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Prevention of Ethanol-Induced Liver Injury in Rats by an Agonist of PPAR-γ, Pioglitazone

Nobuyuki Enomoto, Yoshiyuki Takei, Miyoko Hirose, Akira Konno, Tomoyoshi Shibuya, Shujiro Matsuyama, Satoko Suzuki, Kenichi Ikejima Tsuneo Kitamura, Nobuhiro Sato

Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Abbreviations used in this manuscript

EGTA: ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, ELISA: enzyme-linked immunosorbent assay, FBS: fetal bovine serum, HBSS: Hanks' balanced salt solution, LPS: lipopolysaccharide, TNF-α: tumor necrosis factor-α., PPAR: peroxisome proliferator-activated receptor

Running Title: PPAR γ agonist prevents alcohol-induced liver injury

Corresponding Author
Dr. Nobuhiro Sato
Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.
Phone: +81(3)5802-1058 FAX: +81(3)3813-8862 E-mail: nsato@med.juntendo.ac.jp

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Abstract

Background & Aims: Agonists of peroxisome proliferator-activated receptor (PPAR)-γ have been shown to reduce TNF-α-induced insulin resistance. On the other hand, sensitization of Kupffer cells to lipopolysaccharide (LPS) and their production of TNF-α is 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. **Methods:** Rats were given ethanol (5 g/kg BW) and pioglitazone (500 μg/kg) once every 24 hours intragastrically. **Results:** 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 $[Ca^{2+}]_i$ 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 3-4 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 CD 14 expression, LPS-induced $[Ca^{2+}]_i$ response, and production of TNF-α. **Conclusions:** 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 (Martinez et al., 1992; Stahnke et al., 1991). 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 (Shibayama et al., 1991; Wang et al., 1995; Nolan., 1981; Decker et al., 1989). Among them, TNF-α is likely a critical factor in the progression of alcoholic liver disease since it induces cell death due to apoptosis and necrosis, and stimulates generation of toxic superoxide anion from mitochondrial complex III in parenchymal cells (Shhulze- Osthoff et al., 1993), and expression of factors for neutrophil chemotaxis (IL-8/CINC, MIP, MIP-2) and intracellular adhesion molecule-1 (ICAM-1), leading to microcirculatory disturbance (McCuskey et al., 1993; Hijioka et al., 1991; Oshita et al., 1992). 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 over-production 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 (RXR) and functions as a transcriptional regulator of a wide 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 (Saltiel et al., 1996). The mechanisms of this action remain obscure, although it has been proposed that PPAR-γ ligands crosstalk with several points of signaling pathways evoked by TNF-α (Hofman et al., 1994; Murase et al., 1998; Jiang 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 body weight intragastrically) once every 24 hours (Enomoto et al., 1999). This model achieves inflammatory and necrotic changes in the liver only in 8 weeks, mimicking clinical alcohol 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 while evaluation of Kupffer cell sensitization to LPS was performed at 4 weeks (see below). Female Wistar rats weighing 200-250 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 percent of calories were shown elsewhere (Enomoto et al., 1999). Rats were given one single dose of ethanol (5 g/kg) between 10 to 12 AM via an 18-gauge oral biomedical device every 24 hours (Thurman et al., 1982; Wendell et al., 1979). Two groups of rats received an oral dose of pioglitazone (500 μg/kgBW i.g.) only or concurrently with ethanol.

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

All animals were given humane care in compliance with the institutional guidelines. Sera were stored at -20°C, and aspartate transaminase (AST) and alanine aminotransferase (ALT) were measured by standard enzymatic procedures (Bergmeyer., 1988).

Pathological Evaluation

Liver specimens were obtained from rats 24 hours after final ethanol on 8 weeks of daily single intragastric treatment with ethanol (5 g/kg) and 24 hours 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 Percoll (Pharmacia, Uppsala, Sweden) as described elsewhere with slight modifications (Pertoft et al.). Briefly, the liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution (HBSS) at 37°C for 5 min at a flow rate of 26 ml/min. Subsequently, perfusion was performed with HBSS containing 0.025% collagenase IV (Sigma Chemical Co., St. Louis, MO) 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 450x g for 10 min at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by centrifugation at 50x g for 3 min, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 1000x g 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 (GIBCO Laboratories Life

Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 10 mM HEPES and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin sulfate) at 37°C with 5% CO_2. Non-adherent cells were removed after 1 hour by replacing buffer, and cells were cultured for 24 hours prior to experiments.

Measurement of Intracellular Ca^{2+} ($[Ca^{2+}]_i$) Using a Fluorescence Microscope Imaging System

All of the experiments were performed after cells were incubated at 37ºC for 24 hrs. Fura-2/AM with pluronic F127 was dissolved in phosphate saline solution containing 1.0 mM Ca^{2+}, in which final concentrations of fura-2/AM and pluronic F127 were 4 mM and 0.05 %, respectively. After loading with fura-2/AM solution at 37ºC 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 (MAXYDT2, Mitsubishi, Tokyo, Japan) and fluorescence intensity of fura-2/AM was quantified. Wavelengths of 340nm and 380 nm for excitation and 520 nm for emission were used. $[Ca^{2+}]_i$ was determined by the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = Kd([Ro - Rmin]/[Rmax - Ro])B$$

Kd, the Ca^{2+} dissociation constant for fura-2, was confirmed as 224 nM. R represents fluorescence intensity at 340 nm excitation divided by that at 380 nm excitation (Ro, experimental data; Rmin, R in 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA) and 1mM ionomycin; Rmax is R in 10 mM Ca^{2+} and 1mM ionomycin). B is the ratio of fluorescence intensity at 380 nm in the absence

of Ca^{2+} versus a saturating concentration of Ca^{2+}. Because intracellular Ca^{2+} was calculated from the ratio R, the fading of fluorescence did not interfere with the results.

TNF-α Production by Kupffer Cells

Kupffer cells were seeded onto 24-well plates and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) , 10 mM HEPES and antibiotics at 37°C in the presence of 5% CO_2. Cells were incubated with fresh media containing LPS (100 ng/ml supplemented with 5% rat serum) for an additional 4 hours. In some experiments, cells were pre-incubated for 24 hours 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) and data were corrected for dilution.

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 mM HEPES, pH 7.6, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 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 Na_3VO_4 and 0.5 μg/ml leupeptin. Protein concentration was determined using the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Extracted protein was separated by 10% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked by Tris-buffered saline-Tween

20 (TBS-T) containing 5% skim milk and probed with a mouse anti-rat ED9 monoclonal antibody (Serotec, Oxford, U.K.) and a mouse anti rat TTP monoclonal antibody (Santa cruz Biotechnology, Santa Cruz, CA), followed by an HRP-conjugated secondary antibody as appropriate. Membranes were incubated with a chemiluminescence substrate (ECL reagent, Amersham Life Science, Buckinghamshire, U.K.) 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, 10mM 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 minutes, 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 seconds 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 antibody, 1:200 diluted polyclonal

rabbit anti-CD14 (M-305; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody used was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Samples were evaluated and their appearance was recorded on a Zeiss Axiophot 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.

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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 earlier, ethanol administration once every 24h for 8 weeks caused pronounced steatosis, necrosis and inflammation in the liver (Fig 1C,D). In contrast, these pathological parameters were diminished markedly by the concurrent treatment with pioglitazone (500 μ g/kg/day) (Fig. 1E). Furthermore, while mean value of ALT in the control, non-treated rats was 30 ± 6 IU/L, ALT increased 3-fold to 91 ± 7 IU/L in the 8-week ethanol group (Fig. 1F,G). It is notable that the increase in ALT was blocked almost completely by pioglitazone (41 ± 5 IU/L) (Fig. 1G). Similar results were obtained for AST values (Fig. 1F).

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 microgram/kg) 90 minutes and 6 hours post-administration (485 ± 62 mg/dl vs. 440 ± 23 mg/dl and 350 ± 56 mg/dl vs. 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 as compared

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to those of normal, untreated rats. Further, 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 hours later. LPS caused focal necrosis and neutrophil infiltration in liver from the control, non-treated 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,G).

In the 4-week ethanol group, LPS injection resulted in marked aggravation of these parameters with pronounced steatosis (Fig. 2 C,D). The histological changes were diminished by treatment with pioglitazone (500 μ g/kg/day) (Fig. 2E). As compared to the control group, ALT value 24 hours after LPS challenge was increased 3-fold to 1200 ± 375 IU/L in the 4-week ethanol group. This increase was completely 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,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,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 given ethanol for 4 weeks (Fig. 3 C,E). It was also noted that, after the peak increase, $[Ca^{2+}]_i$ started to decrease but remained elevated over 180 sec (Fig. 3 C). The increased $[Ca^{2+}]_i$ response was blocked completely by co-administration of pioglitazone with ethanol for 4 weeks (Fig. 3 D,E).

Kupffer cell sensitization to LPS was further confirmed by TNF-α production, which demonstrated a 2-fold elevation in the 4-week ethanol group as compared with the control (559 ± 71 vs. 1104 ± 110 pg/mL, $p<0.05$) (Fig. 4). As expected, this increase in TNF-α was blunted by about 70 % with pioglitazone. Kupffer cells obtained from the pioglitazone-only group produced TNF-α that did not differ from control (Fig. 4).

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, eicosanoides and radical species in Kupffer cells, we measured CD14 with Western blotting. Liver from control rats expressed 55kD 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

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enhance CD14 expression was markedly abrogated when pioglitazone was co-administered with ethanol for 4 weeks (lane 4).

Effects of Ethanol and Pioglitazone on Gut Permeability and Portal LPS levels

Since 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, two hours after the final ethanol treatment in the 4weeks 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 pg/ml vs. 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 obvious possibility that may explain the mechanism by which pioglitazone diminished liver injury due to chronic ethanol treatment. We therefore evaluated if 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 % ($p<0.05$ v.s. 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 PPARγ 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 hours 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 if 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 $[Ca^{2+}]_i$ (92 ± 10 nM) in response to 100 ng/ml LPS (Fig. 3 and Fig. 9A). In marked contrast, the LPS-induced $[Ca^{2+}]_i$ response was 2-3 fold greater in Kupffer cells pretreated for 24 hrs with 10 ng/ml TNF-α (Fig. 9 B). When pioglitazone was present

in the culture media, this LPS-induced enhancement of $[Ca^{2+}]_i$ response was almost completely abrogated.

Further, LPS-induced TNF-α production by isolated Kupffer cells was compared between groups cultured for 24 hours in the presence or absence of TNF-α (10 ng/ml). As expected, Kupffer cells cultured in the presence of TNF-α produced 30% more TNF-α in response to LPS (100 ng/ml) than control cells that were cultured in the absence of TNF-α (Fig. 9 C). The addition of pioglitazone in the culture media during the TNF-α stimulation resulted in a complte inhibition of the increase in TNF-α production by Kupffer cells.

Furthermore, pioglitazone increased the amount of tristetraprolin, a CCCH zinc finger protein known to destabilize TNF-α mRNA (Fig. 10).

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Discussion

A PPAR-γ Agonist Pioglitazone Prevents Alcohol-Induced Liver Injury through Supression of TNFα 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 alcohol liver disease (Martinez et al., 1992; Stahnke et al., 1991; 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 since 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. As Kupffer cells, the largest population of macrophage linage 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 since pioglitazone increased expression of tristetraprolin (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 drugs for treatemnt 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 againstor 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 et al., 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 appears to be of primary importance for the pioglitazone action in prevention of alcohol-induced liver damage, given the facts that pioglitazone treatment *in vivo* abrogated ethanol-induced liver

injury. Furthermore, since 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 a efficient way. It is thus postulated that the initial inhibition of TNF-α production, although not complete, culminate 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 to control (Fig. 3). As we reported earlier, 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. 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.

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Alternatively, it appears likely that TNFα itself regulates Kupffer cells senseitization since TNFα upregulated expression of CD 14 protein in Kupffer cells (Fig. 8) and the intracellular calcium response to LPS. Pioglitazone blocked completely the TNFα–induced CD14 upregulation in Kupffer cells as well as the increased intracellular calcium response in 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. Those 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. Further, because non-alcoholic steatohepatitis (NASH) is characterized by an ongoing inflammation associated with overexpression of proinflammatory cytokines such as TNF-α from Kupffer cells (Neuschwander-Tetri BA., et al. 2003), it seems most likely that pioglitazone is also effective in the treatment of NASH.

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Figure Legends

FIGURE 1. Effect of pioglitazone on ethanol-induced liver injury

Photomicrographs of H&E sections of livers from rats treated as described in Materials and Methods: (A) no treatment, (B) 8 weeks pioglitazone (500 μg/kg i.g., once daily), (C) 8 weeks ethanol (5 g/kg) (original magnification, 100 x), (D) 8 weeks ethanol (400 x) and (E) 8 weeks ethanol + pioglitazone (100 x). Typical photomicrographs. Blood samples were collected from the aorta, and AST (F) and ALT (G) were determined as described in Materials and Methods. Results are mean ± SEM (n = 4-8). * , $p<0.05$ vs. control. #, $p<0.05$ vs. 8 weeks of ethanol by ANOVA and Bonferroni's post hoc test.

FIGURE 2. Effect of pioglitazone on ethanol plus LPS-induced liver injury

Photomicrographs of H&E sections of livers from rats treated as described in Materials and Methods. LPS was given at 24 hours after ethanol and rats were sacrificed 24 hours later (48 hours after ethanol), (A) 24 hours after LPS (5 mg/kg i.v.), (B) 4 weeks of daily intragastric pioglitazone (500 μg/kg i.g., once daily) exposure and LPS for 24 hours, (C) 4 weeks of daily intragastric ethanol (5 g/kg) exposure and LPS for 24 hours (original magnification, 100 x), (D) 4 weeks of ethanol and LPS (400 x) and (E) 4 weeks of ethanol + pioglitazone exposure and LPS for 24 hours. (100 x). Typical photomicrographs. (F,G) Blood samples were collected 24 hours after LPS. Results are mean ± SEM for 4 rats per group. *, $p<0.05$ vs. LPS. #, $p<0.05$ vs. 4 weeks of ethanol + LPS by ANOVA and Bonferroni's post-hoc test.

FIGURE 3. Effect of ethanol and pioglitazone on LPS-induced increases in intracellular Ca^{2+} in isolated Kupffer cells.

Isolated Kupffer cells were cultured in 35 mm culture dishes at a density of 5 x 10^5 cells/dish for $[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ was measured using a microspectrometer with the fluorescent indicator, fura-2. Changes in $[Ca^{2+}]_i$ after addition of 100 ng/ml LPS, supplemented with 5% rat serum, are plotted. LPS was added to Kupffer cells from control rats (A,E), to Kupffer cells from rats treated with pioglitazone for 4 weeks prior to isolation (B,E), to Kupffer cells from rats treated with ethanol for 4 weeks prior to isolation (C,E), to Kupffer cells from rats treated with ethanol and pioglitazone for 4 weeks prior to isolation (D,E). Data are representative traces of experiments repeated 4 times (A-D). (E) Results are mean ± SEM, n = 4; *, $p<0.05$ vs. control, #, $p<0.05$ vs. 4 weeks of ethanol by ANOVA and Bonferroni's post-hoc test.

FIGURE 4. Effect of ethanol and pioglitazone on LPS-induced TNF-α production by cultured Kupffer cells.

Isolated Kupffer cells were cultured in 24 well plates at a density of 5 x 10^5 cells/well for TNF-α determination. After 24 hours of preincubation, LPS (final concentration, 100 ng/ml in 5% rat serum) was added, and incubation was continued for 4 hours. TNF-α in media was measured by ELISA. Basal TNF-α release after 4 hours of incubation without LPS was 10 ± 2 pg/ml. Results are mean ± SEM, n = 4; *, $p<0.05$ vs. control, #, $p<0.05$ vs. 4 weeks of ethanol by ANOVA and Bonferroni's post-hoc test.

FIGURE 5. Effect of ethanol and pioglitazone on CD14 expression in liver.

Protein extracts from control liver or livers from rats treated for 4weeks with ethanol alone or with pioglitazone were analyzed by western blotting using an anti-CD14 antibody. Specific bands for CD14 (55kD) are shown. Lane 1; livers from control rats, lane 2; livers from 4weeks of pioglitazone, lane 3; livers from 4weeks of ethanol, lane 4; livers from 4weeks of ethanol with pioglitazone. Data are representative of three individual experiments.

FIGURE 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 horseradish peroxidase (HRP) was detected. Values are mean ± SEM for 4 rats per group. *, $p<0.05$ vs. control by ANOVA and Bonferroni's pot-hoc test.

FIGURE 7. Effect of pioglitazone on LPS-induced TNF-α production in cultured Kupffer cells.

Isolated Kupffer cells were cultured in 24 well plates at a density of 5×10^5 cells/well for TNF-α determination. After 24 hours of preincubation, LPS (final concentration, 100 ng/ml in 5% rat serum) was added, and incubation was continued for 4 hours. TNF-α in media was measured by ELISA. Basal TNF-α release after 4 hours of incubation without LPS was 10 ± 2 pg/ml. Results are mean ± SEM, n = 4; *, $p<0.05$ vs. control, #, $p<0.05$ vs. LPS by ANOVA and Bonferroni's post-hoc test.

FIGURE 8. Effect of TNF-α and pioglitazone on expression of CD14 in cultured Kupffer cells.

Fluorescence micrographs. (A) CD14 staing in control Kupffer cell, (B) CD14 and actin staing in control Kupffer cell, (C) CD14 staing in Kupffer cell treated for 24 hours with TNF-α (10 ng/ml), (D) CD14 and actin staing in Kupffer cell treated for 24 hours with TNF-α. (E) CD14 staing in Kupffer cell treated for 24 hours with TNF-α (10 ng/ml) + pioglitazone, (F) CD14 and actin staing in Kupffer cell treated for 24 hours with TNF-α + pioglitazone. Actin staining was performed to identify the cell contour. Data are representative of 3 individual experiments. Original magnification x 400.

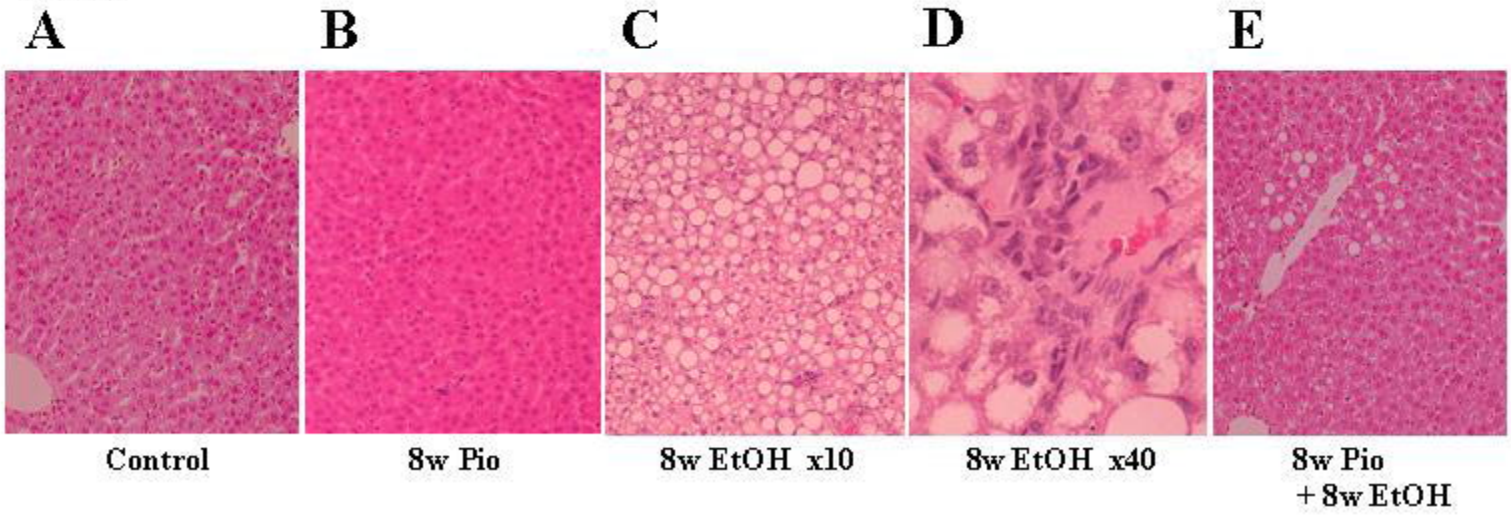
FIGURE 9. Effect of TNF-α on LPS-induced increase in $[Ca^{2+}]_i$ and TNF-α production by cultured Kupffer cells.

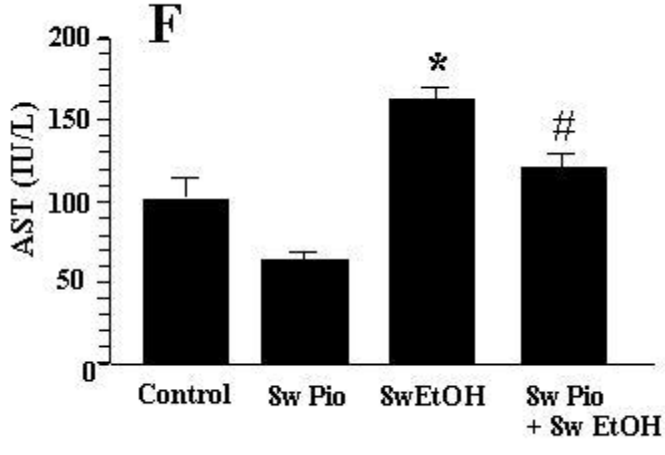
Kupffer cells were isolated and cultured for 24 hours with and without TNF-α (10 ng/ml). Subsequently, cells were washed vigorously with PBS and challenged for 24 hours with LPS (100 ng/ml). (A,B) LPS-induced $[Ca^{2+}]_i$ response was measured as described in materials and methods. (C) TNF-α in media was measured by ELISA. TNF-α release after 4 hours of incubation without LPS was 10 ± 2 pg/ml. Results are mean ± SEM, n = 4; *, $p<0.05$ vs. control (TNF-α(-)), by ANOVA and Bonferroni's post-hoc test.

FIGURE 10. Effect of TNF-α and pioglitazone on TTP by cultured Kupffer cells.

Protein extracts from control Kupffer cell, pioglitazone treated Kupffer cell, TNF-α treated Kupffer cell and pioglitazone plus TNF-α treated Kupffer cell were analyzed by western blotting using an anti-rat TTP antibody. Specific bands for TTP are shown. Lane 1; control Kupffer cells, lane 2; Kupffer cells treated with pioglitazone, lane 3; Kupffer cells treated with TNF-α, lane 4; Kupffer cells treated with pioglitazone and TNF-α. Data are representative of three individual experiments.

Figure 1


A
B
C
D
E
Control
8w Pio
8w EtOH x10
8w EtOH x40
8w Pio
+ 8w EtOH


F
AST (IU/L)
200
150
100
50
0
*
#
Control
8w Pio
8wEtOH
8w Pio
+ 8w EtOH

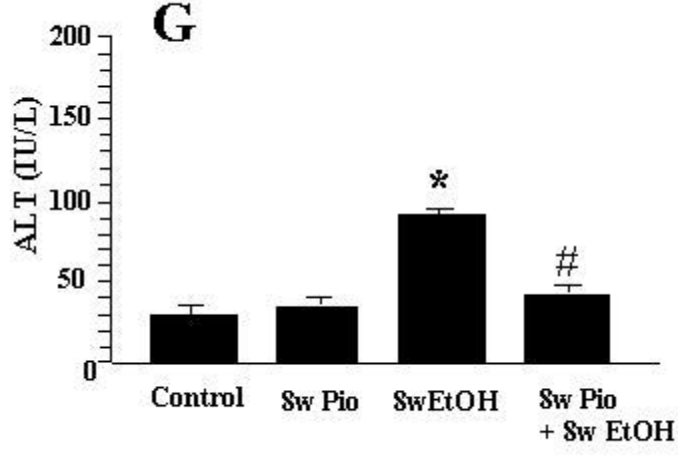
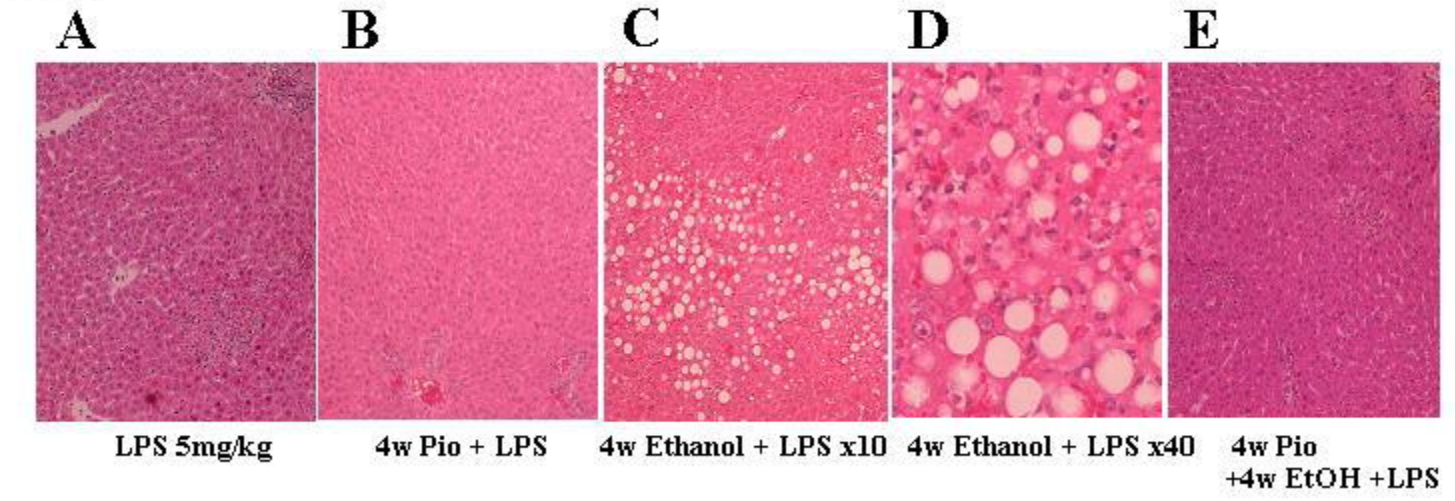
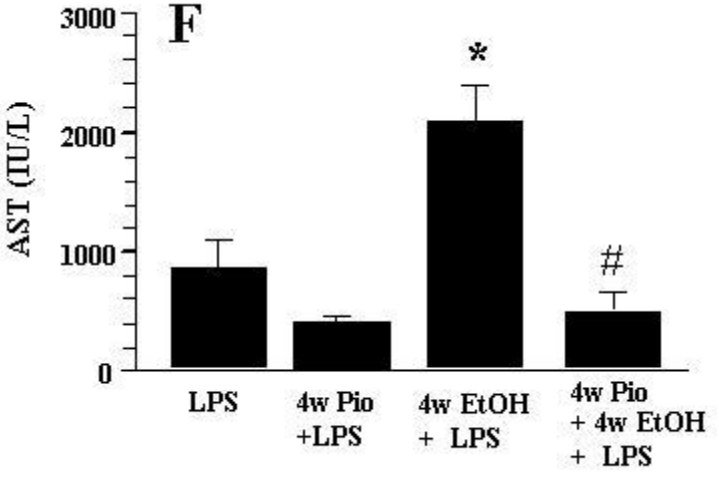

G
ALT (IU/L)
200
150
100
50
0
*
#
Control
8w Pio
8wEtOH
8w Pio
+ 8w EtOH

Figure 2


A
B
C
D
E
LPS 5mg/kg
4w Pio + LPS
4w Ethanol + LPS x10
4w Ethanol + LPS x40
4w Pio
+4w EtOH +LPS


F
AST (IU/L)
3000
2000
1000
0
*
#
LPS
4w Pio
+LPS
4w EtOH
+ LPS
4w Pio
+ 4w EtOH
+ LPS

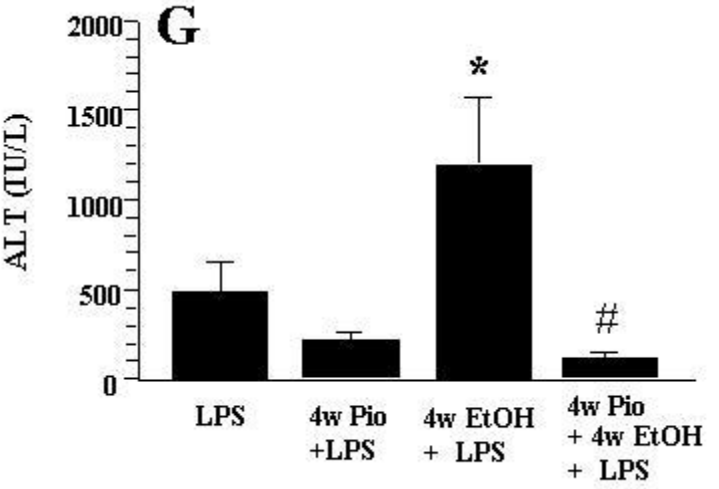

G
ALT (IU/L)
2000
1500
1000
500
0
*
#
LPS
4w Pio
+LPS
4w EtOH
+ LPS
4w Pio
+ 4w EtOH
+ LPS

Figure 3

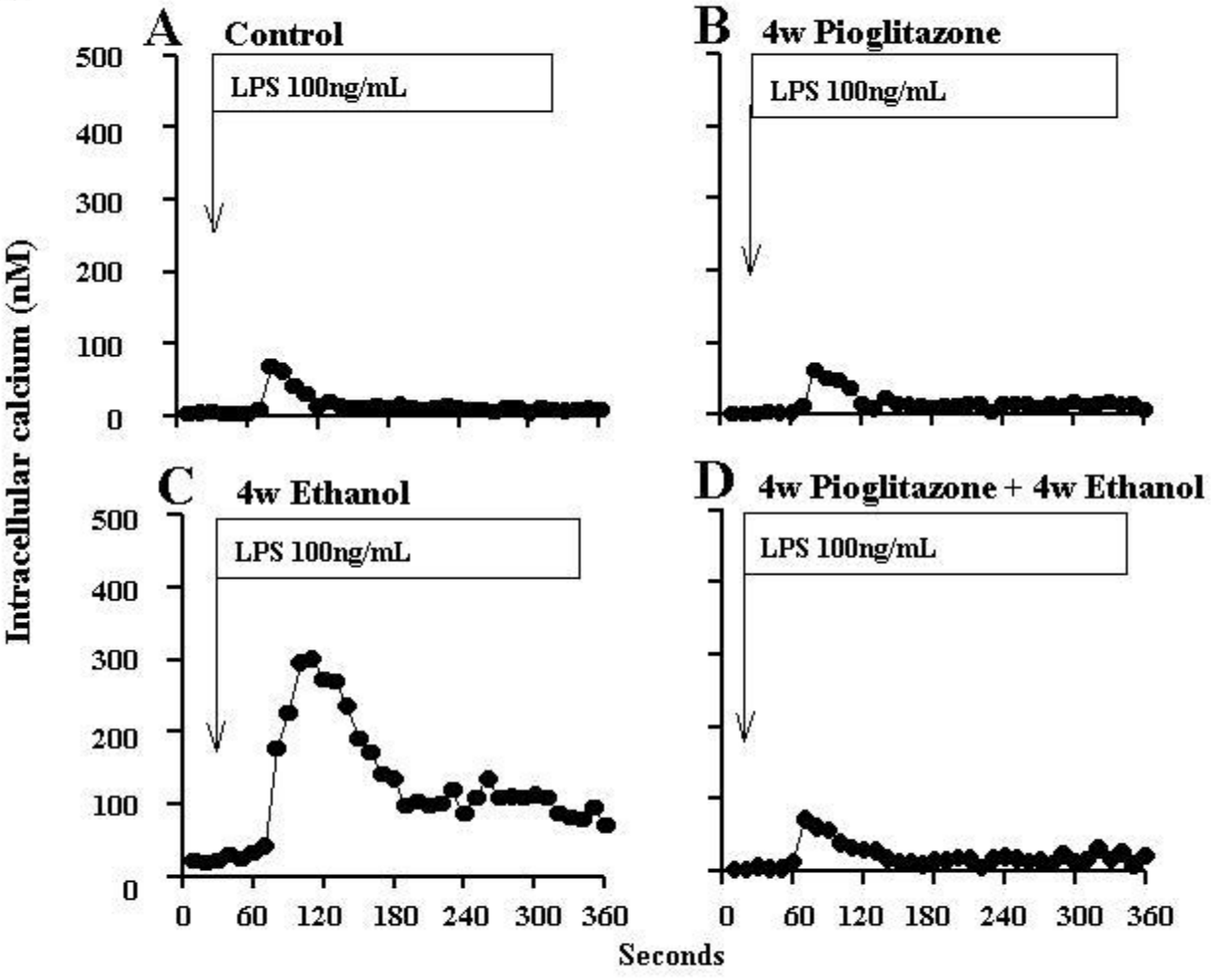

A
Control
LPS 100ng/mL
B
4w Pioglitazone
LPS 100ng/mL
C
4w Ethanol
LPS 100ng/mL
D
4w Pioglitazone + 4w Ethanol
LPS 100ng/mL
Intracellular calcium (nM)
0
100
200
300
400
500
0
60
120
180
240
300
360
Seconds

Figure 3

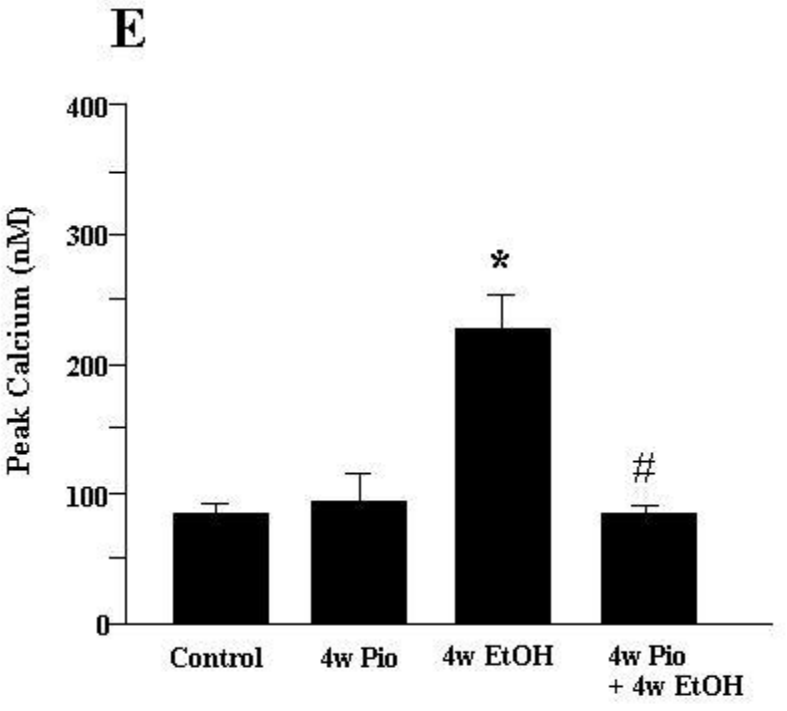

E
Peak Calcium (nM)
400
300
200
100
0
*
#
Control
4w Pio
4w EtOH
4w Pio
+ 4w EtOH

Figure 4

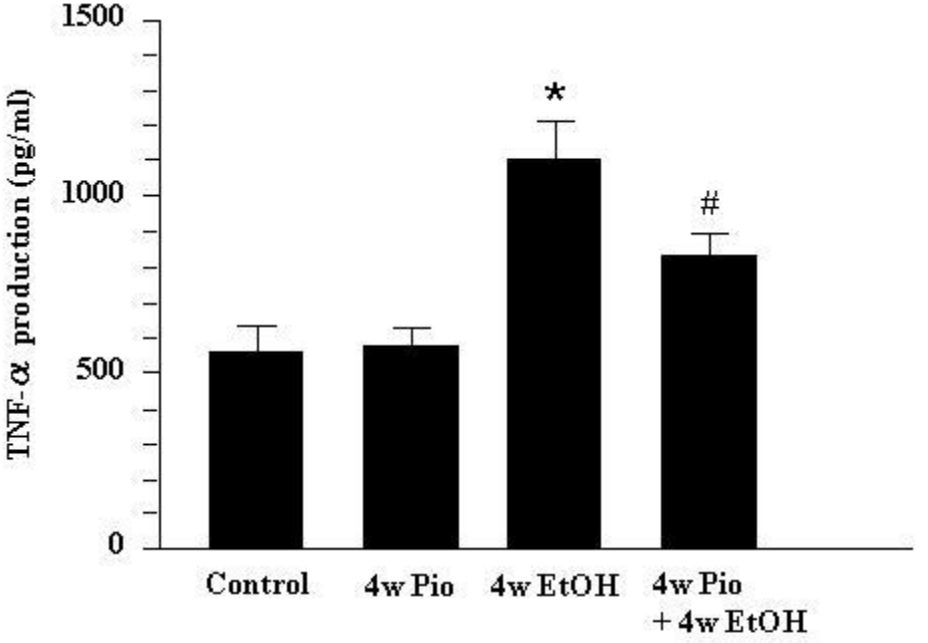

1500
1000
500
0
TNF-α production (pg/ml)
*
#
Control
4w Pio
4w EtOH
4w Pio
+ 4w EtOH

Figure 5

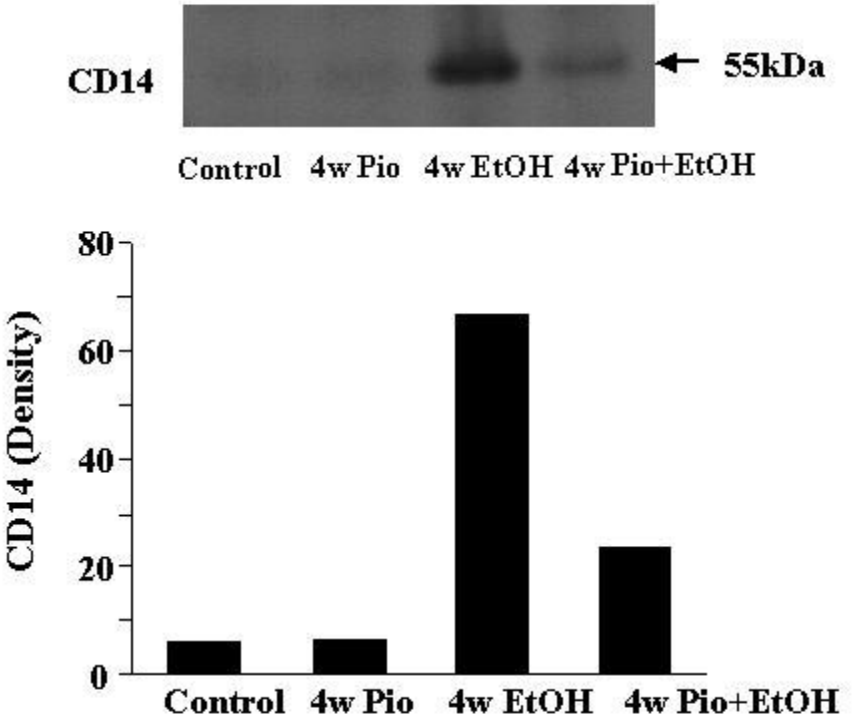

CD14
55kDa
Control
4w Pio
4w EtOH
4w Pio+EtOH
CD14 (Density)
80
60
40
20
0
Control
4w Pio
4w EtOH
4w Pio+EtOH

Figure 6

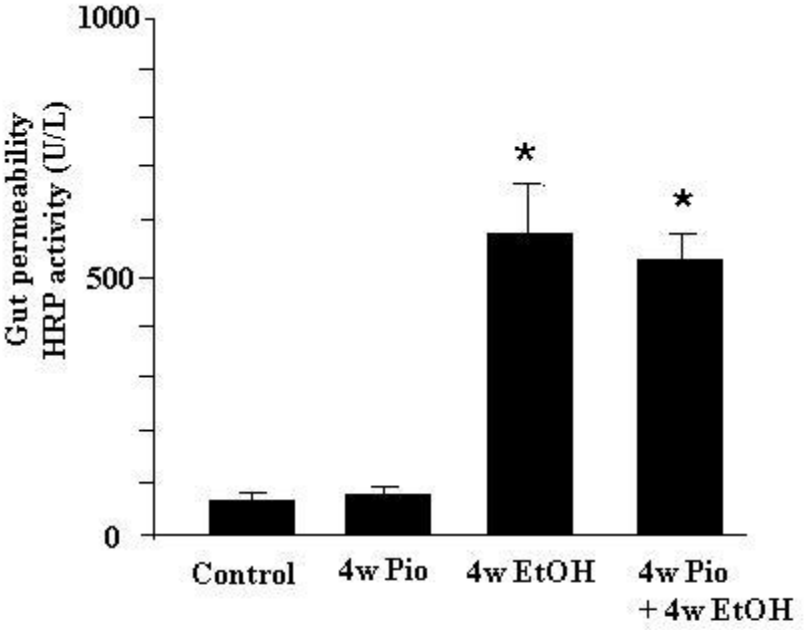

1000
500
0
Gut permeability
HRP activity (U/L)
*
*
Control
4w Pio
4w EtOH
4w Pio
+ 4w EtOH

Figure 7

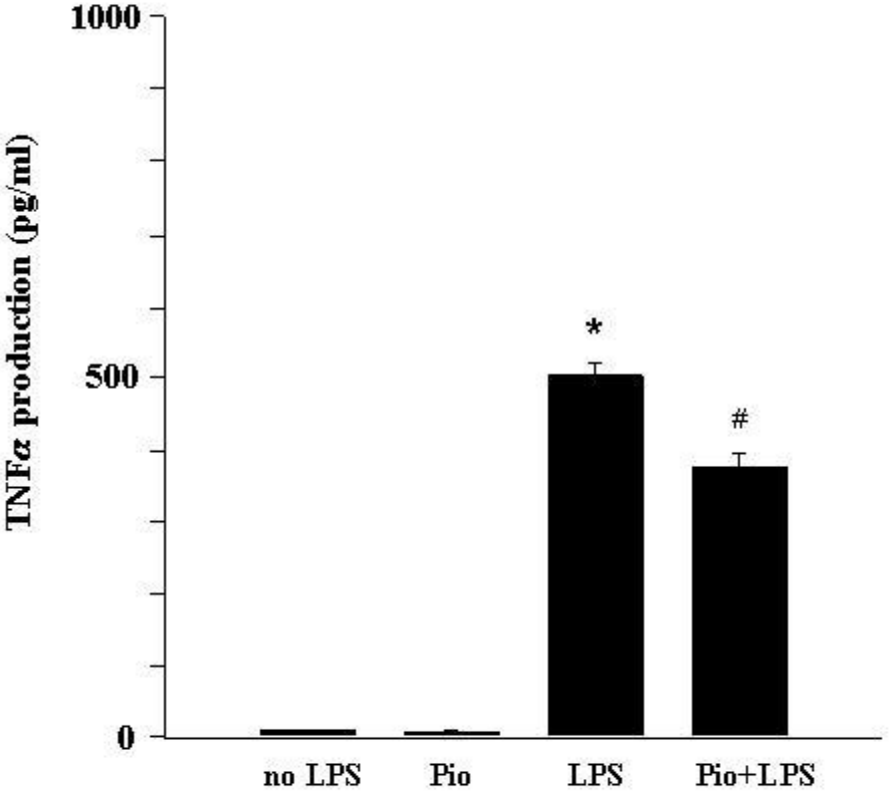


Figure 8

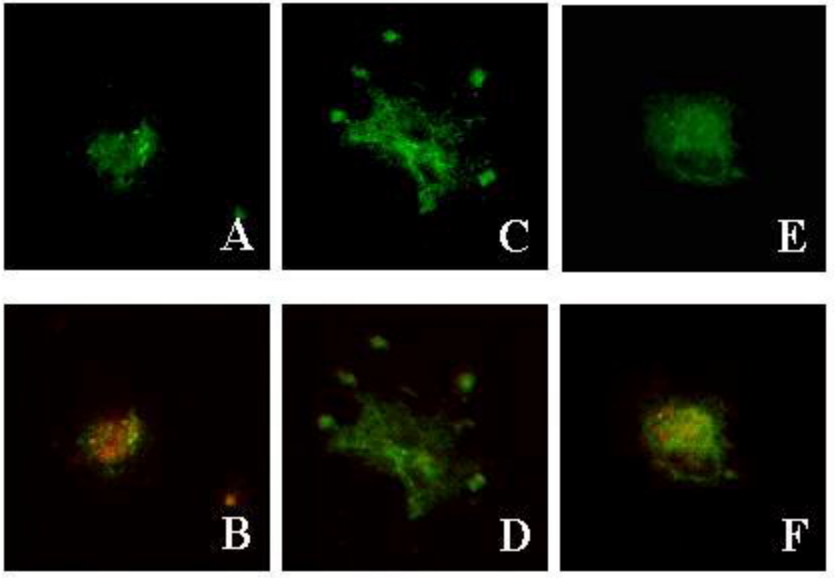


Figure 9

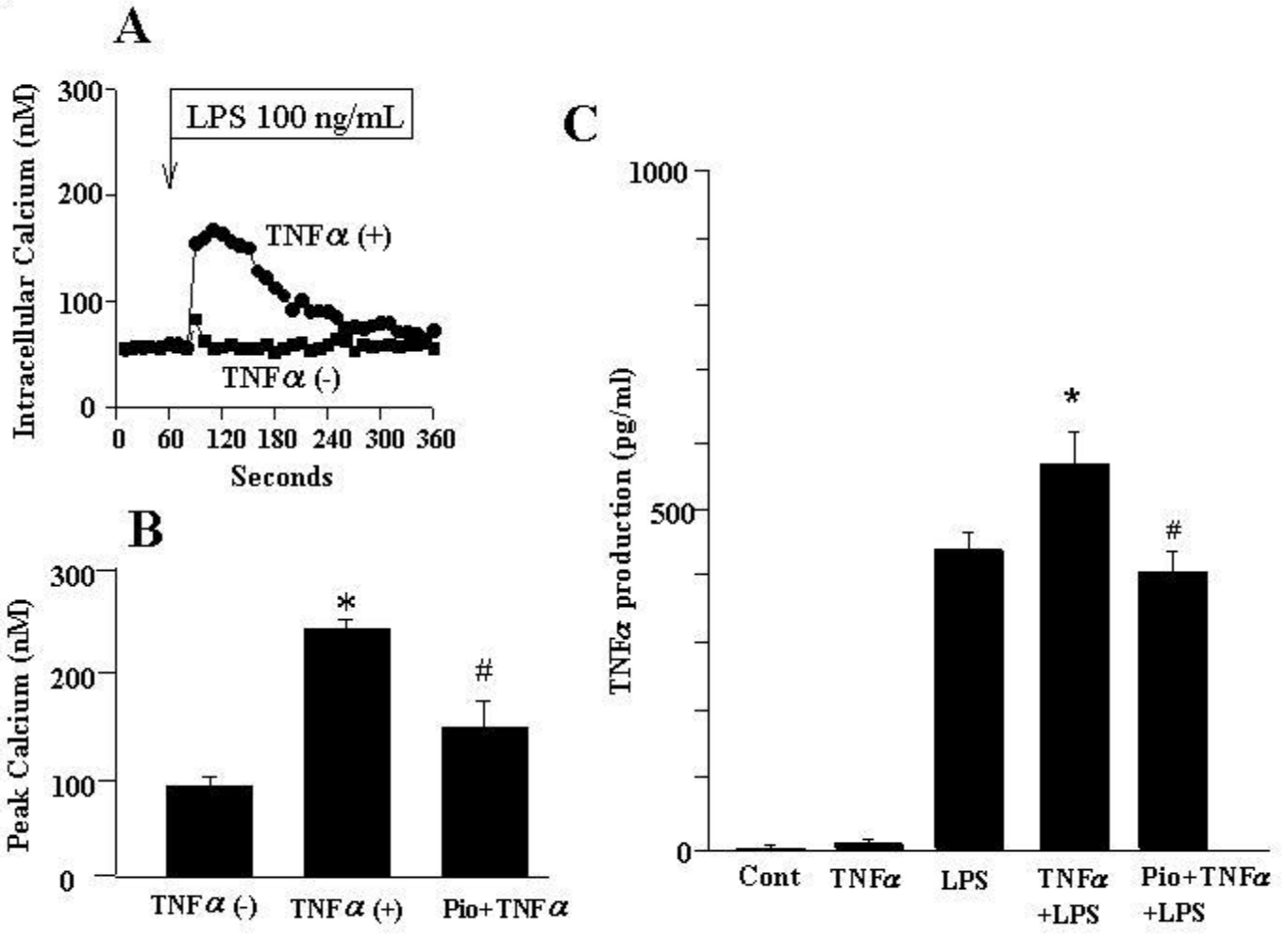
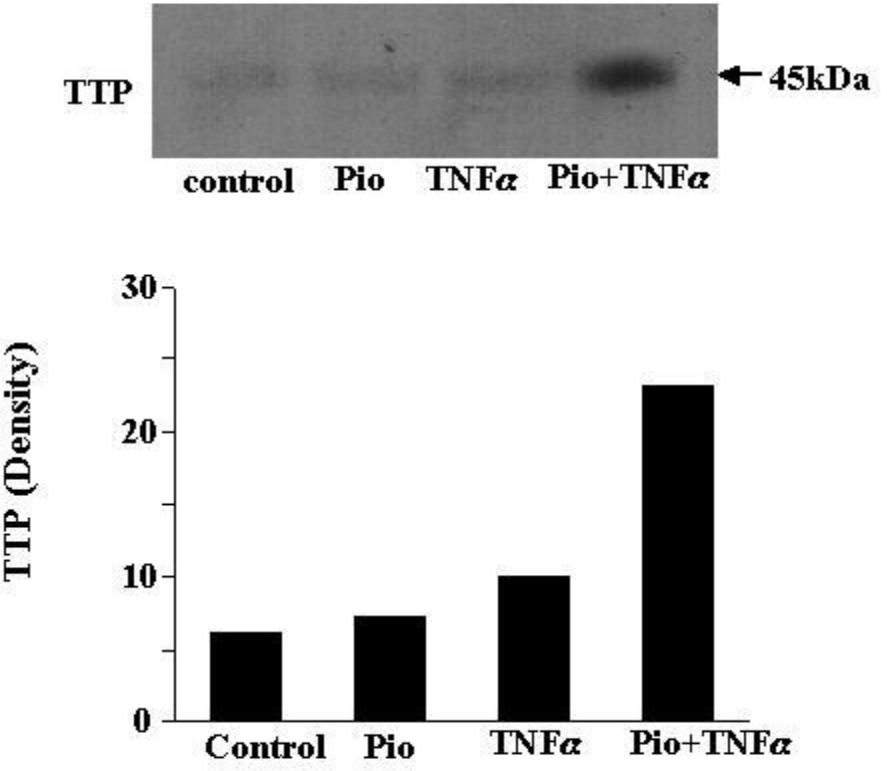

A
LPS 100 ng/mL
TNFα (+)
TNFα (-)
Intracellular Calcium (nM)
300
200
100
0
0 60 120 180 240 300 360
Seconds
B
Peak Calcium (nM)
300
200
100
0
*
#
TNFα (-)
TNFα (+)
Pio+TNFα
C
TNFα production (pg/ml)
1000
500
0
*
#
Cont
TNFα
LPS
TNFα +LPS
Pio+TNFα +LPS

Figure 10


TTP
45kDa
control
Pio
TNFα
Pio+TNFα
TTP (Density)
30
20
10
0
Control
Pio
TNFα
Pio+TNFα