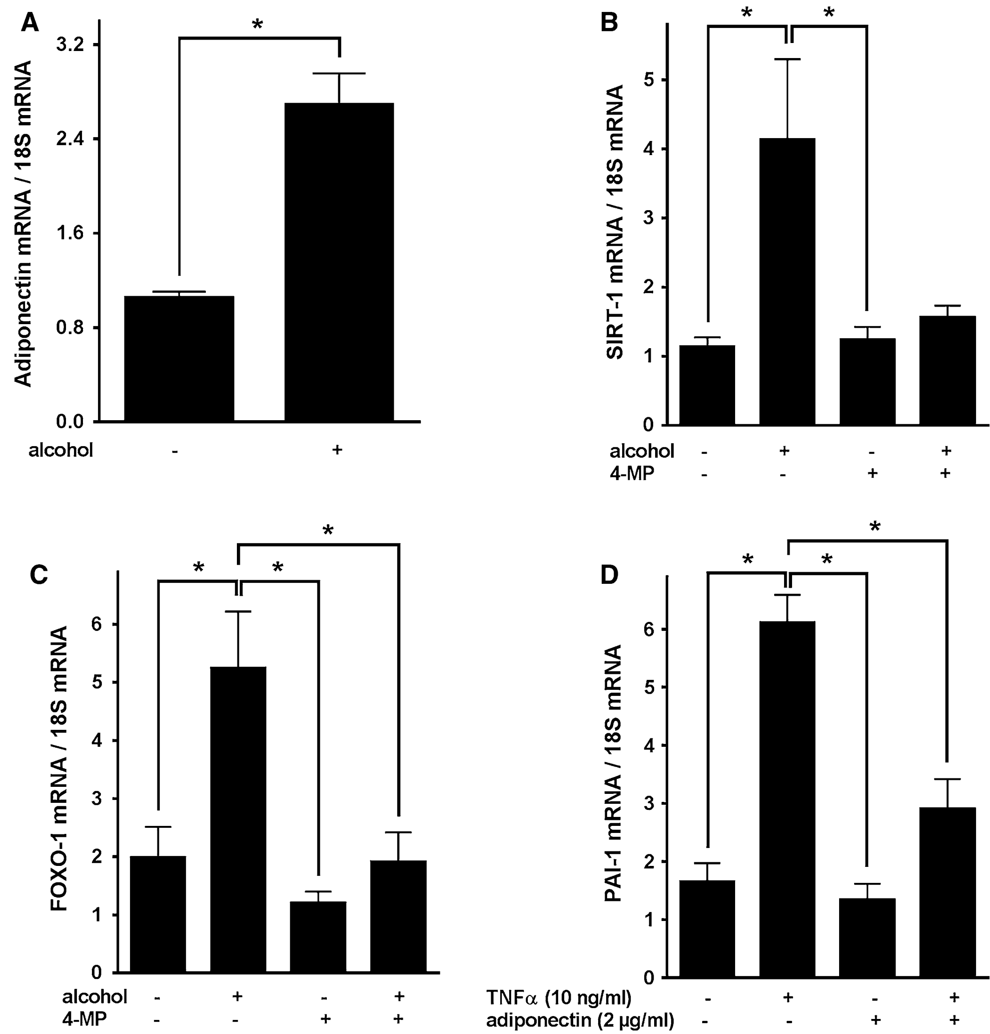


Fig. 4 Effect of moderate alcohol exposure on adiponectin, SIRT1 and FOXO1 mRNA expression in 3T3-L1 cells and effect of TNFα and adiponectin on AML 12 cells. **a** Adiponectin, **b** SIRT1, and **c** FOXO1 mRNA expression in 3T3-L1 cells treated with 0.2 vol% ethanol and 4-methylpyrazole (4-MP; $n = 3$). **d** PAI-1 mRNA expression in AML 12 cells treated with TNFα and adiponectin ($n = 4$). mRNA expression was normalized to 18S mRNA. Data are shown as mean ± SEM. *Data are significantly different, $p < 0.05$

of lipid metabolism, with the exception of SRBEP-1c, were markedly higher in livers of *ob*/*ob* mice than in lean controls. However, only FAS and to a lesser extend SREBP-1c expression was found to be significantly affected by the moderate intake of alcohol. There are only limited data available on the effects of moderate alcohol intake on hepatic lipogenesis yet. However, our data suggest that FAS expression may be markedly suppressed through an adiponectin-dependent down-regulation of SRBEP-1c in livers of *ob*/*ob* mice ingesting moderate amounts of alcohol. This in turn may also add to the less pronounced lipid accumulation found in these mice when compared to *ob*/*ob* mice ingesting only plain water. Indeed, it has been shown that adiponcetin, probably through SREBP-1c-dependent signalling pathways, may alter FAS expression in the liver [45]. Differences between the studies of others and our own in regards to expression of markers of carbohydrate but also lipid metabolism may have resulted from the different Akt subtypes determined. Indeed, in the present study, an antibody detecting phosphorylation status of Akt 1–3 was used to determine phosphorylation status of Akt in liver, whereas for instance, others reported that Akt 2 is the subtype of Akts primarily involved in GLUT4-mediated glucose uptake [44]. Furthermore, several studies suggest that Akt also may affect glucose metabolism through rather direct effects, e.g. through an induction of GLUT4 translocation to cell membranes [46, 47]. Therefore, as in the present study, only mRNA expression of markers of carbohydrate metabolism in the liver was determined, it cannot be ruled out that alterations found in Akt phosphorylation affected carbohydrate metabolism in the liver through other mechanisms. Furthermore, PAI-1 levels but also markers of apoptosis were only found to be increased in livers of *ob*/*ob* mice fed with plain water. Taken together, these data suggest that the protective effects of moderate alcohol intake under the present conditions did not result from alterations at the level of TNFα- and insulin receptor signalling. Rather, our data suggest that moderate alcohol intake “normalizes” Akt-dependent pathways downstream of TNFα and IRS-1/IRS-2. These findings by no means preclude

that moderate alcohol consumption may also affect insulin release in the pancreas or insulin receptor levels in other tissue like the muscle but imply that additional pathways may contribute to the beneficial effects of moderate alcohol intake on the development of NAFLD.

Previous studies have indicated that the protective effects of adiponectin on the development of NAFLD are at least in part mediated through an induction of adenosine monophosphate-activated protein kinase (AMPK) in the liver subsequently leading to a "normalization" of Akt- and PAI-1-dependent pathways [22, 48, 49], while others could not confirm these findings [50]. Results of human studies suggest that moderate alcohol intake is associated with increased circulating adiponectin levels (for overview see [31]). Several animal studies also suggest that alcohol intake might modulate adiponectin release from adipose tissue and subsequently AMPK signalling in the liver [13, 14, 51, 52]; however, alcohol levels given in these studies were rather high (>27.5 % of calories derived from alcohol). In the present study, we found that moderate alcohol intake was associated with a marked induction of SIRT1 and adiponectin in visceral adipose tissue in *ob/ob* mice. In line with these findings, adiponectin levels were markedly higher in differentiated adipocytes exposed to moderate alcohol concentrations (e.g. 0.2 vol %) than in naïve control cells. This was associated with an induction of FOXO1 and SIRT1, shown to be regulators of adiponectin expression in visceral adipose tissue [24]. These effects of alcohol were almost completely blocked when cells were treated with 4-methylpyrazol, an inhibitor of ADH. Our results suggest that an altered $NAD^+/NADH + H^+$ ratio, associated with the metabolism of ethanol, might be the trigger of the induction of SIRT1/FOXO1 and subsequently adiponectin; however, this will have to be addressed in future studies. Furthermore, in AML-12 cells, used as a model of hepatocytes, the challenge with TNFα resulted in a significant induction of PAI-1, an effect almost completely attenuated when cells were concomitantly exposed to adiponectin. Taken together, these data suggest that moderate alcohol intake leads through SIRT1-/FOXO1-dependent signalling pathways to an induction of adiponectin in visceral adipose tissue, which in turn modulates AMPK/Akt signalling in the liver thereby attenuating the TNFα-dependent induction of PAI-1. Earlier studies employing models of chronic high doses of ethanol suggest that adiponectin may also modulate Kupffer cell signalling and herein especially toll-like receptor 4/myeloid differentiation primary response gene 88-dependent signalling cascades [9]. Differences between the findings of the present study and those of others might have resulted from differences in general dietary composition (e.g. standard chow vs. Lieber DeCarli diet [53]), in duration of feeding and in the employed alcohol concentrations.

Conclusion

Taken together, our data suggest that similar to the findings of recent epidemiological studies, moderate alcohol intake in mice diminishes the development of NAFLD. Our data further indicate that these "protective" effects depend upon an induction of the SIRT1-/adiponectin-signalling pathway in visceral adipose tissue subsequently leading to a modulation of the adiponectin/AKT/PAI-1 signalling cascade in the liver. However, when interpreting our data, it has to be kept in mind that *ob/ob* mice develop hyperinsulinemia, insulin resistance but also NAFLD due to their inherited leptin deficiency [54]. This lack of leptin has been shown to be associated with markedly reduced adiponectin levels [55], which in turn may result in alternations not found in other dietary or genetic models of NAFLD or even patients suffering from NAFLD. Therefore, further studies are necessary to determine whether similar molecular mechanisms are also involved in the lower severity of NAFLD found in moderate alcohol consumers versus alcohol abstainers. Furthermore, our data by no means should encourage abstainers to drink alcohol for health reasons and the choice to consume alcohol should always be based on individual considerations (e.g. taking into account life circumstances and general health).

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Conflict of interest All authors declare no conflicts of interest.

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