

# Leptin Deficiency Contributes to the Pathogenesis of Alcoholic Fatty Liver Disease in Mice

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**White adipose tissue (WAT) secretes adipokines, which critically regulate lipid metabolism. The present study investigated the effects of alcohol on adipokines and the mechanistic link between adipokine dysregulation and alcoholic fatty liver disease. Mice were fed alcohol for 2, 4, or 8 weeks to document changes in adipokines over time. Alcohol exposure reduced WAT mass and body weight in association with hepatic lipid accumulation. The plasma adiponectin concentration was increased at 2 weeks, but declined to normal at 4 and 8 weeks. Alcohol exposure suppressed leptin gene expression in WAT and reduced the plasma leptin concentration at all times measured. There is a highly positive correlation between plasma leptin concentration and WAT mass or body weight. To determine whether leptin deficiency mediates alcohol-induced hepatic lipid dyshomeostasis, mice were fed alcohol for 8 weeks with or without leptin administration for the last 2 weeks. Leptin administration normalized the plasma leptin concentration and reversed alcoholic fatty liver. Alcohol-perturbed genes involved in fatty acid  $\beta$ -oxidation, very low-density lipoprotein secretion, and transcriptional regulation were attenuated by leptin. Leptin also normalized alcohol-reduced phosphorylation levels of signal transducer Stat3 and adenosine monophosphate-activated protein kinase. These data demonstrated for the first time that leptin deficiency in association with WAT mass reduction contributes to the pathogenesis of alco-**

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Alcoholic liver disease is traditionally described as three progressive pathologic conditions: steatosis (fatty liver), hepatitis, and cirrhosis.<sup>1</sup> Alcoholic fatty liver is characterized by lipid droplet accumulation in the cytoplasm of hepatocytes, and is one of the earliest pathologic alterations in the liver. Accumulation of lipids in the hepatocytes makes the liver susceptible to inflammatory mediators or other toxic agents, leading to further progression to hepatitis and eventually to fibrosis. Alcohol consumption may affect multiple pathways of hepatic lipid metabolism including *de novo* lipogenesis, fatty acid oxidation, lipid uptake, and lipid export in the form of very low-density lipoproteins.<sup>2,3</sup> However, recent studies have suggested that extrahepatic factors such as adiponectin critically modulate hepatic lipid metabolism.<sup>4,5</sup>

White adipose tissue (WAT) is a major organ for body fat storage, and also functions as an endocrine organ.<sup>6</sup> The hormones secreted by WAT are adipokines, and two of the most important adipokines related to energy homeostasis are adiponectin and leptin. Both adiponectin and leptin critically modulate hepatic lipid homeostasis toward reduction of lipid content in the liver. Adiponectin signaling in the liver leads to activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway via adiponectin receptor.<sup>4–7</sup> AMPK activation negatively regulates the hepatic lipid level by stimulating fatty acid oxidation and suppressing fatty acid influx and *de novo* lipogenesis. Leptin critically regulates whole-body energy homeostasis by inhibiting energy intake and stimulating energy expenditure. Leptin signaling in the liver

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via leptin receptor b (LepRb) activates AMPK and signal transducer Stat3 pathways.<sup>6,7</sup> Mice lacking functional leptin develop not only obesity but also fatty liver. Therefore, adipose tissues via adipokine secretion significantly affect lipid homeostasis in the liver.

Alcohol exposure has been shown to affect adipose mass and adipokine secretion in both humans and animals. Patients with alcoholism have lower body mass index (BMI) and fat mass (FM) but higher liver fat levels.<sup>8–11</sup> Studies in humans have also found that the serum leptin concentration was reduced by either chronic alcohol consumption or acute alcohol abuse.<sup>11–14</sup> Chronic alcohol exposure in rodents reduced adipose tissue weight,<sup>15–17</sup> and serum adiponectin<sup>18–23</sup> and leptin<sup>24,25</sup> concentrations in association with the development of fatty liver. Administration of exogenous adiponectin or stimulation of endogenous adiponectin production attenuated alcoholic fatty liver in mice.<sup>18,19,23,26</sup> These studies indicate that adipokines critically regulate lipid homeostasis at the adipose tissue–liver axis. The present study reports that chronic alcohol exposure caused leptin deficiency, and leptin administration for 2 weeks reversed alcohol-induced fatty liver in mice previously exposed to alcohol for 6 weeks.

## Materials and Methods

### Animals and Alcohol Feeding Experiments

Male C57BL/6 mice were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). All of the mice were treated according to the experimental procedures approved by the Institutional Animal Care and Use Committee. At age 4 months, mice were pair-fed a modified Lieber-DeCarli alcohol or isocaloric maltose dextrin control liquid diet for up to 8 weeks in a stepwise procedure. Caloric content of the alcohol diet was 16% protein, 12% carbohydrate, 34% fat, and 38% ethanol (Sigma-Aldrich Corp., St. Louis, MO). The ethanol content (w/v) in the diet was 4.8% (34% of total calories) for the first 2 weeks, and was increased by 0.2% every 2 weeks, reaching 5.4% (38% of total calories) for the last 2 weeks. On the basis of our observations, this stepwise feeding protocol improves the performance of the mouse model of alcohol feeding, as indicated by reduced mortality. Two sets of animal experiments were performed. Experiment 1 was performed to determine adipokine production in association with development of alcoholic liver disease, and mice were subjected to alcohol exposure for 2, 4, or 8 weeks. Experiment 2 was performed to determine the role of leptin in alcoholic liver disease because leptin deficiency was detected in experiment 1. Mice were subjected to alcohol exposure for 8 weeks, with leptin (purity >95%; ProSpec, East Brunswick, NJ) administration at 0.5 mg/d per kilogram body weight or saline solution as vehicle, via subcutaneous osmotic minipump (ALZET Osmotic Pump; DURECT Corp., Cupertino, CA) for the last 2 weeks.

### Blood Parameters

Blood glucose concentration was measured using a OneTouch Ultra2 Blood Glucose Meter (LifeScan, Inc., Milpitas, CA), and ketone bodies using PTS PANELS Ketone Test Strips (Polymer Technology Systems, Inc., Indianapolis, IN). Plasma alanine aminotransferase activity and triglyceride and cholesterol concentrations were determined using Infinity Reagents (Thermo Fisher Scientific, Inc., Middletown, VA). Plasma free fatty acids (FFAs) were quantified using an FFA Quantification Kit (BioVision, Inc., Milpitas, CA). Plasma leptin and adiponectin concentrations were measured using commercial mouse enzyme-linked immunosorbent assay kits (Millipore Corp., Billerica, MA).

### Liver Histopathologic and Lipid Concentrations

Liver tissues were fixed in 10% formalin and embedded in paraffin. Tissues were cut into 5- $\mu$ m sections and processed for H&E staining and Sirius Red staining. Quantitative assay of lipids was conducted by measuring triglyceride, cholesterol, and FFA concentrations in the liver. Hepatic lipids are extracted by homogenizing liver tissue in chloroform using 1% Triton X-100. The organic extracts were air dried, vacuumed, and dissolved in 1% Triton X-100. Triglyceride, cholesterol, and FFA concentrations in extracts were determined using commercial kits (see *Blood Parameters*).

### Quantitative RT-PCR

The total RNA was isolated from liver or WAT using the TRIzol method (Life Technologies Corp., Grand Island, NY), and reverse transcription was conducted using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Inc., Foster City, CA). The forward and reverse primers (Table 1) were designed using Primer Express Software v3.0.1 (Applied Biosystems), and quantitative RT-PCR (RT-qPCR) analysis with SYBR Green PCR master mix (Qiagen, Inc., Valencia, CA) was performed using a PRISM 7500 Sequence Detection System (Applied Biosystems). Data were normalized to  $\beta$ -actin expression, and are given as fold changes, setting the values of pair-fed mice at 1.

### Immunoblot Analysis

Whole protein lysates of liver were extracted using 10% Nonidet P-40 lysis buffer supplemented with 1% protease inhibitor cocktail and 1% phenylmethylsulfonyl fluoride. Aliquots containing 60  $\mu$ g proteins were loaded onto 10% SDS-PAGE, transblotted onto polyvinylidene difluoride membrane, blocked using 5% nonfat dry milk in Tris-buffered saline solution with 0.1% Tween-20, and incubated with rabbit anti-pStat3, Stat3, pAMPK, AMPK (Cell Signaling Technology, Inc., Danvers, MA), PPAR- $\alpha$  (peroxisome proliferator-activated receptor- $\alpha$ ), HNF-1 $\alpha$  (hepatocyte nuclear factor-1 $\alpha$ ), C/EBP- $\alpha$  (CCAAT/enhancer binding protein), or  $\beta$ -actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then

**Table 1.** Primer Sequences for Real-Time RT-PCR

Gene	GeneBank accession no.	Sequences (Forward/Reverse)
ACADL	NM_007381	5'-TCTTTTCTCGGAGCATGACA-3' 5'-GACCTCTCTACTCACTTCTCCAG-3'
ACOX1	NM_015729	5'-TCCAGACTTCCAACATGAGGA-3' 5'-CTGGGCGTAGGTGCCAATTA-3'
ApoB	NM_009693	5'-TTGGCAAATGCATAGCATCC-3' 5'-TCAAAATGGGACTCTCCTTTAGC-3'
C/EBP- $\alpha$	NM_007678	5'-CAAGAACAGCAACGAGTACCG-3' 5'-GTCACTGGTCAACTCCAGCAC-3'
FABP1	NM_017399	5'-ATGAACTTCTCCGGCAAGTACC-3' 5'-CTGACACCCCTTGATGCC-3'
FATP2	NM_011978	5'-TCCTCCAAGATGTGCGGTA-3' 5'-TAGGTGAGCGTCTCGTCTCG-3'
FATP5	NM_009512	5'-CTACGCTGGCTGCATATAGATG-3' 5'-CCACAAAGTCTCTGGAGGAT-3'
HNF-1 $\alpha$	NM_009327	5'-GACCTGACCGAGTTCGCTAAT-3' 5'-CCGGCTCTTTCAGAATGGGT-3'
HNF-4 $\alpha$	NM_008261	5'-GCCTTCTGCGAAGTCTTCTG-3' 5'-GGGACGATGTAGTCATTGCCT-3'
CPT1a	NM_013495	5'-CTCCGCTGAGCCATGAAG-3' 5'-CACCAGTGATGATGCCATTCT-3'
Leptin	NM_008493	5'-GAGACCCCTGTGTCCGGTTC-3' 5'-CTGCGTGTGTGAAATGTCATTG-3'
LepR-b	NM_146146	5'-TGGTCCCAGCAGCTATGGT-3' 5'-ACCCAGAGAAGTTAGCACTGT-3'
MTPP	NM_008642	5'-TCAAGAGAGGCTTGGCTAGCTT-3' 5'-GCCTGGTAGGCTCACTTTACAATCC-3'
PPAR- $\alpha$	NM_011144	5'-AGAGCCCCATCTGTCTCTC-3' 5'-ACTGGTAGTCTGCAAAACAAA-3'
PPAR- $\gamma$	NM_011146	5'-TCGCTGATGCACTGCCTATG-3' 5'-GAGAGGTCCACAGAGCTGATT-3'
$\beta$ -Actin	NM_007393	5'-GGCTGTATCCCTCCATCG-3' 5'-CCAGTTGGTAACAATGCCATGT-3'

incubated using horseradish peroxidase-conjugated anti-rabbit IgG. The bound complexes were detected via chemiluminescence. The immunoblot bands were quantified via densitometry analysis, and the ratio to  $\beta$ -actin was calculated and is given as fold changes, setting the values of pair-fed mice at 1.

### Statistical Analysis

All data are given as mean  $\pm$  SD. The results were analyzed using the one-sample *t*-test for two groups, and one-way analysis of variance with Turkey post hoc comparison for more than two groups. Correlation coefficients, analyzed using Prime 3, were used to determine linear association between plasma leptin concentration and WAT mass or body weight. In all statistical tests, *P* < 0.05 was considered significant.

## Results

### Chronic Alcohol Exposure Induced Leptin Deficiency in Association With Fatty Liver Disease

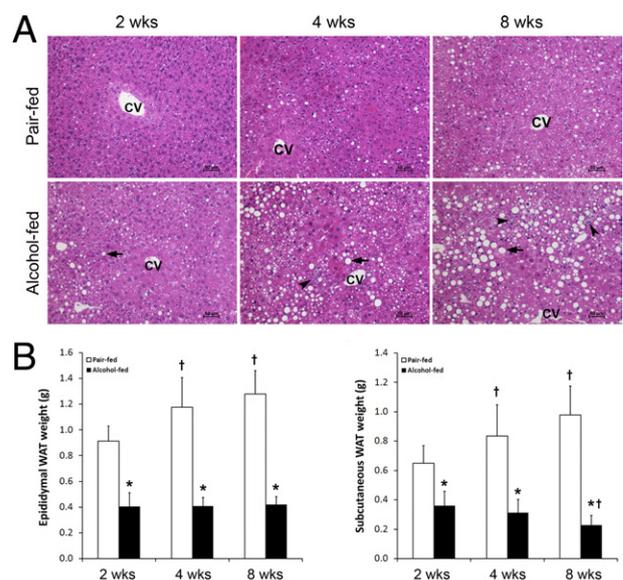
Alcohol exposure for 2, 4, and 8 weeks caused liver damage that was time-dependent (Figure 1A). Lipid droplet accumulation in the liver is the major pathologic change at 2 weeks. At 4 and 8 weeks, the number and

size of the lipid droplets in the hepatocytes gradually increased, and inflammatory cell infiltration was frequently found. In contrast, alcohol exposure significantly reduced epididymal and subcutaneous WAT mass at all three times measured (Figure 1B). In comparison with a gradual increase in WAT weight in pair-fed mice, alcohol-fed mice maintained a low WAT weight. The body weight of alcohol-fed mice was lower than that of pair-fed mice at all three times measured (data not shown).

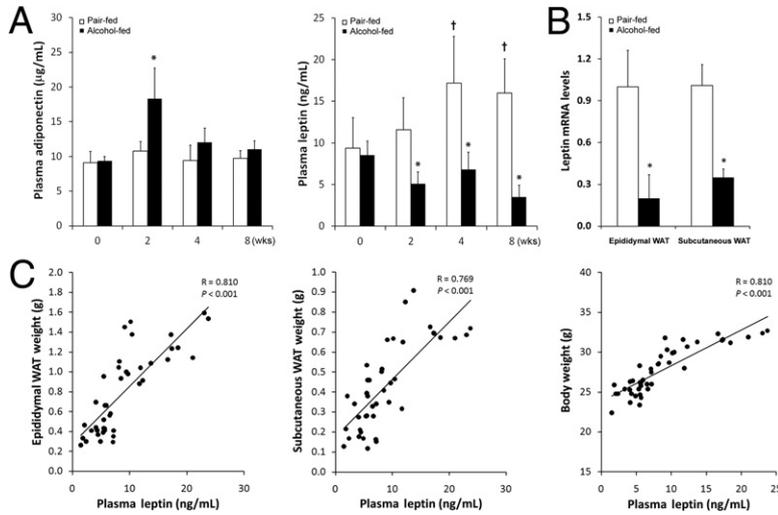
To determine whether alcohol-induced lipodystrophy leads to alterations in adipokine secretion, plasma adiponectin and leptin levels were measured. Plasma adiponectin levels in pair-fed mice did not change at any times measured (Figure 2A). Alcohol exposure for 2 weeks increased the plasma adiponectin concentration, but it declined to normal at 4 and 8 weeks. The plasma leptin concentration in pair-fed mice showed a significant increase at 4 and 8 weeks in comparison with that at 0 and 2 weeks (Figure 2A). Alcohol exposure reduced the plasma leptin concentration by 40%, 20%, and 59% at 2, 4, and 8 weeks, respectively, compared with that at 0 week. RT-qPCR analysis demonstrated that alcohol exposure for 8 weeks dramatically down-regulated leptin gene expression in both epididymal and subcutaneous WATs (Figure 2B). Correlation analysis showed highly positive correlation coefficients between plasma leptin concentration and epididymal and subcutaneous WAT mass and body weight (Figure 2C).

### Effects of Leptin Administration on Body Weight, Liver Weight, Adipose Tissue Weight, and Blood Metabolites

To determine the role of leptin deficiency in the pathogenesis of alcoholic fatty liver, leptin was administered to



**Figure 1.** Alcohol-induced adipose mass reduction in association with liver damage in mice fed with alcohol for 2, 4, or 8 weeks. **A:** Liver histopathologic analysis (H&E) revealed lipid droplets (arrows) and inflammatory cell infiltration (arrowheads). CV, central vein. **B:** WAT mass. Results are given as mean  $\pm$  SD (*n* = 6 to 10). \**P* < 0.05 versus pair-fed mice (*t*-test); †*P* < 0.05 versus 2 weeks (analysis of variance).



**Figure 2.** Effects of alcohol on plasma adiponectin and leptin levels in mice fed with alcohol for 2, 4, or 8 weeks. **A:** Plasma adiponectin and leptin concentrations. **B:** WAT leptin mRNA levels. **C:** Correlation of plasma leptin concentration with WAT mass or body weight. Correlation was analyzed using Prime 3. Results are given as mean  $\pm$  SD ( $n = 6$  to 10). \* $P < 0.05$  versus pair-fed mice (*t*-test); † $P < 0.05$  versus 0 week (analysis of variance).

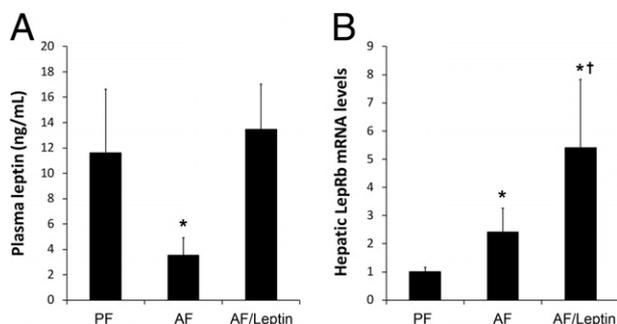
alcohol-fed mice for the last 2 weeks of the study. Leptin administration normalized the alcohol-decreased plasma leptin concentration and stimulated hepatic LepRb gene expression (Figure 3A), although alcohol alone also up-regulated hepatic LepRb (Figure 3B). Effects of leptin administration on routine parameters are given in Table 2. Alcohol exposure with or without leptin administration reduced body weight, whereas liver weight was reduced only by alcohol plus leptin. As a consequence, the liver-body weight ratio was increased by alcohol exposure, which was partially reversed by leptin. Epididymal WAT weight and its ratio to body weight were reduced by alcohol, and the reduction was promoted by leptin. Alcohol exposure significantly elevated the plasma alanine aminotransferase concentration, which was normalized by leptin. Alcohol exposure decreased blood glucose and cholesterol concentrations, and increased the plasma ketone body concentration; these alterations were not affected by leptin. However, leptin administration normalized alcohol-reduced plasma FFA concentrations.

### Leptin Administration Reverses Alcohol Exposure-Induced Lipid Accumulation in the Liver

Alcohol exposure for 8 weeks caused lipid droplet accumulation in the liver, which was attenuated by leptin (Figure 4A). Quantitative assay of hepatic lipids demonstrated that alcohol exposure significantly increased the concentrations of triglycerides, cholesterol, and FFAs in the liver (Figure 4B). Leptin administration normalized the concentrations of hepatic triglycerides and FFAs, but did not affect the alcohol-elevated cholesterol concentration. To exclude the possibility that leptin may affect liver fibrogenesis, the hepatic collagen level was assessed using Sirius Red staining. Neither alcohol feeding alone nor alcohol feeding plus leptin administration caused significant collagen accumulation in the liver (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

### Leptin Administration Improves Alcohol Exposure-Dysregulated Hepatic Genes Related to Lipid Metabolism

The relative mRNA levels of hepatic genes related to lipid metabolism are given in Table 3. Among three fatty acid transport genes, *FABP1* was down-regulated by alcohol regardless of leptin administration, whereas *FATP2* was up-regulated by leptin. Alcohol exposure down-regulated all three genes related to fatty acid  $\beta$ -oxidation, *CPT1a*, *ACADL*, and *ACOX1*, which was normalized by leptin. Leptin administration also normalized alcohol-down-regulated very low-density lipoprotein assembly genes *MTTP* and *ApoB*. Among the five transcription factors related to lipid metabolism regulation, alcohol exposure down-regulated *C/EBP- $\alpha$* , *HNF-1 $\alpha$* , *PPAR- $\alpha$* , and *PPAR- $\gamma$* , and leptin normalized the expression levels of the first three but not *PPAR- $\gamma$* . The *HNF-4 $\alpha$*  gene was not affected by either alcohol or leptin. Immunoblot analysis demonstrated that leptin administration reversed alcohol-reduced protein levels of *PPAR- $\alpha$*  and *HNF-1 $\alpha$*  (Figure 5A). However, the



**Figure 3.** Plasma leptin concentration (A) and hepatic *LepRb* mRNA level (B) in mice fed with alcohol for 8 weeks with or without leptin administration for the last 2 weeks. Results are given as mean  $\pm$  SD ( $n = 6$  to 10 in A;  $n = 4$  in B). \* $P < 0.05$  versus pair-fed mice (PF) (analysis of variance); † $P < 0.05$  versus alcohol-fed mice (AF) (analysis of variance).

**Table 2.** Effects of Chronic Alcohol Exposure and Leptin Administration on Body Weight, Liver Weight, Epididymal WAT Weight, and Blood Metabolites

Measurements	PF	AF	AF/Leptin
Food intake (g/d per mouse)	10.36 ± 0.83	11.02 ± 0.76	10.67 ± 1.47
Alcohol intake (g/d per mouse)	NA	0.59 ± 0.04	0.56 ± 0.07
Body weight (g)	29.77 ± 1.76	24.05 ± 1.40*	22.60 ± 0.52*
Liver weight (g)	1.25 ± 0.08	1.30 ± 0.07	1.09 ± 0.05*
Epididymal WAT weight (g)	1.28 ± 0.21	0.42 ± 0.04*	0.18 ± 0.04*†
Liver–body weight ratio	4.17 ± 0.36	5.41 ± 0.14*	4.83 ± 0.14*
Epididymal WAT–body weight ratio	4.27 ± 0.59	1.74 ± 0.12*	0.78 ± 0.18*†
Plasma ALT (U/mL)	24.36 ± 7.19	65.82 ± 9.60*	31.50 ± 11.18†
Blood glucose (mg/dL)	277.0 ± 45.4	221.0 ± 19.1*	204.7 ± 26.1*
Blood ketone bodies (mg/dL)	4.90 ± 1.59	7.08 ± 1.65*	8.34 ± 1.25*
Plasma triglycerides (mg/dL)	75.45 ± 14.12	76.98 ± 21.32	57.83 ± 20.40
Plasma cholesterol (mg/dL)	91.29 ± 17.88	59.81 ± 11.24*	47.85 ± 9.66*
Plasma free fatty acids (mmol/L)	0.80 ± 0.15	0.43 ± 0.21*	0.72 ± 0.21†

Mice were pair-fed alcohol or isocaloric maltose dextran for 8 weeks in the presence or absence of external leptin for the last 2 weeks.

Data are given as mean ± SD (*n* = 6–10).

\**P* < 0.05 versus PF (ANOVA).

†*P* < 0.05 versus AF (ANOVA).

AF, alcohol-fed; ALT, alanine aminotransferase; ANOVA, analysis of variance; PF, pair-fed; WAT, white adipose tissue.

protein level of C/EBP- $\alpha$  was not affected by alcohol regardless of leptin administration. Immunoblot analysis also showed that the phosphorylation levels of Stat3 and AMPK, the major molecules mediating leptin signaling, were reduced after alcohol exposure, but were normalized by leptin administration (Figure 5B).

### Discussion

Alcohol consumption has been shown to modulate plasma adipokine levels, in particular adiponectin. Alcohol exposure in mice decreased the serum adiponectin concentration in a time-dependent manner, whereas administration with mouse recombinant adiponectin attenuated alcohol-induced fatty liver and inflammation.<sup>18</sup> The decrease in plasma adiponectin concentration was also found in other animal models including rats and micropigs.<sup>19–23</sup> Elevation of the plasma adiponectin concentration was associated with protective effects of dietary supplementation with saturated fat, rosiglitazone, or resveratrol against alcoholic fatty liver.<sup>19,23,26</sup> However,

there are controversial reports on the effect of alcohol on adiponectin. A recent study demonstrated that high dosages of alcohol alone or plus overnutrition increased the plasma adiponectin concentration.<sup>27</sup> Clinical studies have also demonstrated that the serum adiponectin concentration correlated well with daily alcohol consumption and liver injury, but declined during alcohol abstinence.<sup>28</sup> The present study demonstrated that alcohol exposure did not reduce the plasma adiponectin concentration, although a dramatic reduction in adipose mass was found. A previous report showed that adipose tissue reduction in iNOS null mice was associated with hypolep-

**Table 3.** Effects of Leptin Administration on Hepatic Genes Related to Lipid Metabolism

Measurements	PF	AF	AF/Leptin
Fatty acid transport			
<i>FABP1</i>	1.006 ± 0.13	0.41 ± 0.13*	0.33 ± 0.09*
<i>FATP2</i>	1.005 ± 0.12	0.98 ± 0.29	1.50 ± 0.29*†
<i>FATP5</i>	1.003 ± 0.10	0.98 ± 0.10	1.23 ± 0.17
Fatty acid $\beta$ -oxidation			
<i>CPT1a</i>	1.001 ± 0.05	0.53 ± 0.02*	1.06 ± 0.27†
<i>ACADL</i>	1.009 ± 0.16	0.70 ± 0.15*	1.007 ± 0.11†
<i>ACOX1</i>	1.001 ± 0.05	0.58 ± 0.14*	0.86 ± 0.26†
VLDL assembly			
<i>MTTP</i>	1.04 ± 0.35	0.64 ± 0.09*	0.71 ± 0.11
<i>ApoB</i>	1.003 ± 0.10	0.76 ± 0.02*	1.17 ± 0.20†
Transcription factors			
<i>C/EBP-<math>\alpha</math></i>	1.004 ± 0.11	0.67 ± 0.02*	0.93 ± 0.08†
<i>HNF-1<math>\alpha</math></i>	1.008 ± 0.15	0.56 ± 0.12*	0.84 ± 0.12†
<i>HNF-4<math>\alpha</math></i>	1.01 ± 0.21	1.07 ± 0.15	1.27 ± 0.27
<i>PPAR-<math>\alpha</math></i>	1.004 ± 0.11	0.62 ± 0.09*	0.93 ± 0.05†
<i>PPAR-<math>\gamma</math></i>	1.007 ± 0.14	0.27 ± 0.16*	0.16 ± 0.06*

Mice were pair-fed alcohol on isocaloric maltose dextran for 8 weeks in the presence or absence of external leptin for the last 2 weeks.

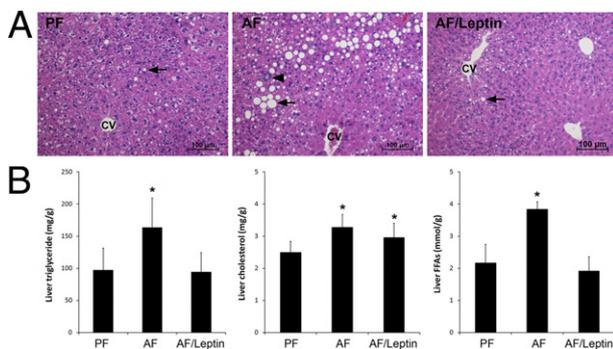
Gene expression was analyzed using quantitative RT-PCR.

Data are given as mean ± SD (*n* = 4).

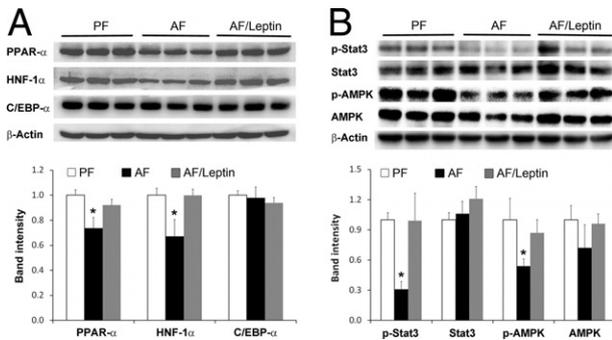
\**P* < 0.05 versus PF (ANOVA).

†*P* < 0.05 versus AF (ANOVA).

AF, alcohol-fed; ANOVA, analysis of variance; PF, pair-fed; VLDL, very low-density lipoprotein.



**Figure 4.** Liver histopathologic and lipid concentrations in mice fed with alcohol (AF) for 8 weeks with or without leptin administration for the last 2 weeks. **A:** Histopathologic analysis (H&E) revealed lipid droplets (arrows) and inflammatory cell infiltration (arrowhead). CV, central vein. **B:** Liver lipid concentrations. Results are given as mean ± SD (*n* = 6 to 10). \**P* < 0.05 versus pair-fed mice (PF) (analysis of variance).



**Figure 5.** Protein levels of PPAR- $\alpha$ , HNF-1 $\alpha$ , and C/EBP- $\alpha$  in the liver of mice fed with alcohol (AF) for 8 weeks with or without leptin administration for the last 2 weeks. **A:** Immunoblot analysis of PPAR- $\alpha$ , HNF-1 $\alpha$ , and C/EBP- $\alpha$ . **B:** Immunoblot analysis of total and pStat3 and p-AMPK. Band intensity was quantified using densitometry analysis, and the ratio to  $\beta$ -actin was calculated by setting the value of pair-fed mice (PF) at 1. Results are given as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  versus PF mice (analysis of variance).

tinemia, although the plasma adiponectin concentration was normal.<sup>29</sup> These data suggest that the plasma adiponectin concentration does not correlate with WAT mass.

Alcohol abuse affected the serum leptin concentration in both human and animal studies. Serum leptin concentrations in patients with alcoholism correlate well with BMI and FM.<sup>11–14,24,25</sup> Patients with alcoholism with lower BMI and FM had decreased serum leptin concentrations,<sup>11,12</sup> whereas those with greater BMI and FM had increased serum leptin concentrations.<sup>30</sup> Of importance, lower BMI and FM in patients with alcoholism were associated with lipid accumulation in the liver.<sup>11,12</sup> An animal study demonstrated that acute alcohol exposure in rats reduced the plasma leptin concentration in a dose-dependent manner.<sup>24</sup> Chronic alcohol exposure in rodents reduced the plasma leptin concentration in association with reduction of WAT mass and accumulation of lipids in the liver or bone marrow.<sup>16,17,25</sup> The present study demonstrated that reduction of the plasma leptin concentration was association with alcoholic fatty liver, and highly positive correlations occur between the plasma leptin concentration and WAT mass and body weight. These findings suggest that hypoleptinemia is associated with WAT reduction and development of alcoholic fatty liver.

Leptin critically regulates hepatic lipid homeostasis, and animals with either leptin mutation, as in *ob/ob* mice, or leptin receptor mutation, as in *db/db* mice and Zucker (*fa/fa*) rats, develop fatty liver in association with early onset of obesity and insulin resistance.<sup>31</sup> Alcohol exposure caused more lipid accumulation and liver injury in Zucker (*fa/fa*) rats than in wild-type rats.<sup>32</sup> Hypoleptinemia in patients with lipodystrophy contributes to fatty liver and insulin resistance.<sup>33–38</sup> Leptin therapy for up to 18 months in these patients significantly reduced hepatic volume and triglyceride contents, and improved pathologic alterations in liver biopsy specimens including steatosis, ballooning injury, and parenchymal inflammation.<sup>33–35</sup> Recent studies also have demonstrated that leptin therapy normalized hepatic triglyceride concentration and reversed deadly consequences in both types I and II diabetes.<sup>36–38</sup> These findings suggest that leptin

has potent antisteatotic and antidiabetic activities. The present study demonstrated that leptin administration ameliorated alcoholic fatty liver and corrected multiple lipid metabolic pathways. While up-regulating genes of fatty acid  $\beta$ -oxidation is likely a major mechanism underlying leptin action, leptin also attenuated alcohol-induced down-regulation of MTP and ApoB. Furthermore, leptin normalized alcohol-induced down-regulation of transcription factors such as PPAR- $\alpha$  and HNF-1 $\alpha$ . Knockout of either PPAR- $\alpha$  or HNF-1 $\alpha$  causes fatty liver.<sup>39,40</sup> The present study and other previously published reports have demonstrated that reactivation of PPAR- $\alpha$  via dietary supplementation with zinc or PPAR- $\alpha$  agonist attenuates alcoholic fatty liver.<sup>16,41</sup> The present study also demonstrated that leptin administration abrogated the inhibitory effects of alcohol on AMPK and Stat3 signal pathways. Inhibition of AMPK has been reported to mediate alcohol action in the liver, and reactivation of AMPK was associated with prevention of alcoholic fatty liver by dietary supplementation with rosiglitazone or resveratrol.<sup>23,26</sup> Recent studies also showed that activation of Stat3 mediates the protective effects of IL-6 and IL-22 on alcoholic liver injury.<sup>42,43</sup> All of these studies suggest that AMPK and Stat3 are important molecular targets for therapeutic intervention in alcoholic fatty liver disease.

Leptin has been implicated in multiple cell functions including innate and adaptive immunity, liver regeneration, and fibrogenesis. Leptin treatment attenuates alcohol-induced apoptosis and production of proinflammatory cytokines and TGF- $\beta$  in HepG2 cells.<sup>44,45</sup> Leptin administration also attenuated TNF- $\alpha$  production and hepatocyte apoptosis in a mouse model of CCl<sub>4</sub> hepatotoxicity.<sup>46</sup> Inhibition of oxidative stress was associated with the protective effects of leptin in both *in vivo* and *in vitro* studies.<sup>47,48</sup> Leptin-deficient rats have lower levels of metallothionein and zinc in the liver, which accounts for the susceptibility to alcohol-induced steatohepatitis.<sup>32</sup> Indeed, our previous studies demonstrated that metallothionein and zinc suppress alcohol-induced oxidative stress, thereby preventing alcoholic liver injury.<sup>16,48,49</sup> Leptin is also involved in liver fibrogenesis. Leptin-deficient *ob/ob* mice failed to develop liver fibrosis in either a methionine/choline deficiency or a CCl<sub>4</sub> model.<sup>50</sup> However, clinical studies with leptin therapy for 4 to 18 months improved the NASH (nonalcoholic steatohepatitis) score without affecting the fibrosis score in liver biopsy.<sup>35</sup> It should be emphasized that leptin administration in the present study was performed on the basis of the pathophysiologic condition of hypoleptinemia. Normalizing the plasma leptin concentration in alcohol-fed mice reversed alcoholic fatty liver without affecting liver fibrogenesis.

In conclusion, leptin deficiency was associated with reduction of WAT mass in a mouse model of alcoholic fatty liver disease. Leptin administration attenuated alcohol-induced hypoleptinemia and hepatic lipid accumulation in association with normalization of alcohol-induced down-regulation of hepatic genes involved in lipid metabolism such as fatty acid oxidation, very low-density lipoprotein secretion, and related transcription factors. Leptin administration reversed alcohol-suppressed phosphorylation of AMPK and Stat3 in the liver. These findings

suggest that leptin deficiency in association with WAT mass reduction may contribute to the pathogenesis of alcoholic fatty liver disease.

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