INTRODUCTION

Septic shock, a serious sequela of acute bacterial infections, remains a significant health problem in the US today.1 Septic shock is the most common cause of death in intensive care units in the US. The incidence of septic shock is not likely to decrease due to increased use of cytotoxic and immunosuppressive drug therapies, invasive devices, antibiotic-resistant organisms and an aging patient population susceptible to sepsis.2 Although the pathogenesis of septic shock is complex, inflammatory mediators such as tumor necrosis factor (TNF) and interleukin 6 (IL-6) play a key role in the outcome of acute bacterial infections.3,4

A number of recent reports have spurred an interest in examining the role of peroxisome proliferator activated receptors (PPARs) in the host immune response. PPARs are a family of steroid receptors belonging to class II of the nuclear steroid receptor superfamily, which also includes the thyroid, retinoic acid, and vitamin D receptors.5 In man and mouse, three types of PPARs have been cloned, α, β/δ, and γ. PPARs heterodimerize with the retinoic X receptor (RXR) which then act as transcription factors by binding to PPAR response elements (PPREs). PPREs are 6-basepair direct repeats separated by a single basepair. PPARα mediates the pleiotropic response to peroxisome proliferators

Pretreatment with troglitazone decreases lethality during endotoxemia in mice

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Troglitazone is an oral antidiabetic drug that is a ligand for peroxisome proliferator activated receptor γ (PPARγ). Based on other studies that have implicated an immunosuppressive role for PPARγ during inflammatory responses, we hypothesized that troglitazone treatment would improve survival in a murine model of endotoxemia and that the protective effect would be mediated by decreased expression of inflammatory mediators. C57Bl/6N x Sv/129 (wild-type [WT]) or PPARα null mice treated for 2 weeks with dietary troglitazone (0.1%) had significantly fewer deaths and a higher LD50 value compared to control-fed mice when challenged with lipopolysaccharide (LPS). PPARα null mice were more sensitive to the lethal effects of LPS as evidenced by a 2-fold lower LD50 (6.6 mg/kg) compared to WT mice (14.6 mg/kg). Troglitazone treatment had no significant effect on LPS-induced plasma TNF, glucose, or nitric oxide levels in WT or PPARα null mice at any of the time points examined. However, troglitazone treatment significantly reduced LPS-induced plasma IL-6 levels in both WT and PPARα null mice. The results of these studies suggest that troglitazone treatment protects mice against a lethal challenge of LPS, but whether or not this effect is mediated through decreased expression of inflammatory mediators remains unclear.
and regulates transcription of genes involved in lipid metabolism. PPARγ induces adipocyte differentiation in fibroblasts and regulates transcription of genes involved in adipogenesis.

We recently reported the effects of a PPARα ligand on TNF expression during endotoxemia in mice. We showed that dietary administration of PPARα ligands, fenofibrate or Wy 14,643, dramatically increased LPS-induced TNF expression and lowered the LD₅₀ for LPS in out-bred CD-1 mice. However, in PPARα null mice, fenofibrate treatment lowered LPS-induced plasma TNF levels compared to wild-type mice. These data suggest that fenofibrate may have acted through another member of the PPAR family, such as PPARγ, to inhibit LPS-induced TNF expression in PPARα-deficient mice. There are also reports from other laboratories that PPARγ has inhibitory effects on the immune system. Jiang et al. tested the effect of natural and synthetic PPARγ agonists on the production of inflammatory cytokines in human peripheral blood monocytes. They reported that troglitazone, a thiazolidinedione (TZD), and 15-deoxy-Δ₁₂,14-prostaglandin J₁ (15d-PGJ₁) inhibited phorbol myristate acetate (PMA)-induced synthesis of TNF, IL-6, and IL-1β, but had no effect on LPS-induced synthesis of these cytokines. In addition, the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin, ibuprofen, and fenoprofen also inhibited PMA-induced synthesis of these cytokines. Ricote et al. also reported that PPARγ is up-regulated in thioglycollate-elicited peritoneal macrophages and that 15d-PGJ₂ and other synthetic PPARγ ligands inhibit synthesis of inducible nitric oxide synthase (iNOS), gelatinase B and scavenger receptor A genes. These data suggest that PPARγ has an immunosuppressive effect on inflammatory processes. However, the role of a PPARγ ligand in endotoxemia has not been fully investigated and is the focus of this work. In order to evaluate the role of PPARγ in septic shock, we utilized a well-characterized model of endotoxemia. Endotoxin or LPS treatment induces a dramatic increase in the expression of inflammatory mediators such as TNF, IL-6, and nitric oxide (NO) among others. These molecules mediate many of the effects of endotoxin and were examined as key indicators of the effect of troglitazone treatment on the host response.

Materials and Methods

Chemicals

All chemicals and supplies were purchased from Sigma (St Louis, MO, USA) or Fisher Scientific (Dallas, TX, USA) unless otherwise noted.

Animals

All animals were housed and cared for under the National Institutes of Health guidelines for the care and use of laboratory animals. The Oklahoma University Health Sciences Center Institutional Animal Care and Use Committee (IACUC) and the Oklahoma Christian University IACUC approved all experiments involving animals. Experiments were also conducted with age- and sex-matched C57Bl/6N x Sv/129 homozygous wild-type or PPARα null mice. Experiments were conducted in which the mice were fed a diet containing no drug or troglitazone (0.1%) ad libitum for 14 days. Troglitazone tablets were pulverized and added to rodent meal as an admixture. The meal was prepared on a weekly basis and stored at 4°C. In all experiments, the animals were injected intraperitoneally (i.p.) with either 0.5 ml of sterile saline, Escherichia coli O111:B4 LPS, or recombinant murine TNF-α (R&D Systems, Minneapolis, MN, USA). At the appropriate time interval, the animals were anesthetized with metofane, weighed and decapitated to remove trunk blood. Livers were removed and weighed. The plasma was separated and stored at –70°C.

Plasma analysis

Plasma samples were assayed for TNF and IL-6 using a sandwich enzyme linked immuno-absorbant assay (ELISA). The capture (rat anti-mouse TNF or IL-6) and detecting (biotinylated rat anti-mouse TNF or IL-6) antibodies were purchased from PharMingen (San Diego, CA, USA). Concentrations of TNF or IL-6 in the plasma samples were calculated from a standard curve determined using recombinant mouse cytokines (Genzyme, Cambridge, MA, USA). The assays were linear between 50–3200 pg/ml. Plasma glucose levels were determined using a colorimetric glucose oxidase assay kit from Sigma. NO levels were determined spectrophotometrically by measuring both nitrite and nitrate in plasma samples as described by Altavilla et al. After stoichiometrically reducing nitrate to nitrite, the plasma samples were assayed in a standard Griess reaction. Nitrite concentrations were determined from a standard curve using sodium nitrite in concentrations from 1.25–100 µM.

Statistical analysis

Statistical analysis of the data was performed by using Student’s t-test for significant differences (P < 0.05) with Minitab statistical software.

Results

Effect of troglitazone treatment on LPS-induced lethality

We tested the effect of dietary troglitazone, a potent PPARγ ligand and antidiabetic drug, on LPS-induced
lethality in WT and PPARα null mice. We used WT and PPARα null mice to compare the response between mice that express the α, β, and γ isoforms of PPAR (WT mice) and mice that only express PPARβ and PPARγ (PPARα null mice), in order to assess the role of PPARγ in regulating inflammatory mediators during endotoxemia. WT mice were fed for 2 weeks with chow containing no drug or 0.1% troglitazone and then challenged with doses of LPS ranging from 6–60 mg/kg. Deaths were monitored for 1 week, although most deaths occurred between 24–72 h. The results are shown in Table 1. The data for WT and PPARα null mice represent three separate experiments with 3–4 mice per dose per experiment. Troglitazone treatment increased the LPS LD50 by 2.4-fold in WT mice and 2.2-fold in PPARα null mice compared to control-fed WT and PPARα null mice, respectively. When WT mice were challenged with 400 µg of LPS (approximately an LD10 dose), troglitazone treatment decreased mortality by 3-fold compared to control-fed mice; P < 0.05). These results also show that PPARα null mice were more sensitive to LPS-induced lethality than WT mice, suggesting a protective role for PPARα.

**Effect of troglitazone treatment on the host response to LPS**

To explore the mechanism of troglitazone-mediated protection from lethal endotoxemia, we examined the effect of troglitazone treatment on LPS and TNF-induced circulating levels of TNF, IL-6, NO, and glucose. WT and PPARα null mice were fed for 2 weeks with control chow or chow containing 0.1% troglitazone and then challenged with 400 µg of LPS. We demonstrated previously that LPS-induced plasma TNF levels peak at 1–2 h and are not detectable by 4 h. Therefore, plasma samples were collected at 1 h for TNF measurements. The results are described in Table 2. No TNF was detectable in saline-challenged WT or PPARα null mice. LPS-challenged control-fed WT and PPARα null mice exhibited a significant increase in plasma TNF levels compared to saline-challenged mice. However, troglitazone treatment did not significantly alter LPS-induced plasma TNF levels in WT or PPARα null mice.

We also measured the effect of troglitazone treatment on LPS-induced plasma IL-6 levels over time. These

![Fig. 1. Time-course of plasma IL-6 levels in control versus troglitazone-fed WT and PPARα null mice during endotoxemia.](image)

Animals were fed chow containing no drug or 0.1% troglitazone for 2 weeks before challenge. Animals were challenged with doses of *E. coli* O111:B4 LPS ranging from 100–1600 µg per animal. The 50% lethal dose (LD50) was calculated based on body weight using the Reed-Muench method.42

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT mice (LD50)</th>
<th>PPARα null mice (LD50)</th>
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<tbody>
<tr>
<td>Control-fed</td>
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<td>6.6 mg/kg</td>
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<tr>
<td>Troglitazone-fed</td>
<td>35.1 mg/kg</td>
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<tr>
<th>Treatment</th>
<th>Wild type Saline</th>
<th>LPS (12 mg/kg)</th>
<th>Knockout Saline</th>
<th>LPS (12 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>11.137 ± 1926</td>
<td>ND</td>
<td>13,904 ± 1222</td>
</tr>
<tr>
<td>Troglitazone (0.1%)</td>
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<td>16,761 ± 3508</td>
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</tr>
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Animals were fed chow containing no drug or 0.1% troglitazone for 2 weeks and then challenged with saline or 12 mg/kg of *E. coli* O111:B4 LPS. Results are expressed as mean ± SEM (n = 11–13). ND, not detected.

![Graph](image)

**Table 1.** Effect of troglitazone treatment on LPS-induced lethality in mice

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**Table 2.** Effect of troglitazone treatment on plasma TNF levels (pg/ml) in WT versus PPARα null mice 1 h after LPS challenge

<table>
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<tr>
<th>Treatment</th>
<th>Wild type Saline</th>
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results are illustrated in Figure 1. Very low levels of IL-6 were detected in saline-challenged animals (0 h). In control-fed WT mice, LPS-induced IL-6 levels were significantly elevated by 1 h, peaked at 2 h, and remained significantly elevated at 18 h compared to saline-challenged mice. LPS-induced IL-6 levels in control-fed PPARα null mice were significantly elevated compared to control-fed WT mice at all time points measured. Troglitazone treatment significantly decreased LPS-induced IL-6 levels at the 18-h time point in WT mice and at the 6-h and 18-h time points in PPARα null mice.

Plasma glucose is a marker of endotoxemia and hypoglycemia is important in the pathogenesis of endotoxemia. Plasma glucose levels were significantly decreased in control-fed PPARα null mice compared to control-fed WT mice at the 6-h time point (Fig. 2). Troglitazone treatment had no significant effect on plasma glucose levels in LPS-challenged WT or PPARα null mice at any time point. NO is a terminal mediator of endotoxemia that is overproduced during septic shock. We found that troglitazone treatment in WT and PPARα null mice had no significant effect on LPS-induced plasma NO levels at any of the time points measured (Fig. 3).

Body and liver weights were also measured. Whereas PPARα activators significantly increased liver weight,9 troglitazone treatment had no significant effect on liver weight (control-fed = 1.8 ± 0.1 g versus troglitazone-fed = 1.7 ± 0.1 g) or body weight (control-fed = 31.1 ± 0.4 g versus troglitazone-fed = 28.9 ± 0.4 g).

### Table 3. Effect of troglitazone treatment on plasma TNF and IL-6 levels in WT mice 1 h after TNF challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline TNF (ng/ml)</th>
<th>TNF</th>
<th>Saline IL-6 (pg/ml)</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-fed</td>
<td>ND</td>
<td>398.4 ± 59.6</td>
<td>ND</td>
<td>5323 ± 518</td>
</tr>
<tr>
<td>Troglitazone-fed</td>
<td>ND</td>
<td>356.4 ± 31.1</td>
<td>ND</td>
<td>3821 ± 445*</td>
</tr>
</tbody>
</table>

Animals were fed chow containing no drug or 0.1% troglitazone for 2 weeks and then challenged i.p. with saline or 2 µg of murine recombinant TNF. Results are expressed as mean ± SEM (n = 6 animals/group).

ND, not detected.

*P < 0.05 as compared to TNF-challenged control-fed animals.
Effect of troglitazone treatment on the host response to TNF

Although troglitazone treatment had no significant effect on LPS-induced plasma TNF levels, it is possible that troglitazone treatment desensitized the host to the downstream effects of TNF. Therefore, we tested the effect of troglitazone treatment on TNF and IL-6 1 h after exogenous administration of murine recombinant TNF. WT mice were fed as described earlier and challenged i.p. with 2 μg of murine recombinant TNF. The results are described in Table 3. No significant difference was observed in TNF-induced plasma TNF levels between control and troglitazone-fed mice. The plasma TNF values may represent not only TNF-induced TNF but absorption of the injected material as well. However, a small, but statistically significant, difference was observed in TNF-induced IL-6 levels between control and troglitazone-fed mice.

DISCUSSION

The major aim of this work was to evaluate the role of a PPARγ ligand on lethality and cytokine expression in a well-characterized mouse model of endotoxemia. We had previously reported that oral administration of fenofibrate, a potent PPARα ligand, dramatically increased LPS-induced TNF levels in mice accompanied by only a modest increase in lethality.9 In the current studies, we used WT and PPARα null mice to examine the contribution of PPARγ to cytokine expression during endotoxemia. However, several recent reports in the literature have indicated that the effects of TZDs and 15d-PGJ2 may be mediated through mechanisms other than activation of PPARγ.23,24 The data presented here must be interpreted in light of these findings. The major findings reported here show that dietary treatment with troglitazone exerted a protective effect from endotoxin-induced lethality in WT (C57Bl/6 x Sc 129) and PPARα null mice. Interestingly, the observation that PPARα null mice (LD90 = 6.6 mg/kg) were more sensitive to LPS lethality than WT mice (LD90 = 14.6 mg/kg) suggests that PPARα has a protective role during endotoxemia. However, it is somewhat paradoxical that CD-1 or WT mice treated with fenofibrate, a PPARα activator, had a 5-fold increase in LPS-induced plasma TNF levels9 and only a 25% increase in mortality in CD-1 mice. Further studies are required to dissect the role of PPARα and their ligands during endotoxemia.

The mechanism of action of troglitazone treatment on increased LPS protection is unclear, although the results of these studies suggest that LPS protection is not mediated via alteration of TNF or NO production. TNF is one of the proximal mediators in the LPS-triggered cytokine cascade and its secretion leads to many of the pathophysiological effects of endotoxin. It is well documented that neutralizing or blocking TNF can abrogate LPS-induced lethality,3,4 thus the focus on TNF as a target in the development of treatment strategies for septic shock. LPS and inflammatory mediators increase iNOS expression resulting in increased levels of NO, a potent vasodilator that contributes to cardiovascular collapse during sepsis.28 Numerous in vitro studies in various macrophage/monocyte cell lines have shown that TZDs and 15d-PGJ2 inhibit TNF and iNOS expression.11,23,26,27 However, in our in vivo model of acute inflammation, troglitazone treatment failed to alter plasma levels of TNF and NO. The discrepancy between our in vivo results and in vitro data could be due to a number of factors, such as doses of troglitazone and/or LPS. Nonetheless, these data suggest that alteration of TNF or NO expression is not responsible for troglitazone-mediated protection from LPS.

Although troglitazone treatment did not modulate expression of TNF or NO during endotoxemia, it did reduce LPS-induced plasma IL-6 levels in both WT and PPARα null mice. IL-6 is a multifunctional cytokine that, during acute inflammation, is primarily responsible for induction of acute phase proteins. There are several conflicting reports for the role of IL-6 in the pathogenesis of endotoxin shock. Depending on the animal model and experimental design, IL-6 has been shown to be a mediator of endotoxin shock,28-30 to be protective against endotoxin shock,31,32 or to have no role in the pathogenesis of endotoxin shock.33 Although troglitazone treatment significantly decreased LPS-induced IL-6 levels at the 18-h time point, it is unlikely that this is the mechanism of protection from endotoxin lethality. Since LPS-induced IL-6 levels are not altered at the early time points (1 h and 2 h) in WT and PPARα null mice, it is likely that the decreases observed at 6 h and 18 h may be due to increased IL-6 clearance rather than decreased expression in troglitazone-treated animals. However, troglitazone treatment did significantly decrease TNF-induced plasma IL-6 levels 1 h after TNF challenge. We cannot discern from these studies what impact this would have on protection from endotoxin lethality, but it does suggest that some downstream effects of TNF may be altered by troglitazone treatment and that these effects may be masked in this model of endotoxemia.

Thieringer et al.34 reported that a 5-day treatment of lean and db/db mice with a potent thiazolidinedione, AD-5075, failed to blunt LPS-induced TNF and IL-6 levels. In fact, they observed a significant increase in LPS-induced TNF levels in lean mice and a significant increase in TNF and IL-6 levels in db/db mice. They also showed that thiazolidinedione-mediated activation of PPARγ in cultured macrophages did not result in anti-inflammatory activity. Taken together with our data,
Trotiglazone-mediated LPS protection does not appear to be due to decreased production of inflammatory cytokines. Alternatively, TZDs such as troglitazone, improve TNF-α-mediated insulin resistance in humans and experimental animals and fatty acid-induced insulin resistance in rats.8 Since endotoxin challenge induces hyperinsulinemia and insulin resistance,39 improving insulin receptor signaling may contribute to the protective effect of troglitazone. It is also well documented that endotoxin challenge induces hypertriglyceridemia.41,42 This is due to increased synthesis of triglyceride-rich lipoproteins by the liver and to decreased uptake of fatty acids from the bloodstream as a result of decreased lipoprotein lipase (LPL) activity.43,44 We showed previously that endotoxin treatment in CD-1 mice decreased LPL mRNA levels at a time co-incident with decreased PPARγ mRNA and protein levels in white and brown adipose tissue.35 Since PPARγ is a transcriptional regulator of LPL, troglitazone treatment may protect LPL levels from being suppressed during endotoxemia and inhibit hypertriglyceridemia. However, Feingold et al.45 have reported that hypertriglyceridemia plays a protective role during sepsis by binding endotoxin, thus reducing its availability to stimulate macrophages and release inflammatory cytokines. However, Thieringer et al.34 reported that TZDs have no effect on serum lipid levels in normal animals. Our data show that troglitazone treatment did not appear to alter the toxicity of exogenously administered LPS as measured by plasma cytokine levels at the early time points. We are investigating further whether troglitazone treatment prevents LPS-induced LPL suppression, hypertriglyceridemia and insulin resistance during endotoxemia in WT and PPARα null mice.

CONCLUSIONS

Troglitazone treatment altered the host response to an acute inflammatory challenge. These findings have potential implications concerning the clinical use of PPAR ligands in patient populations at increased risk for bacteremia and endotoxin shock.

ACKNOWLEDGEMENTS

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REFERENCES

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