Leptin deficiency enhances sensitivity of rats to alcoholic steatohepatitis through suppression of metallothionein

Kengo Tomita,1 Toshifumi Azuma,2 Naoto Kitamura,1 Gen Tamiya,3 Satoshi Ando,3 Hiroshi Nagata,4 Shinzo Kato,1 Sayaka Inokuchi,1 Takeshi Nishimura,1 Hiromasa Ishii,1 and Toshifumi Hibi1

1Department of Internal Medicine, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582; 2Department of Immunology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-8421; and 3Department of Molecular Life Science, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-1193, Japan

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Tomita, Kengo, Toshifumi Azuma, Naoto Kitamura, Gen Tamiya, Satoshi Ando, Hiroshi Nagata, Shinzo Kato, Sayaka Inokuchi, Takeshi Nishimura, Hiromasa Ishii, and Toshifumi Hibi. Leptin deficiency enhances sensitivity of rats to alcoholic steatohepatitis through suppression of metallothionein. Am J Physiol Gastrointest Liver Physiol 287: G1078–G1085, 2004; doi:10.1152/ajpgi.00107.2004.—Oxidative stress is stated to be a central mechanism of hepatocellular injury in alcohol-induced liver injury. Recent reports have shown that Kupffer cell dysfunction in the leptin-deficient state contributes partly to the increased sensitivity to endotoxin liver injury. Here we report that leptin also plays a key role in the development of alcoholic liver injury and that leptin signaling in hepatocytes is involved in cellular mechanisms that mediate ethanol-induced oxidative stress. We found that chronic ethanol feeding in leptin receptor-deficient Zucker (fa/fa) rats for 6 wk resulted in a much more severe liver injury and augmented accumulation of hepatic lipid peroxidation compared with control littersmates. The hepatic induction of stress-response and antioxidant proteins, such as metallothionein (MT)-1 and -2, was significantly suppressed in fa/fa rats after chronic ethanol feeding. Zinc concentration in liver was also decreased in fa/fa rats, compared with control littersmates. In primary cultured hepatocytes from fa/fa rats, incubation with ethanol significantly suppressed MT-1 and -2 expressions. Addition of leptin to leptin-deficient ob/ob mouse primary hepatocytes led to an increase in MT-1 and -2 mRNA levels and a decrease in oxidative stress after incubation with ethanol. In conclusion, leptin deficiency enhances sensitivity of rats to alcoholic steatohepatitis through hepatocyte-specific interaction of MT-1 and -2 and resultant exaggeration of oxidative stress in hepatocytes. These findings suggest that leptin resistance in hepatocytes is an important mechanism of alcohol-induced liver injury.

ALCOHOLIC LIVER DISEASE AFFECTS millions of patients on an annual basis worldwide (3, 30). Although some agents have been evaluated for the prevention and treatment of alcoholic liver disease in experimental models or clinical trials, including ours (20, 34), there are presently no FDA-approved therapies available. Gaps in our knowledge of how the disease progresses must be filled before rational targeted therapy can be developed. One leading putative mechanism by which alcohol is proposed to mediate its effects is oxidative stress. Many studies (4, 7, 11) have indicated that oxidative stress plays a role in the initiation and progression of alcoholic liver disease. Recent studies (4, 7, 11), including the present study, strongly suggest that the mitochondrion may be a significant source of reactive oxygen species (ROS) in hepatocytes when exposed to ethanol acutely or chronically and that the ability of ethanol to increase mitochondrial ROS production is associated with its metabolism and with ethanol-related alterations to the mitochondrial oxidative phosphorylation system, or both. Oxidative stress is also stated to be a central mechanism of hepatocellular injury in nonalcoholic fatty liver disease, which is commonly related to insulin resistance (22). Although hepatic steatosis develops in patients with alcohol abuse and insulin resistance, only a fraction of individuals with steatosis develop steatohepatitis. These results suggest that oxidative stress plays a role in the progression from steatosis to steatohepatitis. Recently, we (34) reported that pioglitazone, a derivative of the antidiabetic reagents, prevents alcohol-induced liver injury through restoration of the ethanol-induced down-regulation of c-Met and upregulation of stearoyl-CoA desaturase. Pioglitazone dramatically attenuated hepatic lipid peroxidation without altering insulin resistance, which suggests that with a better understanding of the mechanisms by which oxidative stress leads to liver damage during alcohol exposure, therapies that are more targeted at the cellular/molecular level may be applied in the clinic with potentially greater success. Obesity is a risk factor for the progression of alcoholic liver disease in humans (21). Leptin-deficient ob/ob mice and leptin receptor-deficient Zucker fa/fa rats are known to be hyperphagic, inactive, obese, and insulin resistant and are often used to study the mechanisms that regulate the progression from steatosis to nonalcoholic steatohepatitis (13). Diehl and colleagues (36) reported that ob/ob mice are more sensitive to endotoxin liver injury. They also showed that ob/ob Kupffer cells produce less IL-15 and more IL-12 after LPS stimulation, which promotes hepatic CD4+ natural killer cell depletion (15). Activation of Kupffer cells after exposure to LPS and the resultant release of proinflammatory cytokines, such as TNF-α, play a pivotal role in the initiation and progression of alcoholic liver disease. Though these results suggest that leptin resistance in Kupffer cells may contribute to the progression of alcoholic liver disease, it is not the only cause of lipid peroxidation and oxidative stress in alcoholic liver disease in the leptin-resistant state. In fact, it has recently been reported (29) that isolated Kupffer cells of Zucker fa/fa rats were less sensitive to LPS treatment, resulting in reduced TNF-α production compared with the control. Clearly, the most obvious
pathological changes to liver during alcohol exposure occur in the hepatocytes. Moreover, the accumulation of indices of oxidative stress is predominantly a hepatocellular event during alcohol administration. Previous reports (4, 7, 11) including the present study have also shown that the metabolism of ethanol leads to excessive production of oxidative stress in hepatocytes and antioxidants are involved in cytoprotective mechanisms that prevent ethanol-associated oxidative injury of hepatocytes. One potential antioxidant is metallothionein (MT). MT is a highly conserved, low-molecular-weight, cysteine-rich protein with antioxidative roles, and is known as an effective agent in cytoprotection against alcohol-induced liver injury (39). It was reported that overexpression of MT in the transgenic mice liver did not affect ethanol metabolism, but significantly inhibited ethanol-associated oxidative stress (38).

Such circumstances led us to examine whether leptin resistance will contribute to the progression of alcoholic liver disease, because it has recently been suggested that local or systemic leptin could be a key factor in the development of hepatic steatosis and steatohepatitis associated with obesity, type 2 diabetes mellitus, and hyperlipidemia (8). We focused on the oxidative mechanism by which leptin resistance in hepatocytes could play a central role in hepatic vulnerability to alcohol administration. Hepatocytes are a reasonable target for examination, because these cells have receptors for leptin (26) and, therefore, could become dysfunctional during liver deficiency. Because our transcriptome analyses by DNA microarray revealed the differential mRNA expression of MT, our attempt to examine the hypothesis in this study has not shown the causal role of leptin deficiency in the severity of alcohol-induced liver injury but also shed light on the significance of leptin-mediated signaling pathway to enhance antioxidants such as MT-1 and -2 in hepatocytes as a potential therapeutic target for treatment of alcohol-induced steatosis and injury.

MATERIALS AND METHODS

Animal feeding. The Lieber-DeCarli ethanol and control diets were purchased from Bio-Serv (Frenchtown, NJ) (17). The calorie distribution of liquid diet components is as follows (in %): 18 protein, 35 fat, 11.5 carbohydrates, and 36 either ethanol or additional carbohydrates in the isocaloric liquid diets. Four-week-old male Zucker (fa/la) rats and their control (fa/−) littersmates were obtained from Charles River (Wilmington, MA). The ingredients of fat include corn, olive, and safflower oil. They were housed in temperature- and light-controlled rooms and were divided into four groups: fa/la rats fed ethanol-containing liquid diet (Lieber-DeCarli diet) for 6 wk (n = 10); fa/la rats pair-fed isocaloric liquid diet without ethanol for 6 wk (n = 10); fa/− rats pair-fed ethanol-containing liquid diet; fa/la rats pair-fed isocaloric liquid diet without ethanol for 6 wk (n = 10); and fa/− rats pair-fed isocaloric liquid diet without ethanol for 6 wk. Rats in all groups were pair-fed daily on an isocaloric basis with each other. Five-week-old C57BL/6j ob/ob male mice and littersmate controls were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals received humane care in compliance with the National Research Council’s criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the U.S. National Academy of Sciences and published by the U.S. National Institutes of Health.

Biochemical and histological analysis. Hepatic triglyceride content was measured as previously described (34). For histological analysis, liver tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Alternatively, hepatic lipids were stained by an oil red O method (Nacalai Tesque, Kyoto, Japan). For RNA analysis, tissue was frozen in liquid nitrogen and stored at −80°C until used. Serum alanine aminotransferase (ALT) activity was measured with a standard clinical autoanalyzer (model 7170; Hitachi, Tokyo, Japan).

Real-time quantitative PCR analysis. Total RNA was extracted from the liver, hepatocytes, or Kupffer cells with Isogen (Nippon Gene, Tokyo, Japan) according to the method of Chomczynski and Sacchi, as previously described (33, 35). For the RT reaction, TaqMan RT reagents (Applied Biosystems, Foster City, CA) were used. Briefly, the RT reaction (final volume of total 50 µl) was conducted for 60 min at 37°C followed by 25°C for 10 min using random hexamer primers. The PCR amplification was performed with TaqMan Universal Master Mix (Applied Biosystems). In brief, reactions were performed in duplicate containing 2× Universal PCR master mix, 2 µl of template cDNA, 900 nM of primers, and 250 nM of probe in a final volume of 50 µl and were analyzed in a 96-well optical reaction plate (Applied Biosystems). Primers and probes were synthesized by Applied Biosystems custom oligosynthesis service: rat MT-1 sense primer, 5′-CTGCTCCACCGGG-3′; rat MT-1 antisense primer, 5′-GCCCTGGGCAATTTGG-3′; rat MT-1 TaqMan probe, 5′-CTCCTGAAGAGGSTCTGCTCTCT-3′; rat MT-2 sense primer, 5′-TCTTGTGGCACAGTATGATC-3′; rat MT-2 antisense primer, 5′-GTCGAAAGCTCTTGGACAG-3′; and rat MT-2 TaqMan probe, 5′-AAAAGCTGTCTCCTGCTGCCC-3′. Probes included a fluorescent reporter dye FAM on the 5′ end and labeled with hydroquinone dye TAMRA on the 3′ end to allow direct detection of the PCR product. Reactions were amplified and quantified by using an ABI 7700 sequence detector and manufacturer’s software (Applied Biosystems). The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The relative quantity of target mRNA was obtained by using the comparative C_T method and was normalized by using predeveloped TaqMan assay reagent rat 18S ribosomal RNA as an endogenous control (Applied Biosystems) (for details, see User Bulletin 2 for the ABI PRISM 7700 Sequence Detection System under www.appliedbiosystems.com/support/tutorials). Briefly, the TaqMan software (Applied Biosystem) was used to calculate a C_T value for each reaction, where the C_T value is the point in the extension phase of the PCR reaction at which the product is distinguishable from the background. The C_T values were then normalized for amplification by subtracting the C_T value calculated for 18S ribosomal RNA from the C_T value for each reaction, where the C_T value is the point in the extension phase of the PCR reaction at which the product is distinguishable from the background. The C_T values were then normalized for amplification by subtracting the C_T value calculated for 18S ribosomal RNA, an endogenous control for the amount of mRNA from the same sample, to obtain a C_T using the following equation: C_T target − C_T 18S ribosomal RNA = C_T. The fold induction was calculated relative to the C_T value obtained in the control rats or cells. The normalized expression was thus expressed as a relative quantity of mRNA (fold induction). The amounts of mRNA of rat Tnf-α and mouse MT-1 and -2, were measured by using Assay-on-Demand TaqMan assay reagent (Applied Biosystems).

Isolation and primary culture of mouse and rat hepatocytes. Parenchymal hepatocytes were isolated from 6-week-old male fa/la rats or ob/ob mice by the in situ collagenase perfusion method and cultured as previously described (36). The isolated hepatocytes were cultured on collagen gel (Cellmatrix; Nitta Zerachin, Osaka, Japan), and the second layer of collagen gel was spread over the cells after 1 day of incubation as previously described (33).

Isolation of rat Kupffer cells. Kupffer cells were isolated by the dish-adhesion technique after collagenase perfusion and pronase digestion as previously described (12, 24, 34). The purity of the cell preparation was >90% as determined by the results of nonspecific esterase staining and Wright staining. The viability of the cell preparation was >95% as determined by trypan blue exclusion. Freshly isolated Kupffer cells were prepared for RNA extraction.

Transcriptome analyses by DNA microarray. Transcriptome analyses were performed by using DNA microarrays (Atlas cDNA expressed sequence tags; Clontech, Palo Alto, CA) as previously described (34). In brief, preparation of [3²P]cDNA samples, hybridization, and washing were carried out with total RNA of the liver tissue according to the manufacturer’s manual. All data sets were normalized to the
signal density of housekeeping genes, such as the gene coding GAPDH and total radioactivity, which represents the total amount of cDNA hybridized to the microarrays. The threshold for determining the significance of changes in the level of gene expression was established by using an algorithm that requires both a significant absolute and significant fold change.

Oxidative stress. To investigate temporal alterations of oxidative stress in individual hepatocytes, 5-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy H₂DCF-DA; Molecular Probes, Eugene, OR) was used according to the methods of Roychowdhury et al. (28) with some modifications as previously described. Briefly, cultured mouse hepatocytes on culture dishes of which the bottom is made of nonfluorescent thin glass (Corning) were incubated with DMEM (pH 7.4) containing 1 μM carboxy H₂DCF-DA for 30 min at 37°C in the dark. The cells were washed three times with DMEM to remove the extracellular carboxy H₂DCF-DA, and the culture dish was mounted onto the stage of the scanning microscope (Zeiss 20X). Digital images of the carboxy H₂DCF-DA fluorescence were obtained at an excitation wavelength of 488 nm (argon laser) using a 515-nm long-pass emission filter.

Image analysis. For confocal imaging, laser attenuation, pinhole diameter, photomultiplier sensitivity, and offset were kept constant for every set of experiments. The obtained images were quantitatively analyzed for changes in fluorescence intensities within regions of interests (boxes drawn over cell soma) using the Zeiss LSM software. Fluorescence values were normalized to the control levels for quantitative analysis and depicted as means ± SE. Data were analyzed by using ANOVA single-factor analysis followed by Student’s t-test. A value of P < 0.05 was considered statistically significant.

Zinc content of liver tissues. Zinc concentration in liver tissues was measured as described elsewhere (18). Briefly, liver tissues were immersed in cold saline to remove adherent blood and blotted, and wet weight was then measured. Tissues were dried at 105°C to constant weight and ashed at 440°C for 24 h. Ash was solubilized with 0.12 N ultrapure HCl. Appropriately diluted samples were analyzed by atomic absorption spectrophotometry (model Z-6160; Hitachi, Tokyo, Japan).

Immunohistochemistry. For detection of 4-hydroxy-2-nonenal (4-HNE) protein adducts and rat MT-1, paraffinized sections were deparaffinized, rehydrated, treated with normal horse serum, and incubated with MAb anti-4-HNE (0.625 μg/ml) (Nippon Rouka Seiyo Kenkyujo, Tokyo, Japan) or MAb anti-MT-1 (0.1 μg/ml) (Transgenic, Kumamoto, Japan) overnight at 4°C. After several washes with PBS, the sections were stained with biotinylated anti-mouse IgG for 1 h (Vectorstain Elite ABC kit; Vector Laboratories, Burlingame, CA). To prevent endogenous peroxidase reactions, the samples were pretreated with 0.3% H₂O₂ in cold methanol for 30 min, and were subsequently incubated with avidin and horseradish peroxidase (HRP)-conjugated biotin for 30 min, followed by detection with 3,3’-diaminobenzidine solution containing 0.003% H₂O₂.

Statistical analysis. All data are expressed as the means ± SE. Statistical analysis was performed by using the unpaired Student’s t-test or by one-way ANOVA.

RESULTS

Chronic ethanol feeding exaggerates liver injury in fa/fa rats compared with fa/− rats. Chronic ethanol feeding increased hepatic triglyceride level. The hepatic triglyceride concentration was significantly higher in fa/fa rats given the ethanol-containing diet than in control rats given the ethanol-containing diet (Fig. 1A). Histological analysis also showed marked fatty accumulation and moderate inflammation in the ethanol-fed fa/fa rats, compared with the ethanol-fed fa/− rats (Fig. 1B and C). Analysis of serum ALT activity to assess the effect of leptin deficiency on liver function showed that the serum concentration of ALT in ethanol-fed fa/fa rats was elevated dramatically, compared with ethanol-fed fa/− rats (Fig. 1D). The aggravation was associated with histological findings in the liver. Ethanol feeding caused an increase in the liver weight-to-body weight ratio, and the ratio was significantly higher in ethanol-fed fa/fa rats than ethanol-fed fa/− rats (Fig. 1E).

Transcriptome analyses for mining genes responsible for anti-inflammatory effects of leptin. To elucidate mechanisms by which leptin deficiency exaggerates alcoholic liver disease,
we applied DNA microarrays to reveal differential mRNA expression between the livers of ethanol-fed fa/fa and ethanol-fed fa/- rats. The transcriptome analysis allowed us to reveal a much smaller amount of MT-1 in the livers of fa/fa rats (data not shown). These results were confirmed by real-time PCR analysis (Fig. 2A).

We could not find any differences in c-Met mRNA expression at this differential analysis (data not shown). Hepatic MT-1 and -2 mRNA levels and zinc concentration were significantly lower in ethanol-fed fa/fa rats. To elucidate the mechanism by which leptin deficiency enhanced sensitivity to alcohol-induced liver injury, we examined hepatic MT-1 and -2 mRNA levels among the treated groups by real-time PCR analysis (Fig. 2A). Though chronic ethanol feeding increased hepatic MT-1 and -2 levels, they were significantly lower in ethanol-fed fa/fa rats than in ethanol-fed fa/- rats. The zinc concentration in liver was also significantly lower in ethanol-fed fa/fa rats than in ethanol-fed fa/- rats (Fig. 2B).

Increased TNF-α mRNA level in Kupffer cells from ethanol-fed fa/fa rats. We examined TNF-α mRNA level in Kupffer cells from each treated group by real-time PCR analysis. Chronic ethanol feeding caused a significant increase in TNF-α mRNA level of Kupffer cells from fa/fa rats, compared with that of fa/- rats (Fig. 3). We also examined hepatic TNF-α mRNA level among the treated groups by real-time PCR analysis. Chronic ethanol feeding enhanced hepatic TNF-α level, and it was significantly increased in ethanol-fed fa/fa rats, compared with ethanol-fed fa/- rats (data not shown).

Immunohistochemistry for MT-1. After chronic ethanol feeding, staining of MT-1 protein was much more intense in livers from fa/- rats than fa/fa rats (Fig. 4). MT-1 stain was seen in the parenchymal cells of the liver. Chronic ethanol feeding caused a significant increase in hepatic lipid peroxidation in fa/fa rats compared with fa/- rats. After chronic ethanol feeding, the staining of 4-HNE-adducted protein as product of lipid peroxidation reaction was much more intense in livers of fa/fa rats than fa/- rats (Fig. 5).

Leptin deficiency caused a significant decrease in MT-1 and -2 mRNA levels in primary cultured hepatocytes after addition of ethanol. We found that primary cultured mouse hepatocytes on a single collagen gel gradually lost hepatocyte-specific morphology, and another collagen gel overlay remarkably recovered it (33). Because this culture device is a good method to maintain differentiated hepatocytes in a long-term culture, we used this culture system to investigate MT-1 and -2 mRNA levels in primary cultured hepatocytes from ob/ob mice or fa/fa rats by real-time PCR method. The ob/ob mice hepatocytes were treated with ethanol (20 mM) or not in the absence or presence of leptin (100 ng/ml) for 30 days. Addition of ethanol significantly increased MT-1 and -2 mRNA levels from the cultured hepatocytes in the presence of leptin (Fig. 6, A and C). The hepatocytes from fa/fa rats and fa/- rats were also treated with ethanol (20 mM) or not for 30 days. Addition of ethanol significantly decreased MT-1 and -2 mRNA levels in fa/fa rats hepatocytes but not in fa/- rats hepatocytes (Fig. 6, B and D).

Leptin deficiency caused a significant decrease in MT-1 and -2 expression levels and zinc concentration among the treated groups. Real-time PCR analysis was carried out as described in MATERIALS AND METHODS. Results (means ± SE) from 5 rats/group at the end of the feeding period are shown. All real-time quantitative PCR reactions were carried out in duplicate. *P < 0.05 vs. the control groups; **P < 0.05 vs. the control fa/fa rats group and the EtOH-fed fa/- group. Zinc concentration in liver tissues was measured as described in MATERIALS AND METHODS. Results (means ± SE) from 5 rats/group at the end of the feeding period are shown. *P < 0.05 vs. the other groups; **P < 0.05 vs. the control fa/- rats group. Control, rats pair-fed isocaloric liquid diet without EtOH.
We could not find any significant difference in TNF-α mRNA level among the treated cultures (data not shown).

Carboxy H$_2$DCF-DA fluorescence in ob/ob mice hepatocytes enhanced by ethanol treatment was inhibited in the presence of leptin. The ob/ob mice hepatocytes were cultured with or without ethanol (20 mM) in the absence or presence of leptin (100 ng/ml). Cultures with ethanol were incubated with ethanol (50 mM) for another 15 min in the absence or presence of leptin (100 ng/ml). After the incubation, analysis using the ROS-sensitive fluorescent probe carboxy H$_2$DCF-DA showed a clear fluorescence signal in ethanol-treated cultures without addition of leptin, and carboxy H$_2$DCF-DA fluorescence inhibition in the presence of leptin (Fig. 7).

**DISCUSSION**

This study is the first to suggest that leptin deficiency causes a much more severe steatohepatitis after chronic ethanol exposure. Differential transcriptome analyses comparing mRNA expression in the chronic ethanol-exposed livers in fa/fa rats and fa/− rats led us to pinpoint a critical role of the stress-response and antioxidant proteins such as MT-1 and -2. Furthermore, several lines of evidence provided in this study suggest that the possible mechanisms include the suppression of MT-1 and -2 induction in leptin-resistant liver, leading to the enhancement of oxidative stress in liver.

MT is a highly conserved, low molecular weight, cysteine-rich, metal-binding protein with antioxidative roles. Many studies have demonstrated that MT expression provides effective protection against hepatotoxicity induced by various stresses, such as acetaminophen, carbon tetrachloride, doxorubicin, and glutathione (GSH) depletors (39). Recent studies (38) have also demonstrated that MT plays a beneficial role in inhibition of ethanol-induced oxidative hepatotoxicity in vivo. The increased level of MT in ethanol-fed rats in our study
been suggested that chronic lipid peroxidation could represent a mechanism of alcohol-induced hepatotoxicity. It has been suggested that chronic ethanol feeding augments oxidative stress and hypoxia (16). It is reported that chronic ethanol feeding also caused a significant increase in TNF-α synthesis by fa/fa rat Kupffer cells, compared with that from fa/fi rats. These results suggest that the exaggeration of alcohol-induced liver injury in fa/fa rats may be, in part, due to increased TNF-α synthesis by fa/fa rat Kupffer cells. Notably, Kupffer cells are the primary source of hepatic TNF-α. Several lines of evidence indicate that Kupffer cells, the resident hepatic macrophage, play an important role in alcohol-induced liver damage. Kupffer cell activation is evoked by endotoxin or LPS, which was elevated in the blood due to endotoxemia after infection or the missing link between chronic steatosis and steatohepatitis.}

In this study, we also showed that chronic ethanol consumption led to the significant increase of hepatic TNF-α levels in fa/fa rats, compared with those of control rats. Chronic ethanol feeding also caused a significant increase in TNF-α synthesis of Kupffer cells from fa/fa rats, compared with that from fa/fi rats. These results suggest that the exaggeration of alcohol-induced liver injury in fa/fa rats may be, in part, due to increased TNF-α synthesis by fa/fa rat Kupffer cells. Notably, Kupffer cells are the primary source of hepatic TNF-α (31). Several lines of evidence indicate that Kupffer cells, the resident hepatic macrophage, play an important role in alcohol-induced liver damage. Kupffer cell activation is evoked by endotoxin or LPS, which was elevated in the blood due to

**Fig. 6.** MT-1 and -2 expression levels in primary cultured hepatocytes. A and C: ob/ob mice hepatocytes were treated with EtOH (20 mM) or not in the absence (Lep−) or presence (Lep+) of leptin (100 ng/ml) for 30 days on a collagen gel sandwich system. Values are means ± SE. Each experiment was performed at least 4 times. Real-time PCR analysis was carried out as described in MATERIALS AND METHODS. **P < 0.05 vs. the other groups.** B and D: hepatocytes from fa/fa rats or fa/fi rats were treated with EtOH (20 mM) or not (Cont) for 30 days on a collagen gel sandwich system. Values are means ± SE. Each experiment was performed in triplicate. Similar experiments were performed at least 4 times. Real-time PCR analysis was carried out as described in MATERIALS AND METHODS. **P < 0.05 vs. the control group.**

**Fig. 7.** 5-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy H2DCF-DA) in ob/ob mice hepatocytes after EtOH fluorescence. The ob/ob mice hepatocytes were cultured with EtOH (20 mM) or not in the absence or presence of leptin (100 ng/ml). Cultures with EtOH were incubated with EtOH (50 mM) for another 15 min in the absence or presence of leptin, and prepared for carboxy H2DCF-DA loading. A: shown are representative images of carboxy H2DCF-DA-loaded cultures. Top: confocal imaging of carboxy H2DCF-DA; bottom: transilluminated images. Ethanol, cultured with EtOH in the absence of leptin; Ethanol + Leptin: cultured with EtOH in the presence of leptin. B: graph depicts the fluorescence values digitally quantified from the images. Data are presented as means ± SE of 3 different cultures for each treatment described in MATERIALS AND METHODS. **P < 0.05 vs. the other groups; **P < 0.05 vs. the control groups. Cont, without addition of ethanol; Ethanol, with addition of ethanol; Lep−, without addition of leptin; Lep+, with addition of leptin.
alteration of gut permeability after chronic ethanol exposure (32). In fact, inactivation of Kupffer cells with gadolinium chloride or decreasing the amount of gut-derived endotoxin by sterilizing the intestine with antibiotics has been found to decrease ethanol-induced steatohepatitis (1, 2). On the other hand, isolated Kupffer cells of Zucker fa/fa rats showed significantly reduced LPS uptake as well as TNF-α production compared with control rats (29). Recent reports (40) also showed that oxidative stress mediates endotoxin-triggered TNF-α production in the alcohol-intoxicated liver. Antioxidants are known to persistently suppress LPS activation in Kupffer cells (5). These observations suggest that, in our study, activation of Kupffer cells of ethanol-fed fa/fa rats was enhanced by much more oxidative stress produced through suppression of MT. Yin et al. (37) found no evidence of alcohol-induced steatohepatitis in type-1 TNF-receptor-deficient mice, suggesting an essential role of TNF-α in the pathogenesis of alcoholic liver disease. There is accumulating evidence that TNF-α signaling in the hepatocyte causes an increase in mitochondrial ROS generation through ubiquinone cycling via the electron transport chain (14). Increased TNF-α production could contribute to exaggeration of liver damage in ethanol-fed fa/fa rats.

The low hepatic zinc content found in ethanol-fed rats in our study confirms the conclusions of previous studies (10, 19, 27). Patients with alcoholic cirrhosis are reported to have an abnormal zinc metabolism with low serum zinc, increased urinary zinc excretion, and decreased hepatic zinc levels, whereas the absorption of zinc seems unaffected. We suggest that these mechanisms could partly explain low hepatic zinc content in our ethanol-fed rats. On the other hand, MT plays a critical role in maintaining high levels of zinc in the liver, especially under stress conditions. Though the protective action of zinc could be independent of MT, zinc is released from MT under oxidative stress (39). Increasing evidence indicates that zinc has antioxidant properties by inhibition of HO• formation through antagonism of redox-transition metals (25). These observations suggest that in our study upregulation of MT could exert antioxidant action partly by maintaining zinc levels.

In summary, we found that leptin deficiency enhanced sensitivity of rats to alcohol-induced steatohepatitis through suppression of hepatic MT level and resultant exaggeration of oxidative stress. The role of leptin resistance in the severity of liver injury remains to be elucidated. The report that overexpression of leptin in the liver caused a small liver with a marked decrease in lipid storage could suggest the beneficial role of leptin signaling in the treatment of fatty liver disease (23). Our present study is the first to suggest the pathophysiological and therapeutic implications of leptin in alcoholic steatohepatitis, which merits further evaluation.

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