EXPERIMENTAL

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Pioglitazone reduces systematic inflammation and improves mortality in apolipoprotein E knockout mice with sepsis

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Abstract *Objective:* To determine
whether peroxisome proliferator-
activated receptor (PPAR) γ ligands
improve survival of patients with
septic shock we treated a mouse
model of sepsis [apolipoprotein (Apo)
E) knockout mice] with pioglitazone,
a PPAR-γ ligand. ApoE knockout
mice have a high mortality rate due
to sepsis because the endotoxin is
not cleared. *Design and setting:*
Prospective study in a university
laboratory. *Subjects:* We assorted
87 male ApoE knockout mice and 60
wild-type C57/B6 mice randomly into
three groups (sepsis, pretreatment,
posttreatment). *Interventions:* Cecal
ligation and puncture (CLP) was
carried out in the sepsis and treatment
groups. Mice were injected with
pioglitazone (5 mg/kg per day) on the
day before CLP or 6 h after surgery.
Measurements and results: Both pre-
and post-CLP treatment with piogli-
tazone improved survival of ApoE
knockout and wild-type mice. Serum
levels of cytokines and chemokines
and myeloperoxidase activity in lung
and liver were suppressed in the
pioglitazone-treated group. Piogli-
tazone also suppressed monocyte
adhesion to vascular endothelium
under flow conditions. *Conclusions:*
Pioglitazone improved survival of
ApoE knockout mice after onset of
septic shock through suppression of
inflammatory responses.

Keywords Peroxisome proliferator-
activated receptor γ · Sepsis · Inflam-
mation

Introduction

Severe sepsis and septic shock are the second com-
mon causes of death in the intensive care unit. Despite
significant advances in intensive care therapy and an-
tibiotics the mortality rate due to severe sepsis has not
decreased significantly over the past 10 years [1]. In
fact, the number of patients suffering from severe sepsis
has increased. Sepsis is a complex clinical syndrome
that results from a damaging host response to microbial
infection due to dysregulation of the innate immune
system. Sepsis is characterized by excessive production
of cytokines such as tumor necrosis factor (TNF) α
and interleukin (IL) 6. Many anti-inflammatory drugs
have been tested for the treatment of severe sepsis.
Some have improved the survival rate of animal models
when administered after onset of sepsis. These drugs
include antagonists of high-mobility group box 1 [2],
statins [3], soluble Flt-1 [4], adrenomedullin [5], and
β-glucan [6].

It was recently reported that lipid metabolism has a strong relationship to the progression of severe sepsis [7, 8]. Some lipoproteins have been shown to inhibit inflammation in vivo and in vitro [9]. Moreover, an apolipoprotein (Apo) E polymorphism also has a close association with the risk and mortality of severe sepsis [10]. ApoE is a ligand for the low-density lipoprotein (LDL) receptor and plays a significant role in lipid metabolism, and ApoE knockout (ApoE$^{-/-}$) mice show hyperlipidemia. It was reported recently that ApoE$^{-/-}$ mice are highly susceptible to endotoxinemia [11]. This can be explained by the fact that under normal circumstances ApoE binds and neutralizes endotoxins. Interestingly, administration of ApoE to ApoE$^{-/-}$ mice suppresses inflammation and improves the rate of survival of mice with sepsis [12]. On the basis of these facts we hypothesized that ApoE$^{-/-}$ mice could be a model system for high mortality in response to sepsis.

Peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear receptor superfamily and is expressed in various cells, including monocyte-macrophages, T cells, endothelial cells (ECs), and other cells involved in the progression of sepsis. PPAR-γ ligands have anti-inflammatory effects and improve outcomes in various situations such as transplantation-associated arteriosclerosis [13], rheumatoid arthritis [14], allergic reaction [15], and inflammatory bowel disease [16]. Recent studies have shown that administration of ciglitazone, a thiazolidinedione and PPAR-γ ligand, 1 h after cecal ligation and puncture (CLP) suppresses inflammation and improves survival in a rat model of sepsis [17]. The statins improve survival rate through their pleiotropic effects, such as inhibition of monocytes–endothelial cell interaction and anti-inflammatory activity.

There are reports that the PPAR ligands have the same anti-inflammatory effects as statins. Therefore in the present study we investigated whether pioglitazone, a member of the thiazolidinediones and PPAR-γ ligand, improves the survival of septic ApoE$^{-/-}$ and wild-type (WT) mice 6 h after CLP.

Methods

Animals

The study used 10- to 12-week-old male C57BL/6 mice from Japan Clea and male C57BL/6 ApoE$^{-/-}$ mice from Prof. S. Ishibashi (Jichi Medical School, Tochigi, Japan). Animals were maintained at least 1 week in our animal facility and weighed 25–35 g. The present study conformed to the Guide for the Care and Use of Laboratory Animals of Tokyo Medical and Dental University.

CLP model

Mice were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) given intraperitoneally. The cecum was exposed through an abdominal incision and subjected to ligation of the distal half followed by two punctures with a 21-gauge needle. A small amount of stool was expelled into the peritoneal cavity, and the incision was closed. After the surgery 0.5 ml 0.9% NaCl was administered by subcutaneous injection twice each day for volume restoration. Pioglitazone (Takeda Pharmaceutical, Tokyo, Japan) was dissolved with 0.5% methylcellulose and administered by intraperitoneal injection. One group of mice ($n = 20$) received pioglitazone 18 h and 6 h before surgery and received twice injections each day (total 5 mg/kg per day) until 7 days after CLP; this group was called the Pre-Pio group. The other group of mice ($n = 20$) received pioglitazone 6 h after surgery and received injections twice each day (total 5 mg/kg per day); this group was called the Post-Pio group. Control group mice ($n = 20$) received 0.5% methylcellulose only. The daily dose of 5 mg/kg pioglitazone used in the present study is parallel to the dose used for therapy in humans and used in many studies [13, 18, 19]. Six mice in each CLP (vehicle, preadministration, and postadministration) and sham-operated (vehicle, preadministration, and postadministration) group were used in the following experiments. Three mice in each CLP and sham-operated group were used for histopathology. In total, 87 ApoE$^{-/-}$ mice and 60 C57BL/6 mice were subjected to CLP, and 27 ApoE$^{-/-}$ mice were used as sham controls.

Hemodynamic measurements

Blood pressure (BP) was measured in six mice each group by a noninvasive method (BP-98A, Softron, Tokyo, Japan). Mice were placed in a warm, dark sack, and BP was measured in the tail as described previously [20].

Measurement of cytokines and chemokines by enzyme-linked immunosorbent assay

The blood samples obtained and heparinized 18 h after CLP were centrifuged at 5,000 rpm for 5 min to separate the plasma. Serum IL-6, TNF-α, IL-10, and monocyte chemoattractant protein (MCP) 1 levels in ApoE$^{-/-}$ mice ($n = 6$, each group) 18 h after CLP were measured with an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions. The levels exceeded the upper measurement limits of the kits, and therefore we diluted each serum 100-fold and repeated the assay.

Measurement of liver function

Blood aspartate transaminase (AST) and alanine transaminase (ALT) levels were determined by MDH-UV method and Hitachi 7350 (Hitachi, Tokyo, Japan) in the same serum samples used for ELISA.

Measurement of myeloperoxidase activity in lung and liver

Myeloperoxidase (MPO) activity in ApoE$^{-/-}$ mice ($n = 6$) 18 h after surgery was determined as described previously [21]. Briefly, tissues were weighed and washed with 0.9% NaCl immediately after excision. Tissues were homogenized in a potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA). Homogenates were reacted with a 50-mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine hydrochloride (Sigma) and 0.0005% hydrogen peroxide (Wako, Osaka, Japan). The change in absorbance at 460 nm was measured with a microplate reader (Ultramark, Bio-Rad, Hercules, CA, USA), and the results are expressed as units of MPO/100 mg tissues. One unit of MPO was defined as that degrades 1 μmol peroxidase/min at 25°.

## Histology and immunohistochemistry

Lungs and livers from ApoE$^{-/-}$ mice were dissected 18 h after surgery. Lung tissues were inflated with formalin at a pressure of 20 cmH$_2$O. Tissues were fixed in formalin for 3 days and then embedded in paraffin. Tissues were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin. Liver tissues were frozen in liquid nitrogen. Frozen sections (5 μm) were fixed in acetone for 10 min at 4°. Sections were washed with phosphate-buffered saline and then incubated with anti-CD11b rat antibody (1:400, BD Biosciences, NJ, USA) overnight at 4°. Sections were then incubated with biotinylated secondary antibody for 30 min at room temperature. Immunocompleted slides were visualized with avidin-biotin-horseradish peroxidase complex (Nichirei, Tokyo, Japan) according to the manufacturer's protocol and then counterstained with hematoxylin.

Monocyte adhesion assay

The monocyte/macrophage cell line THP-1 was obtained from Riken Bioresource Center (Tsukuba, Ibaragi, Japan) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies Oriental, Tokyo, Japan). Human umbilical vein ECs (HUVECs) were isolated from normal-term umbilical cords and cultured as described previously [22]. The protocols for the adhesion assay under static flow and flow conditions were described previously [22]. Briefly, for the flow assays HUVEC monolayers were stimulated with 10 ng/ml TNF-α (Genzyme, Boston, MA, USA) for 4 h on coverslips and then positioned in a flow chamber mounted on an inverted microscope (IX70, Olympus, Tokyo, Japan). THP-1 cells (1×10^6/ml) were preincubated with pioglitazone (2 and 20 μM) for 12 h and drawn through the chamber at a wall shear stress of 1.0 dyne/cm^2 for 10 min. Those dose of pi-

Fig. 1 Pioglitazone (*Pio*) administration improves survival of both ApoE$^{-/-}$ (*ApoE KO*) and wild-type (*WT*) mice: survival curves. WT mice: daily preadministration of 5 mg/kg pioglitazone (*filled squares*), daily postadministration of 5 mg/kg pioglitazone mice (*filled triangles*), and control (*filled diamonds*); * $p < 0.05$ vs. control; $n = 20$ in each group. ApoE$^{-/-}$ mice: daily preadministration of 5 mg/kg pioglitazone (*open circles*), daily postadministration of 5 mg/kg pioglitazone mice (*filled circles*), and control (×); # $p < 0.05$ vs. control; $n = 20$ in each group
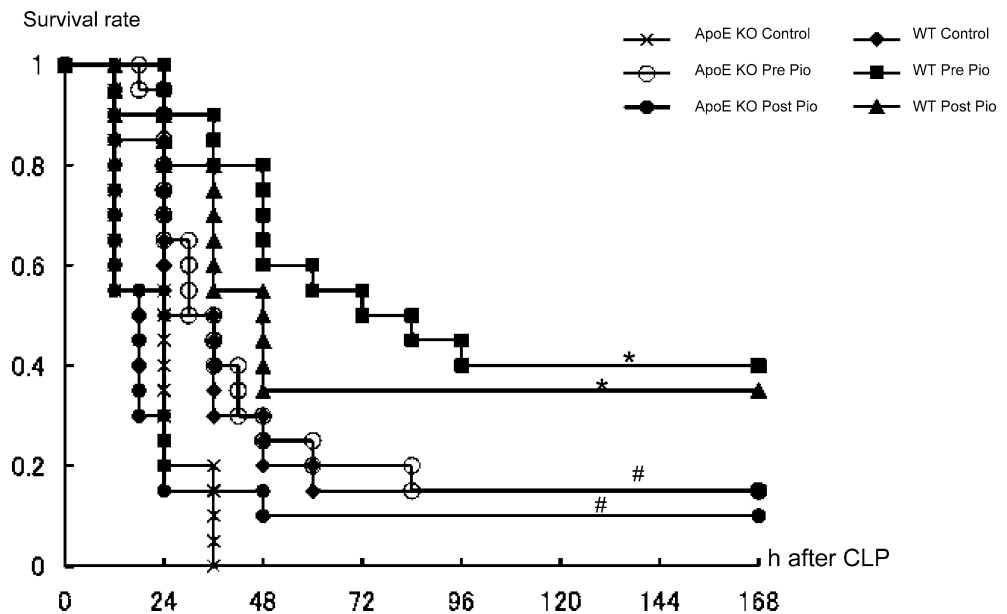
oglitazone were used previously in many experiments [13, 23]. The entire perfusion period was recorded on video. The number of rolling and adherent THP-1 cells present on HUVEC monolayers was counted in ten randomly selected 20 × microscope fields.

Statistical analysis

All data are expressed as mean ± SEM. Kaplan–Meier analysis was used to evaluate mice survival, and the Mann–Whitney U test was used to examine differences in survival between two groups. Analysis of variance with Dunnett's post-hoc test for comparisons between groups ELISA and MPO assay results. Differences at $p < 0.05$ were considered statistically significant. All statistical analyses were performed with JMP 5.1 software (SAS, Cary, NC, USA).

Results

Pioglitazone improved survival and BP in an experimental model of sepsis

After undergoing CLP the mice displayed signs of severe sepsis, including reduced mobility, apathy, and falling BP. Both ApoE$^{-/-}$ and WT mice treated with pioglitazone ei-
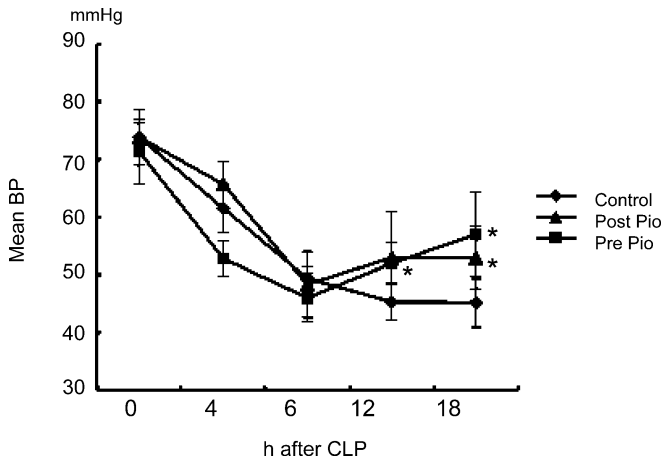
Fig. 2 Pioglitazone (*Pio*) restores mean BP in CLP ApoE$^{-/-}$ mice. Mean BP progression in mice treated with 5 mg/kg pioglitazone per day for 1 day before CLP (*filled squares*), for 6 h after CLP (*filled triangles*), and injected with control vehicle alone (*filled diamonds*); * $p < 0.05$ vs. control; $n = 6$, each group

Fig. 3 Pioglitazone (*Pio*) treatment significantly decreases plasma cytokine levels in ApoE$^{-/-}$ *mice*. Plasma levels of TNF-α, IL-6, IL-10, and MCP-1 18 h after CLP. Levels of all cytokines in mice treated with 5 mg/kg pioglitazone per day 1 day before CLP were reduced significantly. TNF-α, IL-6, and MCP-1 levels were reduced significantly in mice that received postsurgical pioglitazone; ** $p < 0.01$, * $p < 0.05$ vs. control; $n = 6$, each group
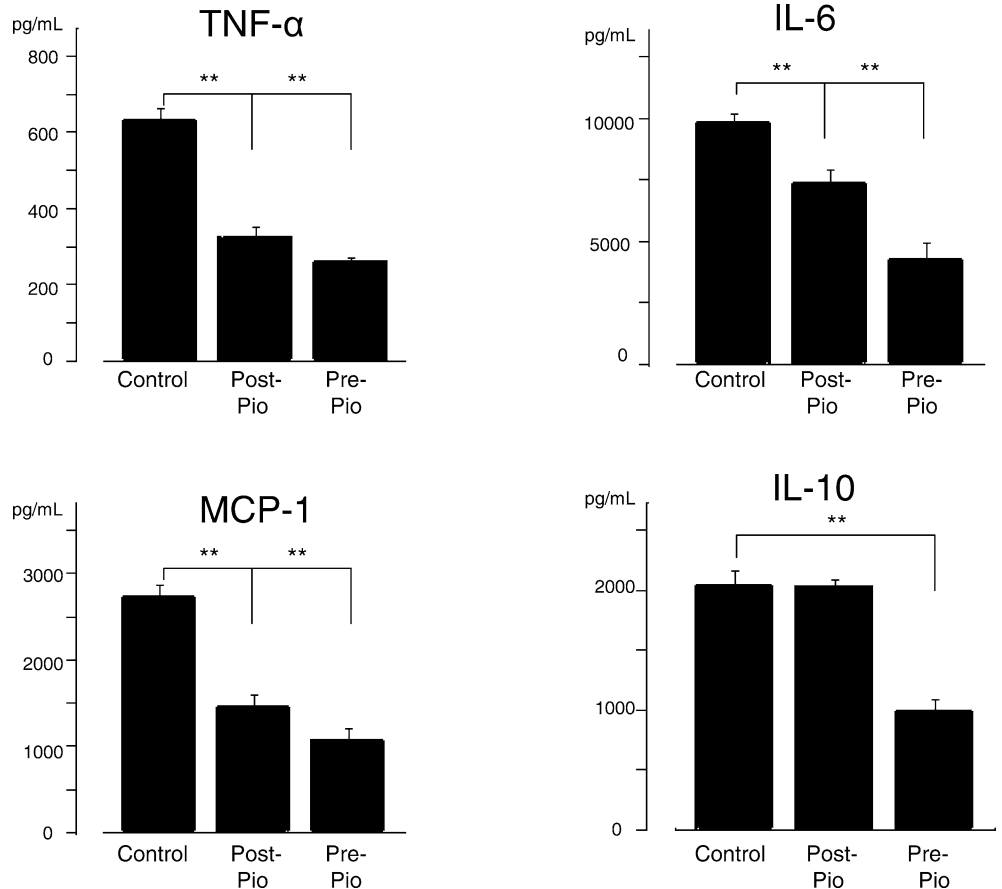
Fig. 4 a MPO activity 18 h after CLP. MPO activity in both lung and liver of mice treated with pioglitazone (*Pio*) before CLP was reduced significantly. MPO activity in mice treated with pioglitazone after CLP was also reduced; ** $p < 0.01$, * $p < 0.05$ vs. control; $n = 6$, each group. **b** Serum AST and ALT levels of mice treated with pioglitazone were also reduced in mice treated with pioglitazone; ** $p < 0.01$, * $p < 0.05$ vs. control; $n = 6$, each group. **c** Representative immuno-histochemical staining of CD11b in liver 18 h after CLP. Invasion by CD11b^{+} cells was significantly lower in mice treated with pioglitazone than in control mice; original magnification $\times 400$
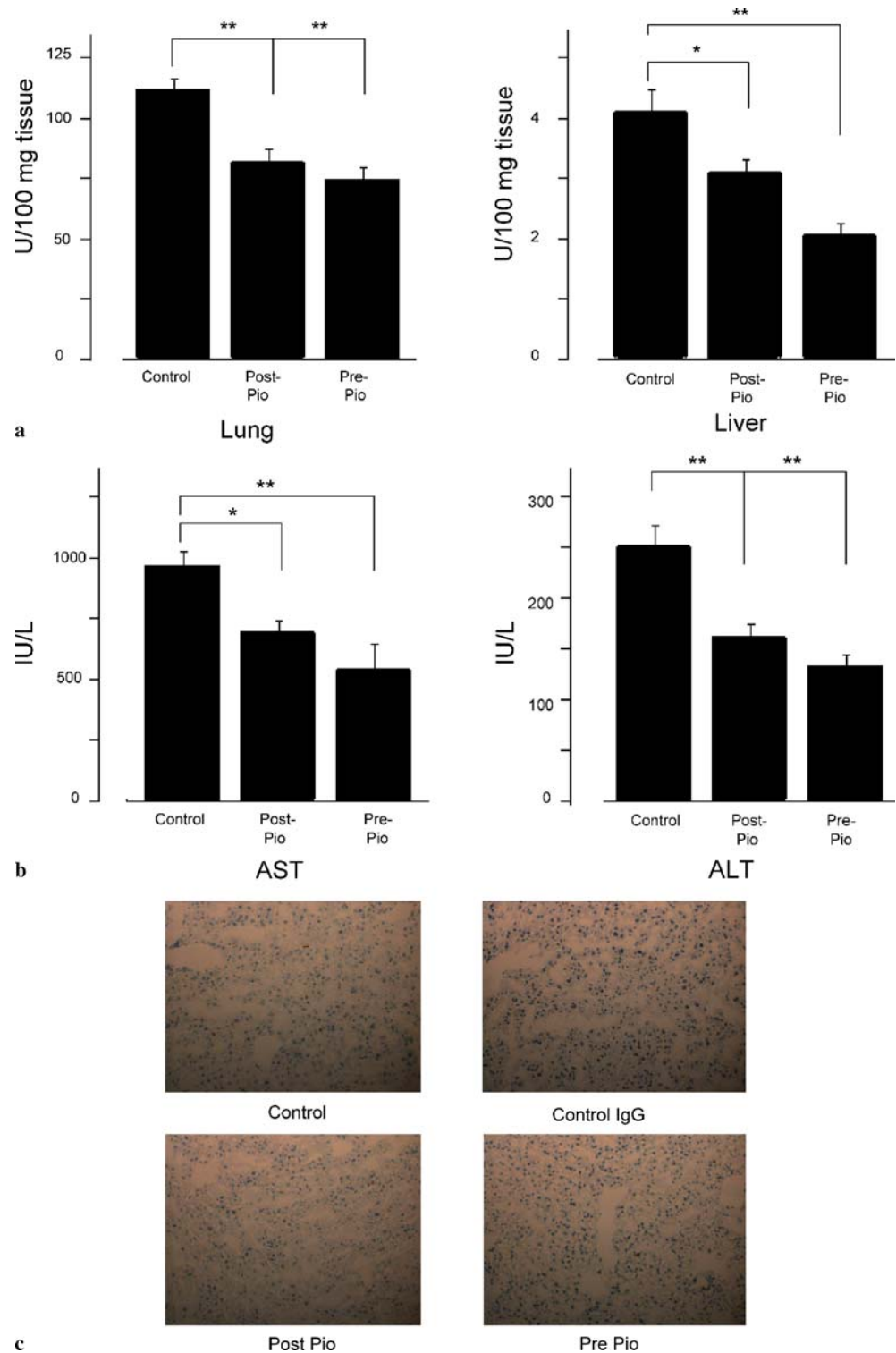
ther on the day prior to CLP or 6 h showed significantly better survival rates than control mice (Fig. 1; $p < 0.05$). In contrast, ApoE$^{-/-}$ mice deceased within 36 h after CLP. Pioglitazone treatment also prevented the drop in mean BP in ApoE$^{-/-}$ mice (Fig. 2; $p < 0.05$). Mean BP was lower in sham-operated mice given pioglitazone than in mice given vehicle; however, this drop was not statistically significant 18 h after CLP (data not shown).

Pioglitazone suppressed the systemic release
of inflammatory cytokines and chemokines
in ApoE$^{-/-}$ mice

Serum levels of TNF-α, IL-6, IL-10, and MCP-1 in
ApoE$^{-/-}$ mice ($n = 6$) 18 h after CLP were measured.
As expected, CLP significantly increased levels of all
proteins examined. Treatment with pioglitazone sig-
nificantly decreased plasma levels of proinflammatory
cytokines TNF-α and IL-6, anti-inflammatory cytokine
IL-10, and proinflammatory chemokine MCP-1 (Fig. 3).
Serum levels of TNF-α, IL-6, IL-10, and MCP-1 were
measured six times for each of the three animal groups
(preadministration, postadministration, and control).

Pioglitazone suppressed inflammation of the organs
in ApoE$^{-/-}$ mice

We next examined whether pioglitazone suppresses in-
flammation in the lungs and liver of septic mice. Increased
MPO activity is correlated with neutrophil invasion of
the organs. MPO activity was reduced significantly in
lungs and liver from animals treated with pioglitazone
(Fig. 4a). Lung MPO activity (IU/100 mg tissue) in mice
treated with pioglitazone prior to surgery, after surgery,
and not treated ($n = 6$) was 74.7 ± 5.1, 82.3 ± 5.0, and
111.6 ± 4.4, respectively. Liver MPO activity values were
2.1 ± 0.3, 3.1 ± 0.2, and 4.1 ± 0.3, respectively. Next we
checked blood AST and ALT levels as an indication of
liver damage (Fig. 4b). The AST (IU/l) in mice ($n = 6$,
each group) treated with pioglitazone prior to surgery,
after surgery, and with vehicle were 545.0 ± 100.1,

693.8 ± 44.6, and 966.8 ± 56.6, while the ALT (IU/l,
$n = 6$) values were 133.0 ± 10.9, 162.3 ± 12.5, and
252.2 ± 19.7. Immunohistochemistry revealed that piogli-
tazone treatment suppressed invasion of CD11b$^+$ cells into
the liver (Fig. 4c). Morphological analysis indicated that
pioglitazone treatment inhibited leukocyte infiltration into
the interstitial space and alveolar wall (data not shown).

Pioglitazone suppressed monocytes adhesion to ECs

To clarify the mechanisms of the protective effects of
pioglitazone on the organs we studied the interaction of
monocytes with ECs. We examined the effect of piogli-
tazone on adhesion of monocytic THP-1 cells to TNF-α
activated HUVECs under flow conditions at a laminar
shear stress level of 1.0 dyne/cm^2. THP-1 cells were
pretreated with 2 and 20 μM pioglitazone for 12 h prior
to the adhesion assay. As shown in Fig. 5, the numbers of
adhered and rolling THP-1 cells were significantly lower
after pioglitazone treatment.

Discussion

Sepsis is a complex clinical syndrome that results from
a harmful host response to infection. Sepsis progresses
without the initial, appropriate host responses to inflam-
mation. In the presence of endotoxin white blood cells
such as monocytes and neutrophils become activated and
produce proinflammatory cytokines. Excessive production
of these proinflammatory cytokines causes irreversible
multiorgan failure. There is an urgent need to identify sub-

Fig. 5 Pioglitazone reduces adhesion and rolling of THP-1 cells on HUVECs under physiological flow conditions. *Black bars* Adhesion; *gray bars* number of rolling cells/HPF. THP-1 cells were treated with 2 or 20 μM pioglitazone for 12 h and then passed over HUVECs activated with TNF-α; ** $p < 0.01$, * $p < 0.05$ vs. control vehicle only
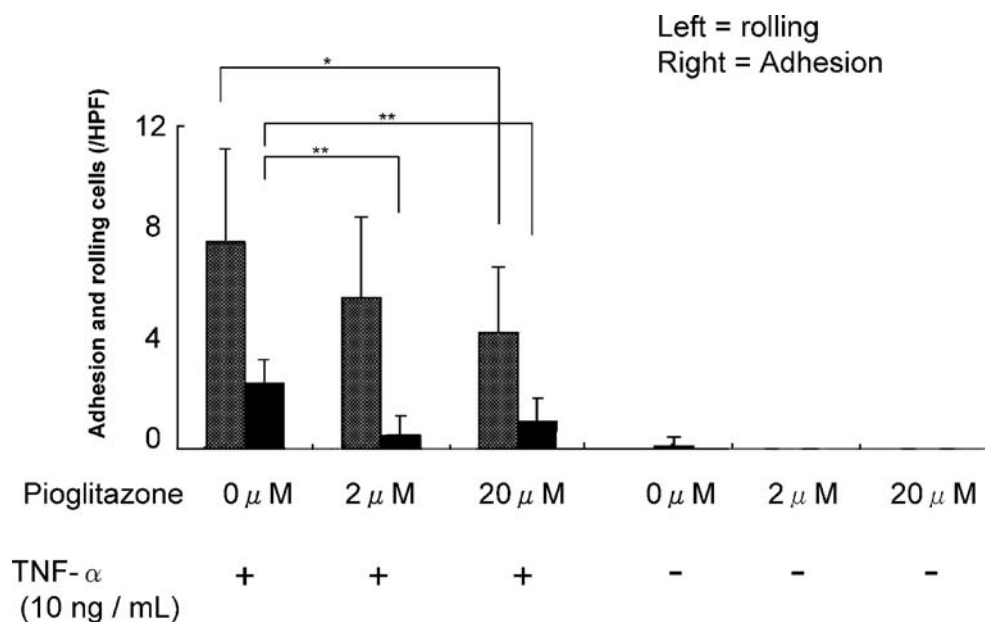
stances that improve survival of patients with modulating the immune response under such conditions.

We used ApoE$^{-/-}$ mice subjected to CLP to demonstrate that pioglitazone improves survival under conditions of severe sepsis through suppression of inflammatory responses. Ours is the first study showing that administration of a PPAR-γ ligand, pioglitazone, after onset of severe sepsis improves survival in a clinically relevant model of sepsis [24]. It was previously reported that PPAR-γ ligands, ciglitazone, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ reduced systemic inflammation and improved survival in animal model [17], indicating that PPAR-γ ligands are effective treatment for severe sepsis. Zingarelli B et al. [17] found that BP in rats had fallen significantly 4 h after CLP procedure in their study. We therefore administered pioglitazone at 6 h after the drop in BP. We also found that administration of pioglitazone before CLP improved survival of WT mice, as reported previously. In a separate study the administration of the phytochemical curcumin 5 h after CLP suppressed inflammatory responses and improved survival through activation of PPAR-γ. These effects were abolished by administration of a PPAR-γ antagonist [25]. PPAR-γ ligands improve the survival rate in various models of shock including hemorrhagic shock [26], zymosan-induced nonseptic shock [27], and endotoxin shock [28]. Moreover, a recent report clarified that the acute anti-inflammatory effects of statins were mediated by PPAR-α, an isotype of PPAR expressed by macrophages and ECs during progression of sepsis [29]. The PPAR-α ligand improved severe sepsis in a rabbit model of shock [30]. These reports support our finding that pioglitazone has strong anti-inflammatory effects and improves survival of mice with severe sepsis.

It was reported previously that many PPAR-γ ligands, including pioglitazone, suppress inflammation through suppression of mitogen-activated protein kinase (MAPK) and nuclear factor (NF) κB activation by lipopolysaccharide in monocytes/macrophages. We also checked the suppressive effects of pioglitazone on MAPK and NF-κB stimulated by LPS in THP-1 cells (data not shown). Suppression of cytokines and chemokines may occur through these MAPK and NF-κB pathways. However, there are many reports that anti-inflammatory treatment increases IL-10 expression, and IL-10 is thought to be a marker of good positive change effect. We did not observe an increase in IL-10 ApoE$^{-/-}$ mice. A previous report indicated that serum IL-10 levels differ in mild vs. moderate and severe cases, whereas IL-6 levels differ between each group [31]. We believe that IL-10 caused the ApoE$^{-/-}$ mice to have such severe inflammation, and that the IL-10 level was not affected. It was recently reported that serum cytokine levels sometimes vary from survival and other inflammatory indices [32].

The present study also shows that pioglitazone inhibits adhesion of monocytes to ECs similarly to statins [3, 22]. This suppression may decrease invasion of white blood cells into organs such as lung and liver and may attenuate lung injury [33]. This would explain the decreased MPO activity observed in lungs and liver of pioglitazone-treated mice. PPAR-γ was initially described as an insulin sensitizer; however, it has many functions in other cells and situations. The PPAR-γ ligand pioglitazone improves EC function [34] and suppresses neointimal formation after stent implantation [35], even in nondiabetic patients.

We also found that pioglitazone treatment improves survival of both septic ApoE$^{-/-}$ mice and WT mice. ApoE has received a great deal of attention in the fields of neurology, endocrinology, and cardiology where it is believed to have direct links to some diseases. ApoE is a ligand of the LDL receptor and plays an essential role in lipid metabolism and atherosclerosis progression. In addition, an ApoE polymorphism is a significant predictor of Alzheimer's disease occurrence. Furthermore, an ApoE polymorphism is linked to the incidence of severe sepsis [10]. ApoE also modulates the type I inflammatory responses; a recent study indicated that cytokine levels in ApoE$^{-/-}$ mice cytokine are higher than those in WT mice [9]. In addition, ApoE regulates clearance of apoptotic bodies involved in systemic inflammation [36]. ApoE has multiple functions in progression of inflammatory responses. For these reasons the CLP model with ApoE$^{-/-}$ mice can reproduce one type of severe sepsis in human beings. Our findings suggest that treatment with PPAR-γ agonist may be a strategy to treat severe sepsis.

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