Modulation of LPS-mediated Inflammation by Fenofibrate via the TRIF-dependent TLR4 Signaling Pathway in Vascular Smooth Muscle Cells

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Key Words
Peroxisome proliferator-activated receptor α agonists • Toll-like receptor • Inflammation • Vascular smooth muscle cells

Abstract
Lipopolysaccharide (LPS) induced-vascular inflammation plays a central role in vasculitis and atherosclerosis. The stimulation of toll-like receptor 4 (TLR4) by LPS elicits the release of major proinflammatory cytokines that aggravates cardiovascular disorders. Peroxisome proliferator-activated receptor α (PPARα) agonists have been shown to reduce cardiovascular events by controlling lipid metabolism as well as inflammation. However, the role of PPARα agonist fenofibrate in modulating LPS-mediated inflammatory responses in vascular smooth muscle cells (VSMCs) remains elusive. The present study demonstrated that fenofibrate exerted a potent anti-inflammatory action through reducing interleukin-1(IL-18), tissue inhibitor of metalloproteinase-1(TIMP-1), TLR4 and enhancing PPARα in LPS-stimulated VSMCs. Additionally, treatment of VSMCs with the TLR4 inhibition or TLR4 small-interfering RNA illustrated that the modulatory effects of fenofibrate on LPS-mediated inflammatory responses in VSMCs were reliant on TLR4. Especially, the results suggested that beneficial effects of fenofibrate on LPS-stimulated inflammatory responses in VSMCs were mediated through interference of TLR4 and its downstream signaling components such as Toll-interleukin-1(IL-1) receptor domain- containing adaptor inducing interferon-β (TRIF), interferon regulatory factor 3 (IRF3) and interferon-gamma inducible protein 10 (IP-10). In conclusion, PPARα agonist fenofibrate exerts anti-inflammatory property by antagonizing LPS-mediated inflammatory responses in VSMCs. More importantly, the modulation of the TRIF-dependent signaling pathway (TLR4/TRIF/IRF3/IP-10) might be a useful and novel anti-inflammatory strategy of fenofibrate.

Introduction
Lipopolysaccharide (LPS), a component of the outer membrane of the cell walls of gram-negative bacteria, stimulates generations of cytokines from host cells and
PPAR metabolism [17, 18]. In addition, previous study has shown that acids, thus playing a pivotal role in the regulation of lipid metabolism [16]. PPARα controls expression of a variety of genes involved in the transport and catabolism of fatty acids, thus playing a pivotal role in the regulation of lipid metabolism [17, 18]. In addition, previous study has provided the evidence of an anti-inflammatory activity of PPARα in atherosclerosis [19]. PPARα agonist fenofibrate has the capability to decrease serum levels of pro-inflammatory cytokines in atherosclerotic patients [20, 21]. Recent study also demonstrates that fenofibrate suppresses the production of IL-12 family cytokines by inhibiting the MyD88-dependent TLR4 signaling [22]. However, the role of fenofibrate in modulating vascular inflammation remains to be thoroughly identified. Hence, the aim of the present study was to investigate the effects of PPARα agonist fenofibrate on LPS-mediated inflammatory responses and the TRIF-dependent TLR4 signaling pathway involved in VSMCs and to explore the novel mechanisms of the anti-inflammatory action of fenofibrate.

**Materials and Methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). LPS from Escherichia coli 0111:B4 was produced by Sigma (St. Louis, MO, USA). Fenofibrate was from Cayman (Ann Arbor, MI, USA). Polyclonal anti-rat TLR4, anti-α-smooth muscle actin and anti-β-smooth muscle actin antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against TIMP-1, PPARα, PPARγ, TRIF, IP-10 and phospho-IRF3 and were supplied by ABCAM (Cambridge, UK). Monoclonal anti-TLR4 antibody (MT5S10) was from eBioscience (San Diego, CA, USA). Rat IL-18 ELISA kit was purchased from Invitrogen (Invitrogen Corp., Carlsbad, CA, USA). siRNA specific for TLR4 (siGENOME SMARTpool, M-090819-00) and negative control siRNA (siGENOME Non-Targeting siRNA Pool, D-001206-13-05) and DharmaFECT 2 transfection reagent (T-2002-02) were obtained from Dharmacon (Lafayette, CO, USA).

**Cell culture**

The experimental protocols were approved by the Animal Ethical Committee of Xi’an Jiaotong University. Male Sprague-Dawley rats (weight 120-160 g) were obtained from the Laboratory Animal Institute of School of Medicine of Xi’an Jiaotong University. VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats by the explant technique as previously described [23]. The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml streptomycin, and 100 μg/ml penicillin in 5% CO2 at 37°C. The cells were used between passages 3 and 10 for all experiments. The cells exhibited the typical “hill and valley” growth morphology and were confirmed by smooth muscle α-actin immunostaining. When the cells were grown to confluence, the medium was changed to serum free medium for an additional 24 h before the experiments.

**Small-interfering RNA**

VSMCs (5×10⁵) were seeded into 6-well plates and were grown until 60%-80% confluent. The cells were transiently

transfected with 150 pM of TLR4 small-interfering RNA (siRNA) or negative control siRNA (NC siRNA) using DharmaFECT 2 transfection reagents according to the manufacturer’s instructions. After 48 h, protein expression and mRNA levels of TLR4 were detected by western blot, quantitative real-time-PCR and RT-PCR. Transfection rates of 60%-70% of the cells were accepted for all the experiments.

**Western blot analysis**

As described previously [24], protein samples (20 µg) were separated on 12% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and incubated with specific antibodies against TLR4 (1:200), TIMP-1 (1:400), PPARα (1:100), PPARγ (1:400), TRIF (1:600), phospho-IRF3 (1:500), IP-10 (1:5000), and β-actin (1:400). The expression of β-actin was used as a loading control. Reagents (Pierce Corp., Rockford, IL, USA) for the enhanced chemiluminescence were applied to the blots, and the light signals were detected by X-ray film. Optical densities of the bands were scanned and quantified with the Gel Doc 2000 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Three independent experiments were carried out to study protein expressions.

**Quantitative real-time PCR and RT-PCR**

mRNA levels were determined by our previous method [24]. Total RNA was isolated using a TRIZol Kit (Invitrogen Corp., Carlsbad, CA, USA). cDNA was synthesized from 1 µg samples of total RNA using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) following the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) was performed with the SuperScript III Platinum SYBR-Green One-Step qRT-PCR kit (Invitrogen Corp., Carlsbad, CA, USA) on a Mx3000P QPCR System (Stratagene, LaJolla, CA, USA) following the manufacturer’s instructions. The samples were run in triplicate. Primers for rat TLR4, TIMP-1, PPARα, PPARγ, TRIF, IRF3, IP-10 and β-actin were designed with Beacon designer v 4.0 (Premier Biosoft, CO, USA) (see Table 1 for the sequences). β-actin was used as an endogenous control. Traditional PCR was performed according to the manufacturer’s instructions. The RT-PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. Quantization of relative gene expression was calculated by the comparative Ct method (2−ΔΔCT) as described by the manufacturer. Data were normalized to rat β-actin mRNA levels. Three independent experiments were carried out to study mRNA levels.

**Enzyme-linked immunosorbent assay (ELISA)**

VSMCs were seeded into 6-well plates at a density of 5×10^5 cells/well, and were pretreated for 1 h with different concentrations of Fenofibrate (25, 50 and 100 µM) before exposure to LPS (100 ng/ml) for 9 h. In another experiment, the cells were pretreated with anti-TLR4 antibody (5 µg/ml) for 1 h prior to the addition of fenofibrate (100 µM) for 1 h , and

<table>
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<td>β-actin</td>
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<td>NM_031144</td>
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Table 1. Primers used for real-time PCR analysis

**Fig. 1.** Effect of fenofibrate (Feno) on LPS-induced IL-1β production in rats VSMCs. VSMCs were pretreated for 1 h with different concentrations of Feno (25, 50 and 100 µM) before exposure to LPS (100 ng/ml) for 9 h. The culture medium was then collected, and TIMP-1 was determined with ELISA. Data are expressed as mean±SEM from three independent experiments. ***P<0.001 vs. control; **P<0.05, ##P<0.01 vs. LPS.
subsequently stimulated with LPS (100 ng/ml) for 9 h. Moreover, after application of NC siRNA or TLR4 siRNA for 48 h, VSMCs were exposed to fenofibrate (100 µM) for another 1 h, and subsequently stimulated with LPS (100 ng/ml) for 9 h. IL-18 in the culture supernatant of VSMCs was measured by ELISA kits according to the manufacturer’s instructions.

**Immunofluorescence staining**

To detect the effect of fenofibrate on TLR4 and TIMP-1 expressions in LPS-induced VSMCs, the cells were pretreated with fenofibrate (25, 50 and 100 µM) for 1 h, and then exposure to LPS (100 ng/ml) for 9 h. After the treatment, the cells were fixed with 4% formaldehyde-PBS for 15 min. The cell membranes were fenestrated with 0.3% Triton-100-PBS, and nonspecific binding sites were blocked with 10% goat serum. The cells were incubated with rabbit anti-rat TLR4 (1:200) or anti-rat TIMP-1 antibody (1:400) and then incubated with the secondary antibody conjugated to fluorescein isothiocyanate (FITC). The immunolabeled cells were observed under fluorescence confocal microscopy (Leica TCS SP2-AOB, GER), and then fluorescent intensity of TLR4 and TIMP-1 were detected. Data were expressed as relative to control (%).

**Statistical analysis**

Data were expressed as mean±SEM. Differences between two groups were determined either by unpaired Student’s t-test or by one-way ANOVA followed by post hoc Dunns multiple-comparison test. A value of $P < 0.05$ was considered statistically significant.

**Results**

**Effect of fenofibrate on LPS-induced IL-18 production in VSMCs**

To evaluate effects of fenofibrate on LPS-induced IL-18 production, VSMCs were pretreated with different concentrations of fenofibrate (25, 50 and 100 µM) for 1 h, and then stimulated with LPS (100 ng/ml) for...
The results indicated that fenofibrate concentration-dependently reduced LPS-stimulated IL-18 production in VSMCs (Fig. 1).

Effects of fenofibrate on LPS-induced immunofluorescent expressions of TLR4 and TIMP-1 in VSMCs

The immunolabeled VSMCs were observed under fluorescence confocal microscopy, and fluorescent intensity of TLR4 and TIMP-1 were also measured. The results showed that fenofibrate attenuated LPS-induced immunofluorescent expressions of TLR4 and TIMP-1 in VSMCs in a concentration-dependent manner (Fig. 2).

Effects of fenofibrate on LPS-mediated protein and mRNA expressions of TLR4, TIMP-1, PPARα and PPARγ in VSMCs

On the basis of the above-mentioned results, effects of fenofibrate on LPS-mediated protein and mRNA expressions of TLR4, TIMP-1, PPARα and PPARγ in VSMCs were further determined. As shown in Fig. 3, fenofibrate concentration-dependently downregulated protein and mRNA expressions of TLR4 and TIMP-1, but showed no effect on PPARγ expression in LPS-stimulated VSMCs.

Association between effects of fenofibrate on LPS-mediated inflammatory responses in VSMCs and TLR4

To investigate whether fenofibrate depresses LPS-induced IL-18 generation via TLR4, VSMCs were pretreated with anti-TLR4 antibody (5 µg/ml) for 1 h prior to the addition of fenofibrate (100 µM) for 1 h, and subsequently stimulated with LPS (100 ng/ml) for 9 h. As shown in Fig. 4A, compared with the control, stimulating the cells with LPS resulted in IL-18 elevation, whereas the TLR4 inhibition and fenofibrate remarkably reversed the LPS-induced effect on IL-18 in VSMCs. Furthermore, treatment of the cells with the combination of the TLR4 blocker and fenofibrate synergistically reversed the effect elicited by LPS in comparison with the treatment of the TLR4 inhibition or fenofibrate alone. Considering that the TLR4 inhibition antagonizes effect of LPS on IL-18 and fenofibrate directly downregulates LPS-induced TLR4 expression in VSMCs, the inhibitory
effect of fenofibrate on LPS-induced IL-18 production in VSMCs is associated with TLR4.

In addition, the results demonstrated that the TLR4 inhibition reduced TIMP-1 protein expression and enhanced PPARα and PPARγ protein expressions in LPS-stimulated VSMCs. Fenofibrate also exerted the similar effects on TIMP-1 and PPARα protein expressions to the TLR4 inhibition, but showed little effect on PPARγ protein expression. Treatment of the cells with the combination of the TLR4 inhibition and fenofibrate also synergistically reversed LPS-mediated changes of TIMP-1 and PPARα protein expressions (Fig. 4B). Consequently, the effects of fenofibrate on LPS-mediated inflammatory responses in VSMCs are dependent on TLR4.

Effects of TLR4 siRNA on anti-inflammatory action of fenofibrate in LPS-stimulated VSMCs

To further confirm the role of TLR4 in LPS-mediated inflammatory responses in VSMCs and in anti-inflammatory mechanisms of fenofibrate, VSMCs were transiently transfected with TLR4 siRNA for 48 h, and then protein expression and mRNA levels of TLR4 were detected (Fig. 5A and 5B). Knock down efficiency of TLR4 was 62.5% or 60% as determined by Western blot or quantitative real-time PCR. The transfected VSMCs were pretreated with fenofibrate (100 µM) for 1 h prior to stimulation with LPS (100 ng/ml) for 9 h. As described in Fig. 5C, compared with the negative control, stimulating the cells with LPS led to IL-18 increase, whereas lack of TLR4 markedly impaired LPS-induced IL-18 generation in VSMCs, thus suggesting that LPS elicited IL-18 production via TLR4. Fenofibrate decreased LPS-induced IL-18 production in negative control, but the effect of fenofibrate was almost abrogated in TLR4 siRNA control, implying that inhibition by fenofibrate of LPS-induced IL-18 production in VSMCs depended on the existence of TLR4. The similar results to IL-18 were achieved for protein expression and mRNA levels of TIMP-1(Fig. 5D and 5E). Moreover, TLR4 siRNA also abolished LPS-elicited reduction of protein and mRNA expressions of PPARα and PPARγ compared to negative control, thus manifesting that LPS downregulated PPARα and PPARγ expressions in VSMCs via TLR4. Fenofibrate upregulated PPARα protein and mRNA expressions in negative control in LPS-stimulated VSMCs, but TLR4 siRNA did not obviously antagonize the increased effect of fenofibrate on PPARα protein and mRNA expressions (Fig. 5D and 5E), indicating that the elevation of PPARα expression by fenofibrate does not completely depend on TLR4. In addition, fenofibrate did not produce significant effect on PPARγ protein and mRNA expressions in negative control and TLR4 siRNA control (Fig. 5D and E).

Inhibition by fenofibrate of LPS-mediated inflammatory responses in VSMCs via interference with the TRIF-dependent signaling pathway

As mentioned above, TLR4 is involved in the regulatory effects of fenofibrate on LPS-mediated inflammatory responses in VSMCs. Accumulating
evidence indicates that LPS signaling through TLR4 utilizes both MyD88 and TRIF pathways. The TRIF-dependent pathway also results in activation of another transcription factor, IRF-3, further leading to expression of IP-10 [25]. Hence, we further investigated the effects of fenofibrate on the TRIF/IRF3/IP-10 pathway. As seen from Fig. 6A and 6B, treatment of VSMCs with LPS (100 ng/ml) for 9 h significantly enhanced protein and mRNA expressions of TRIF and IP-10, and fenofibrate produced the potent inhibitory effects on expressions of TRIF and IP-10 in LPS-stimulated VSMCs. Meanwhile, IRF3 phosphorylation and mRNA expression induced by LPS (100 ng/ml) treatment for 30 min or 9 h were evidently blunted by fenofibrate. The results also showed that
fenofibrate did not change basal TRIF, IRF3 and IP-10 levels in the unstimulated VSMCs. Taken together, all results in the present study strongly suggest that fenofibrate exerts its regulatory action on LPS-mediated inflammatory responses in VSMCs via interfering with TLR4/TRIF/IRF3/IP-10 signaling pathway.

Discussion

LPS-induced vascular inflammation plays a central role in vasculitis and atherosclerosis with the contribution of monocytes/macrophages, lymphocytes and vascular resident cells, including VSMCs [26, 27]. The stimulation of TLR4 by LPS elicits the release of major proinflammatory cytokines that aggravates cardiovascular disorders [28]. The present study demonstrates that PPARα agonist fenofibrate exerts a potent anti-inflammatory action through reducing IL-18, TLR4, TIMP-1, and enhancing PPARα in LPS-stimulated VSMCs. The investigations also illustrate that the modulatory effects of fenofibrate on LPS-mediated inflammatory responses in VSMCs are reliant on TLR4. More importantly, our findings suggest that fenofibrate is able to suppress LPS-mediated inflammatory responses in VSMCs via interfering with the TRIF-dependent signaling pathway (TLR4/TRIF/IRF3/IP-10).

Inflammation is a critical event in the development of cardiovascular diseases such as hypertension, atherosclerosis, and restenosis. IL-18, a pleiotropic proinflammatory cytokine, is widely expressed in various cell types [29]. IL-18 enhances the expression of certain inflammatory cytokines and MMPs, thus contributing to atherosclerotic diseases [30]. Conversely, IL-18 deficiency reduces the extent of atherosclerosis in apolipoprotein E-knockout mice [31]. A great amount of evidence confirms that MMPs are both effectors and regulators of inflammatory responses [32]. And tissue activity requires a balance between MMPs activation and TIMPs inhibition, which is important in tissue remodeling and inflammation [33]. TIMP-1, the typical inhibitor of MMP-9, has been shown to predict future cardiovascular risk in a variety of clinical settings [34]. Previous report has proved that PPARα-deficient mice can demonstrate enhanced vascular inflammatory responses to LPS administration [35]. In the current study, fenofibrate decreased LPS-induced generations of IL-18 and TIMP-1 in VSMCs, further supporting a potential role of PPARα activation in the retard of vascular inflammation. Additionally, the present results seem to be consistent with our previous findings showing that LPS leads to downregulation of PPARα and PPARγ [24], however, treatment with fenofibrate evidently antagonized LPS-reduced PPARα expression, but showed little effect on PPARγ expression in VSMCs. Consequently, PPARα agonist fenofibrate may have a direct effect on inflammation through its ability to modulate inflammatory cytokines production by VSMCs.

LPS aggravate atherosclerosis in humans and rodents by inducing inflammation via TLR4 [36]. TLR4 is critically involved in the pathogenesis of arteriosclerosis, and therefore might make a fundamentally significant contribution to the pathophysiological relationship between inflammation and cardiovascular disorders [37]. Specially, TLR4 signaling is becoming more of interest in the inflammatory and atherosclerotic field. In accordance with our and others’ studies [4, 9], we found that TLR4 in VSMCs was activated by LPS. Additionally, deficiency of the PPARα gene in macrophages remarkably accelerated LPS-mediated activation of the inflammatory response genes [38]. More importantly, the present study provided the direct evidence that PPARα agonist fenofibrate inhibited LPS-induced TLR4 expression in VSMCs, thus implying that activation of PPARα may interrupt the impact of LPS on the TLR4. Although PPARα has attracted considerable attention for its anti-inflammatory effects, the association between effect of fenofibrate on LPS-mediated inflammatory responses in VSMCs and TLR4 is poorly understood. Therefore, both the TLR4 inhibition and TLR4 siRNA were applied to VSMCs to elucidate whether TLR4 is involved in the modulatory effect of fenofibrate on LPS-mediated inflammatory responses in VSMCs. The present results manifested that the TLR4 inhibition obviously antagonized the LPS-mediated inflammatory responses in VSMCs, which were potentiated by fenofibrate. In addition, inflammatory responses in VSMCs elicited by LPS require TLR4 at least in part, as productions of IL-18, TIMP-1, PPARα and PPARγ were regulated by the TLR4 inhibition and TLR4 siRNA. Hence, the results seem to further corroborate the fact that the effects of LPS are mediated via TLR4 [39]. Since LPS is a special TLR4 ligand, fenofibrate also ameliorated LPS-mediated inflammatory responses and directly downregulated LPS-induced TLR4 expression in VSMCs, further revealing that TLR4 is involved in the modulatory effect of fenofibrate on LPS-mediated inflammatory responses in VSMCs. In combination of the finding that lack of TLR4 almost abrogates the inhibitory effect of fenofibrate on LPS-induced inflammatory responses, TLR4 may be a new target for PPARα agonists in the regulation of LPS-
mediated inflammatory responses in VSMCs.

TLR4 has two major downstream signaling pathways, MyD88- and TRIF-dependent pathways leading to the activation of NF-κB and IRF3, and the expression of inflammatory mediators [40]. Evidence is accumulating that the TRIF-dependent TLR4 signaling pathway plays a crucial role in inflammatory responses and development of certain chronic diseases. It was also reported that LPS induced the production of a greater proportion of genes via the “TRIF-dependent” than the “MyD88-dependent” pathway, thus suggesting the significant contribution of TRIF-signaling to TLR4-mediated immune and inflammatory responses [15]. PPAR agonist fenofibrate suppresses the production of IL-12 family cytokines by inhibiting the MyD88-dependent TLR4 signaling [22]. However, it is not known whether fenofibrate is able to regulate the TRIF-dependent signaling following LPS stimulation. On the basis of these considerations, we further investigated if the TRIF-dependent signaling was involved in anti-inflammatory effects of fenofibrate in LPS-mediated inflammatory responses in VSMCs. In the present study, treatment of VSMCs with LPS dramatically enhanced TRIF expression, and fenofibrate produced the potent inhibitory effects on TRIF expression in LPS-stimulated VSMCs, supporting that the TRIF-dependent signaling pathway (TLR4/TRIF/IRF3/IP-10) might be a useful and novel anti-inflammatory strategy of fenofibrate.

In conclusion, PPAR agonist fenofibrate exerts anti-inflammatory property by antagonizing LPS-mediated inflammatory responses in VSMCs. More importantly, the modulation of the TRIF-dependent signaling pathway (TLR4/TRIF/IRF3/IP-10) might be a useful and novel anti-inflammatory strategy of fenofibrate.

Acknowledgements

This study was supported by a grant from National Natural Science Foundation of China (No. 30772567).

References


