Prevention of Ethanol-Induced Liver Injury in Rats by an Agonist of Peroxisome Proliferator-Activated Receptor-γ, Pioglitazone

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ABSTRACT
Agonists of peroxisome proliferator-activated receptor (PPAR)-γ have been shown to reduce tumor necrosis factor-α (TNF-α)-induced insulin resistance. On the other hand, sensitization of Kupffer cells to lipopolysaccharide (LPS) and their production of TNF-α are critical for progression of alcoholic liver injury. This study was intended to determine whether pioglitazone, a PPAR-γ agonist, could prevent alcohol-induced liver injury. Rats were given ethanol (5 g/kg b.wt.) and pioglitazone (500 μg/kg) once every 24 h intragastrically. Ethanol for 8 weeks caused pronounced steatosis, necrosis, and inflammation in the liver. These pathological parameters were diminished greatly by pioglitazone. Kupffer cells were sensitized to LPS after ethanol for 4 weeks as evidenced by aggravation of liver pathology induced by LPS (5 mg/kg) and enhancement of LPS (100 ng/ml)-induced intracellular Ca2+ concentration elevation in Kupffer cells. The parameters were diminished by treatment with pioglitazone. LPS-induced TNF-α production by Kupffer cells from the 4-week ethanol group was 3 to 4 times higher than control. This increase was blunted by 70% with pioglitazone. Gut permeability was 10-fold higher in the 4-week ethanol group, and pioglitazone treatment did not change the value. Inclusion of TNF-α in culture media of Kupffer cells enhanced CD14 expression, LPS-induced intracellular Ca2+ concentration response, and production of TNF-α. These results indicate that pioglitazone prevents alcoholic liver injury through abrogation of Kupffer cell sensitization to LPS.

Sensitization of Kupffer cells is a prominent event in the initiation of alcoholic liver disease (Stahnke et al., 1991; Martinez et al., 1992). Sensitized Kupffer cells are activated by endotoxin (lipopolysaccharide, LPS), leading to a rapid increase in intracellular calcium (Watanabe et al., 1996) followed by release of inflammatory mediators (e.g., cytokines and lipid metabolites), as well as reactive oxygen intermediates (Nolan, 1981; Decker et al., 1989; Shibayama et al., 1991; Wang et al., 1995). Among them, TNF-α is likely a critical factor in the progression of alcoholic liver disease because it induces cell death due to apoptosis and necrosis and stimulates generation of toxic superoxide anion from mitochondrial complex III in parenchymal cells (Schulze-Osthoff et al., 1993) and expression of factors for neutrophil chemotaxis (interleukin-8/cytokine-induced neutrophil chemoattractant, macrophage inflammatory protein, macrophage inflammatory protein-2) and intracellular adhesion molecule-1, leading to microcirculatory disturbance (Hijioka et al., 1991; Oshita et al., 1992; McCuskey et al., 1993). This scenario is supported by the fact that early injury in the Tsukamoto-French enteral model of alcohol induced liver injury, in which high-fat liquid diet is infused continuously intragastrically, is diminished by an anti-TNF-α antibody (Iimuro et al., 1997) and is prevented in TNF-α receptor-knockout mice (Yin et al., 1999). Accordingly, it is postulated that sensitization of Kupffer cells to LPS and overproduction of TNF-α by Kupffer cells are critical for progression of alcoholic liver injury.

On the other hand, peroxisome proliferator-activated receptor (PPAR)-γ is a member of the nuclear hormone receptor superfamily that heterodimerizes with the retinoid X receptor and functions as a transcriptional regulator of a variety of genes. The thiazolidinedione class of antidiabetic drugs was identified as ligands for PPAR-γ, and subsequently they have been shown to reduce TNF-α-induced insulin resistance (Sulliel and Olefsky, 1996). The mechanisms of this action remain obscure, although it has been proposed that PPAR-γ ligands cross talk with several points of signaling pathways evoked by TNF-α (Hofman et al., 1994; Jiang et al., 1998; et al., 1991; Oshita et al., 1992; McCuskey et al., 1993). This scenario is supported by the fact that early injury in the Tsukamoto-French enteral model of alcohol induced liver injury, in which high-fat liquid diet is infused continuously intragastrically, is diminished by an anti-TNF-α antibody (Iimuro et al., 1997) and is prevented in TNF-α receptor-knockout mice (Yin et al., 1999). Accordingly, it is postulated that sensitization of Kupffer cells to LPS and overproduction of TNF-α by Kupffer cells are critical for progression of alcoholic liver injury.

1. ABBREVIATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; PPAR, peroxisome proliferator-activated receptor; i.e., intragastrically; AST, aspartate transaminase; ALT, alanine aminotransferase; [Ca2+]i, intracellular calcium concentration; AM, acetoxymethyl ester; ELISA, enzyme-linked immunosorbent assay; TTP, tristetraprolin; ANOVA, analysis of variance; HRP, horseradish peroxidase.
Murase et al., 1998). The property of PPAR-γ ligands to oppose TNF-α actions suggests that they might be used for treatment of alcoholic liver disease. Accordingly, this study was intended to determine whether pioglitazone, a PPAR-γ agonist, could prevent alcohol-induced liver injury.

**Materials and Methods**

**Animals and Treatments.** In this study, we used a model of alcoholic liver injury based on the sensitization of Kupffer cells, in which rats are given ethanol (5 g/kg b.wt. intragastrically) once every 24 h (Enomoto et al., 1999). This model achieves inflammatory and necrotic changes in the liver only in 8 weeks, mimicking clinical alcoholic liver injury (Enomoto et al., 1999). These histological manifestations are preceded by sensitization of Kupffer cells to LPS. Accordingly, liver damage was evaluated after 8 weeks of ethanol treatment, whereas evaluation of Kupffer cell sensitization to LPS was performed at 4 weeks (see below). Female Wistar rats weighing 200 to 250 g were fed a liquid diet (Oriental, Tokyo, Japan) in which 35% of the calories were from corn oil and 47% were from maltose-dextrin ad libitum. Elements of this diet and percentage of calories were shown elsewhere (Enomoto et al., 1999). Rats were given one single dose of ethanol (5 g/kg) between 10:00 AM and 12:00 PM via gavage, with ethanol. Dextrin ad libitum. Elements of this diet and percentage of calories were shown elsewhere (Enomoto et al., 1999). Rats were given one single dose of ethanol (5 g/kg) between 10:00 AM and 12:00 PM via gavage, with ethanol.

To assess the sensitization of Kupffer cells to LPS in vivo, LPS (5 mg/kg, Escherichia coli serotype 0111:B4; Sigma-Aldrich, St. Louis, MO) was administered i.v. into either control or ethanol-treated rats, and liver histology was evaluated 24 h later.

**Pathological Evaluation.** Liver specimens were obtained from rats 24 h after final ethanol on 8 weeks of daily single intragastric treatment with ethanol (5 g/kg) and 24 h after LPS (5 mg/kg) on 4 weeks of ethanol. Livers were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin for assessment of steatosis, inflammation, and necrosis (Nanji et al., 1989).

**Kupffer Cell Preparation and Culture.** Kupffer cells were isolated by collagenase digestion and differential centrifugation using a tissue AB. Uppsala AB). As described elsewhere with slight modifications (Pertot et al., 1982). Briefly, the liver was perfused through the portal vein with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution at 37°C for 5 min at a flow rate of 26 ml/min. Subsequently, perfusion was performed with Hanks’ balanced salt solution containing 0.025% collagenase IV (Sigma-Aldrich) at 37°C for 5 min. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze mesh and the filtrate was centrifuged at 450g for 10 min at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by centrifugation at 50g for 3 min, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 1,000g for 15 min, and the Kupffer cell fraction was collected and washed with buffer again. Viability of cells determined by trypan blue exclusion was >90%. Cells were seeded onto 35-mm glass bottom culture dish (YSI Japan, Tokyo, Japan) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 10 mM HEPES and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin sulfate) at 37°C with 5% CO₂. Nonadherent cells were removed after 1 h by replacing buffer, and cells were cultured for 24 h before experiments.

**Measurement of Intracellular Ca²⁺ ([Ca²⁺]) Using a Fluorescence Microscope Imaging System.** All of the experiments were performed after cells were incubated at 37°C for 24 h. Fura-2/AM with Fluoronic F127 was dissolved in phosphate saline solution containing 1.0 mM Ca²⁺, in which final concentrations of Fura-2/AM and Fluronic F127 were 4 nM and 0.05%, respectively. After loading with Fura-2/AM solution at 37°C for 30 min, Kupffer cells on coverslips were installed in a fluorescence microscope (Diaphot; Nikon, Tokyo, Japan) with a 100-W xenon arc lamp as a light source. The objective lens was a Nikon Fluor X100. A silicon-intensified target camera (C-2400; Hamamatsu Photonics, Hamamatsu, Japan) was linked to a computer (MAXXDT2; Mitsubishi, Tokyo, Japan) and fluorescence intensity of Fura-2/AM was quantified. Wavelengths of 340 and 380 nm for excitation and 520 nm for emission were used. [Ca²⁺], was determined by the following equation (Grynkiewicz et al., 1985): [Ca²⁺] = Kd ([Rmax – Rmin]/[Rmax – Rmin]B). Kd is the Ca²⁺ dissociation constant for Fura-2, was confirmed as 224 nM. R is the ratio of fluorescence intensity at 380 nm divided by that at 380-nm excitation (Rmax, experimental data; Rmin, R in 2 mM EGTA and 1 mM iomycin; B = R in 10 mM CaCl₂ and 1 mM iomycin). B is the ratio of fluorescence intensity at 380 nm in the absence of Ca²⁺ versus a saturating concentration of Ca²⁺. Because intracellular Ca²⁺ was calculated from the ratio R, the fading of fluorescence did not interfere with the results.

**TNF Production by Kupffer Cells.** Kupffer cells were seeded onto 24-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, and antibiotics at 37°C in the presence of 5% CO₂. Cells were incubated with 10 ng/ml TNF-α before challenge with LPS. Samples of media were collected and kept at −80°C until assay. TNF-α in the culture media was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Cambridge, MA).

**Western Blotting for CD14 and Tristetraprolin (TTP).** Total protein extracts of liver and cultured Kupffer cells were obtained by homogenizing samples in a buffer containing 10 m M HEPES, pH 7.6, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 40 µg/ml bestatin, 20 mM β-glycerophosphate, 10 mM 4-nitrophenylphosphate, 0.5 mM pefabloc, 0.7 µg/ml pepstatin A, 2 µg/ml aprotinin, 50 µM NaVO₃, and 0.5 µg/ml leupeptin. Protein concentration was determined using the Bradford assay kit (Bio-Rad, Hercules, CA). Extracted protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked by Tris-buffered saline (TBS) containing 10% w/v nonfat dry milk, incubated with a mouse anti-rat CD14 monoclonal antibody (Serotec, Oxford, UK) and a mouse anti rat TTP monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by a horseradish peroxidase (HRP)-conjugated secondary antibody as appropriate. Membranes were incubated with a chemiluminescence substrate (ECL reagent; Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and exposed to X-OMAT films (Eastman Kodak, Rochester, NY).

**Gut Permeability.** Gut permeability was measured in isolated segments of ileum from translocation of horseradish peroxidase as described previously (Carter et al., 1987). Briefly, 8-cm segments of ileum were everted, filled with 1 ml of Tris buffer (125 mM NaCl, 10 mM fructose, and 30 mM Tris; pH 7.5), and ligated at both ends. The filled gut segments were incubated in Tris buffer containing 40 mg/100 ml horseradish peroxidase. After 45 min, gut sacs were removed and blotted lightly to eliminate excess horseradish peroxidase and the contents (~750 µl) of each sac were collected carefully using a 1-ml syringe. Horseradish peroxidase activity in the contents of each sac was determined spectrophotometrically.

**Fluorescence Staining of CD14.** Kupffer cells were fixed on a plastic dish using cold pure ethanol for 30 s, and the phalloidin rhodamine method (Watanabe et al., 1990) was used for staining actin. Indirect immunofluorescence staining was performed for CD14. They were then incubated overnight with the primary anti-
body, 1:200 diluted polyclonal rabbit anti-CD14 (M-305; Santa Cruz Biotechnology, Inc.). The secondary antibody used was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.). Samples were evaluated and their appearance was recorded on an Axioshot microscope (Carl Zeiss, Oberkochen, Germany) with Ektachrome Dyna 400 films (Eastman Kodak).

Statistical Analysis. All results were expressed as mean ± S.E.M. Statistical differences between means were determined using analysis of variance (ANOVA) and Bonferroni's post hoc test. p < 0.05 was selected prior to the study to reflect significance.

Results

Effect of Pioglitazone on Ethanol-Induced Liver Injury. There are no differences in body weight growth among control, pioglitazone, ethanol, and ethanol + pioglitazone groups during 8 weeks of ethanol treatment. All animals survived until the end of the 8-week experiment. Animals given pioglitazone only showed completely normal liver histology (Fig. 1B). As reported previously, ethanol administration once every 24 h for 8 weeks caused pronounced steatosis, necrosis, and inflammation in the liver (Fig. 1, C and D). In contrast, these pathological parameters were diminished markedly by the concurrent treatment with pioglitazone (500 μg/kg/day) (Fig. 1E). Furthermore, although mean value of ALT in the control, nontreated rats was 30 ± 6 IU/l, ALT increased 3-fold to 91 ± 7 IU/l in the 8-week ethanol group (Fig. 1, F and G). It is notable that the increase in ALT was blocked almost completely by pioglitazone (41 ± 5 IU/l) (Fig. 1G).

To exclude the possibility that the protective effect observed is due to alterations of absorption and/or elimination of ethanol, we measured blood ethanol concentration. There were no statistically significant differences in blood ethanol concentrations between groups given either ethanol only or ethanol + pioglitazone (500 μg/kg) 90 min and 6 h postadministration (485 ± 62 versus 440 ± 23 mg/dl and 350 ± 56 versus 367 ± 32 mg/dl, respectively; N.S.).

Effect of Pioglitazone on Ethanol Plus LPS-Induced Liver Injury. ALT/AST values after 4 weeks of ethanol remained unchanged compared with those of normal, nontreated rats. Furthermore, there were no significant difference in ALT/AST values among the 4-week ethanol, 4-week pioglitazone, and 4-week ethanol + pioglitazone groups (data not shown).

To assess the sensitization of Kupffer cells to LPS in vivo, LPS (5 mg/kg) was administered i.v. into either control or ethanol-treated rats, and liver histology was evaluated 24 h later. LPS caused focal necrosis and neutrophil infiltration in liver from the control, nontreated rats (Fig. 2A). In the group treated with pioglitazone for 4 weeks, liver histology displayed only slight infiltration of inflammatory cells, but lacking overt necrosis (Fig. 2B). This result was in line with serum transaminase levels, which were slightly lower in the LPS-only group (Fig. 2, F and G).

In the 4-week ethanol group, LPS injection resulted in marked aggravation of these parameters with pronounced steatosis (Fig. 2, C and D). The histological changes were diminished by treatment with pioglitazone (500 μg/kg/day) (Fig. 2E). Compared with the control group, ALT value 24 h after LPS challenge was increased 3-fold to 1,200 ± 375 IU/l in the 4-week ethanol group. This increase was completely

Fig. 1. Effect of pioglitazone on ethanol-induced liver injury. Photomicrographs of H&E sections of livers from rats treated as described under Materials and Methods: no treatment (A), 8-week pioglitazone (500 μg/kg i.g., once daily) (B), 8-week ethanol (5 g/kg) (original magnification, 100 ×) (C), 8-week ethanol (400 ×) (D), and 8-week ethanol + pioglitazone (100 ×) (E). Typical photomicrographs. Blood samples were collected from the aorta, and AST (F) and ALT (G) were determined as described under Materials and Methods. Results are mean ± S.E.M. (n = 4–8). *, p < 0.05 versus control. #, p < 0.05 versus 8 weeks of ethanol by ANOVA and Bonferroni's post hoc test.
blunted by pioglitazone (Fig. 2G). Similar results were obtained with AST values (Fig. 2E).

**Effect of Pioglitazone and Ethanol on LPS-Induced Increases in [Ca^{2+}]_{i} and TNF-α Production in Isolated Kupffer Cells.** To evaluate the effect of pioglitazone on ethanol-induced Kupffer cell sensitization to LPS, we measured the LPS-induced increase of [Ca^{2+}]_{i} and production of TNF-α, as reported elsewhere (Enomoto et al., 1999). LPS (100 ng/ml) elicited a transient increase in [Ca^{2+}]_{i} of Kupffer cells isolated from control rats from basal level (36 ± 8 nM) to 81 ± 13 nM (Fig. 3, A and E). After the peak increase, [Ca^{2+}]_{i} declined rapidly returning to basal value. Pioglitazone treatment did not change the [Ca^{2+}]_{i} response (Fig. 3, B and E). In contrast, the peak [Ca^{2+}]_{i} elevation elicited by LPS was about 2- to 3-fold greater (227 ± 26 nM) in Kupffer cells obtained from rats treated with ethanol for 4 weeks (Fig. 3, C and E). Furthermore, the effect of ethanol to enhance CD14 expression was markedly abrogated when pioglitazone was coadministered with ethanol for 4 weeks (lane 3).

**Effect of Ethanol and Pioglitazone Treatment on CD14 Expression in Liver.** Because CD14, a functional LPS/LBP receptor, is critical for signaling pathways leading to expression of cytokines, eicosanoids, and radical species in Kupffer cells, we measured CD14 with Western blotting. Liver from control rats expressed 55-kDa CD14 (Fig. 5, lane 1). Pioglitazone only did not alter CD14 level (lane 2). In marked contrast, the band was about 8-fold more intense in Kupffer cells from rats treated with ethanol for 4 weeks (lane 3). Furthermore, the effect of ethanol to enhance CD14 expression was markedly abrogated when pioglitazone was coadministered with ethanol for 4 weeks (lane 4).

**Effect of Ethanol and Pioglitazone on Gut Permeability and Portal LPS Levels.** Because Kupffer cell sensitization is caused by LPS (Enomoto et al., 1999), we then examined the gut permeability, assessed by HRP (Fig. 6). Pioglitazone alone did not change the basal level of gut permeability. In marked contrast, 2 h after the final ethanol treatment in the 4-week group, gut permeability was increased dramatically, levels being about 10-fold higher than values from control rats; however, the ethanol-induced increase in gut permeability was not affected by treatment with pioglitazone (Fig. 6). LPS levels in portal blood did not differ between the ethanol-only and ethanol + pioglitazone groups (140 ± 51 versus 152 ± 80 pg/ml; N.S.).

**Effect of Pioglitazone on LPS-Induced TNF-α Production in Cultured Kupffer Cells.** Because TNF-α plays a critical role in the pathogenesis of alcoholic liver injury, inhibition of TNF-α production from Kupffer cells is an obvi-
ous possibility that may explain the mechanism by which pioglitazone diminished liver injury due to chronic ethanol treatment. We therefore evaluated whether pioglitazone acted directly on Kupffer cells, thereby inhibiting TNF-α production.

After addition of LPS (100 ng/ml) to Kupffer cells isolated from normal rats, TNF-α production by Kupffer cells was increased (Fig. 7). Pioglitazone at 5 μM reduced the TNF-α production by Kupffer cells by about 25% (p < 0.05 versus LPS group).

Effect of Pioglitazone and TNF-α on Sensitization of Kupffer Cells to LPS in Vitro. It has been reported that specific agonists for PPAR-γ diminish insulin resistance in target cells. This effect is likely elicited by blockade of TNF-α–induced signaling pathways that interfere with the insulin-induced intracellular signal transduction. Analogously, we hypothesized that TNF-α potentiates Kupffer cell sensitization to LPS and that pioglitazone intervenes in the signaling pathways downstream of TNF-α receptors in Kupffer cells that control sensitization to LPS. To explore this possibility, a series of in vitro experiments using cultured Kupffer cells were performed.

First, to determine whether CD14 expression in Kupffer cells was regulated by TNF-α, immunocytochemical staining with anti-CD14 antibody was performed. As depicted in Fig. 8, Kupffer cells from control rats displayed a constitutive expression of CD14. Treatment with 10 ng/ml TNF-α for 24 h resulted in a pronounced increase in intensity of CD14 staining in Kupffer cells. Interestingly, inclusion of pioglitazone in the culture media during the TNF-α stimulation led to a diminished expression of CD14 to a level almost comparable to control expression (Fig. 8).

Next, we evaluated whether TNF-α potentiates the LPS-induced increase in intracellular calcium response, a critical event leading to TNF-α production by Kupffer cells. As shown earlier, Kupffer cells from control rats exhibited a transient
increase of \([\text{Ca}^{2+}]\), \((92 \pm 10 \text{ nM})\) in response to 100 ng/ml LPS (Figs. 3 and 9A). In marked contrast, the LPS-induced \([\text{Ca}^{2+}]\), response was 2- to 3-fold greater in Kupffer cells pretreated for 24 h with 10 ng/ml TNF-\(\alpha\) (Fig. 9B). When pioglitazone was present in the culture medium, this LPS-induced enhancement of \([\text{Ca}^{2+}]\), response was almost completely abrogated.

Furthermore, LPS-induced TNF-\(\alpha\) production by isolated Kupffer cells was compared between groups cultured for 24 h in the presence or absence of TNF-\(\alpha\) (10 ng/ml). As expected, Kupffer cells cultured in the presence of TNF-\(\alpha\) produced 30% more TNF-\(\alpha\) in response to LPS (100 ng/ml) than control cells that were cultured in the absence of TNF-\(\alpha\) (Fig. 9C). The addition of pioglitazone in the culture media during the TNF-\(\alpha\) stimulation resulted in a complete inhibition of the increase in TNF-\(\alpha\) production by Kupffer cells. In addition, pioglitazone increased the amount of tristetraprolin, a CCCH zinc finger protein known to destabilize TNF-\(\alpha\) mRNA (Fig. 10).

**Fig. 4.** Effect of ethanol and pioglitazone on LPS-induced TNF-\(\alpha\) production by cultured Kupffer cells. Isolated Kupffer cells were cultured in 24-well plates at a density of 5 \(\times\) 10^5 cells/well for TNF-\(\alpha\) determination. After 24 h of preincubation, LPS (final concentration, 100 ng/ml in 5% rat serum) was added and incubation was continued for 4 h. TNF-\(\alpha\) in medium was measured by ELISA. Basal TNF-\(\alpha\) release after 4 h of incubation without LPS was 10 \(\pm\) 2 pg/ml. Results are mean \(\pm\) S.E.M., \(n = 4; \# , p < 0.05\) versus control; \#, \(p < 0.05\) versus 4 weeks of ethanol by ANOVA and Bonferroni’s post hoc test.

**Fig. 5.** Effect of ethanol and pioglitazone on CD14 expression in liver. Protein extracts from control liver or livers from rats treated for 4 weeks with ethanol alone or with pioglitazone were analyzed by Western blotting using an anti-CD14 antibody. Specific bands for CD14 (55 kDa) are shown. Lane 1, livers from control rats; lane 2, livers from 4 weeks of pioglitazone; lane 3, livers from 4 weeks of ethanol; and lane 4, livers from 4 weeks of ethanol with pioglitazone. Data are representative of three individual experiments.

**Fig. 6.** Effect of ethanol and pioglitazone treatment on gut permeability. Rats were treated with ethanol and pioglitazone for 4 weeks before experiments. Two hours after administration of final ethanol, segments of ileum were isolated and permeability to HRP was detected. Values are mean \(\pm\) S.E.M. for four rats per group. *, \(p < 0.05\) versus control by ANOVA and Bonferroni’s post hoc test.

**Fig. 7.** Effect of pioglitazone on LPS-induced TNF-\(\alpha\) production in cultured Kupffer cells. Isolated Kupffer cells were cultured in 24-well plates at a density of 5 \(\times\) 10^5 cells/well for TNF-\(\alpha\) determination. After 24 h of preincubation, LPS (final concentration, 100 ng/ml in 5% rat serum) was added, and incubation was continued for 4 h. TNF-\(\alpha\) in media was measured by ELISA. Basal TNF-\(\alpha\) release after 4 h of incubation without LPS was 10 \(\pm\) 2 pg/ml. Results are mean \(\pm\) S.E.M., \(n = 4; \#, p < 0.05\) versus control; \#, \(p < 0.05\) versus LPS by ANOVA and Bonferroni’s post hoc test.
**Discussion**

A PPAR-γ Agonist Pioglitazone Prevents Alcohol-Induced Liver Injury through Suppression of TNF-α Production. It has been established that sensitization of Kupffer cells to LPS and consequent overproduction of TNF-α play a pivotal role in the pathogenesis of alcoholic liver disease (Stahnke et al., 1991; Martinez et al., 1992; Enomoto et al., 1998). Therefore, in this study, we used a model of alcohol-induced liver injury based on sensitization of Kupffer cells (Enomoto et al., 1999). This model achieves pathological changes in the liver (e.g., steatosis, inflammation, and necrosis) that resemble alterations found in clinical alcoholic liver disease (Fig. 1). In this setting, Kupffer cells isolated from rats exposed to ethanol chronically were sensitized to LPS as evidenced by enhanced transient increase in [Ca^{2+}]_i and TNF-α production (Fig. 3). In addition, CD14 expression in livers of the ethanol-treated rats was greatly enhanced (Fig. 5). Consequently, LPS administration into rats treated with ethanol for 4 weeks led to marked aggravation of liver injury (Fig. 2).

It was shown that a PPAR-γ agonist, pioglitazone, prevented ethanol-induced liver injury almost completely (Fig. 1). This effect is at least in part attributable to reduced TNF-α production because pioglitazone blunted markedly TNF-α production by Kupffer cells from ethanol-treated animals (Fig. 4). PPAR-γ is a member of the nuclear receptor family of transcription factors. Because Kupffer cells, the largest population of macrophage lineage in the body, contain PPAR-γ (Ricote et al., 1999), we explored the possibility that pioglitazone directly acted on Kupffer cells, thereby preventing TNF-α production. As shown in Fig. 7, pioglitazone suppressed TNF-α production in Kupffer cells. This result agrees with an earlier work by Uchimura showing that PPAR-γ ligands inhibited TNF-α production from macrophages (Uchimura et al., 2001). They suggested that this inhibition occurred at the transcriptional level. Our results may add to a new mechanism for the action of pioglitazone to inhibit TNF-α production because pioglitazone increased expression of TTP, a CCCH zinc finger protein shown to inhibit TNF-α-induced TNF-α production from macrophages by destabilizing its mRNA (Carbollo et al., 1998). Furthermore, the fact that pioglitazone destabilizes TNF-α mRNA gives a good basis for the use of this type of drug for treatment of alcohol-induced liver injury because it was recently reported that chronic ethanol results in stabilization of TNF-α mRNA (Kishore et al., 2001).

One could, however, argue that the preventive effect of pioglitazone against alcoholic liver could not be attributable solely to the direct suppression of TNF-α because the inhibition was not perfect (25% reduction as shown in Fig. 7). TNF-α has a wide range of bioactivity, and the regulatory mechanisms of TNF-α production and its intracellular signaling have been studied extensively (Papadakis and Targan, 2000). It is suggested that TNF-α acts on macrophages/monocytes to promote its own synthesis and secretion (Carbollo et al., 1998). Indeed, the results of this study indicate that in the presence of TNF-α, the production of TNF-α from Kupffer cells was about 30% higher than that in its absence, confirming our recent observation (Fig. 9C; Enomoto et al., 2002).

The autocrine acceleration of TNF-α production seems to be of primary importance for the pioglitazone action in prevention of alcohol-induced liver damage, given the fact that pioglitazone treatment in vivo abrogated ethanol-induced liver injury. Furthermore, because Kupffer cells reside strategically in the narrow sinusoidal space, one can envision that the liver microenvironment favors this autocrine activation to operate and perpetuate in an efficient way. It is thus postulated that the initial inhibition of TNF-α production, although not complete, culminates in sufficient suppression of TNF-α synthesis during a long-term ethanol load that might account for the hepatoprotective effect of pioglitazone.

**Pioglitazone Prevents Kupffer Cell Sensitization to LPS.** It is notable that Kupffer cell sensitization to LPS was almost completely prevented in the group treated for 4 weeks with ethanol and pioglitazone, given that Kupffer cell response to LPS, as evaluated by intracellular calcium increase, was diminished to a level comparable with control (Fig. 3). As we reported previously, ethanol-induced sensitization of Kupffer cells is caused by gut-derived endotoxin and that sensitization in Kupffer cells is caused by an increase in CD14 (Watanabe et al., 1990). To support this notion, Kupffer cell sensitization caused by long-term ethanol treatment was blocked by antibiotics in vivo (Enomoto et al., 1999). Moreover, Nanji et al. (1993) reported that a good correlation between blood endotoxin and liver pathology was observed in the Tsukamoto-French model (Ricote et al., 1999). Therefore, we investigated the effect of pioglitazone on gut permeability. Pioglitazone, however, did not alter gut permeability (Fig. 6). Furthermore, LPS concentrations in portal blood of the both groups did not differ. These data indicate that Kupffer cells in both groups were exposed to similar concentrations of LPS, making it unlikely that pioglitazone elicited its effect through regulation of portal LPS concentrations.

Alternatively, it seems likely that TNF-α itself regulates Kupffer cells sensitization because TNFα up-regulated expression of CD 14 protein in Kupffer cells (Fig. 8) and the...
intracellular calcium response to LPS. Pioglitazone blocked completely the TNF-α–induced CD14 up-regulation in Kupffer cells as well as the increased intracellular calcium response to LPS (Fig. 9).

The precise mechanisms by which PPAR-γ ligands/agonists interfere with the TNF-α–induced signal transduction, thereby abolishing sensitization to LPS and TNF-α secretion, are yet to be elucidated. The possibilities that pioglitazone may oppose TNF-α-induced reduction of its receptor PPAR-γ and/or that pioglitazone acted on hepatocytes to enhance resistance to TNF-α may also be taken into account.

These results show that pioglitazone prevents alcoholic liver injury through suppression of TNF-α production and Kupffer cell sensitization to LPS. PPAR-γ agonists are now widely used for treatment of diabetes mellitus, and they may prove useful as a therapeutic modality in the treatment of alcohol-induced liver injury. Furthermore, because nonalcoholic steatohepatitis is characterized by an ongoing inflammation associated with overexpression of proinflammatory cytokines such as TNF-α from Kupffer cells (Neuschwander-Tetri and Caldwell, 2003), it seems most likely that pioglitazone is also effective in the treatment of nonalcoholic steatohepatitis.

References


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