Fenofibrate inhibits tumor necrosis factor-α-induced expression of CD40 and matrix metalloproteinase in human vascular endothelial cells

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Abstract: **Objective** To investigate the regulatory effects of fenofibrate on TNF-α-induced CD40 expression and matrix metalloproteinase (MMP) activity in human vascular endothelial cells (HUVECs). **Methods** Quantitative RT-PCR and flow cytometry were employed to evaluate the effect of fenofibrate on TNF-α-induced CD40 mRNA and cell surface CD40 expression in HUVECs, and gelatin zymography was used to determine the effect of fenofibrate on the gelatinolytic activities of MMP-2 and MMP-9 in TNF-α-stimulated HUVECs. **Results** Fenofibrate at the concentrations of 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/L significantly reduced TNF-α-induced increment of CD40 mRNA and cell surface CD40 expressions ($P<0.01$), with the maximal inhibition achieved at the concentration of 1×10^{-4} mol/L. Fenofibrate at 2×10^{-4} mol/L did not further decrease CD40 expression induced by TNF-α. Fenofibrate significantly inhibited the stimulatory effect of TNF-α on MMP-2 and MMP-9 activities in HUVECs. **Conclusion** Fenofibrate reduces TNF-α-induced increment of CD40 expression and MMP-2 and MMP-9 activities in HUVECs.

Key words: fenofibrate; CD40; matrix metalloproteinase-2; matrix metalloproteinase-9

Inflammatory response plays an important role in the onset, development and evolution of atherosclerotic lesions [1], and the inflammatory pathways have been suggested as potential targets for therapy of this prevalent human disease. Evidences obtained from recent studies indicate that the CD40-CD40L system acts as an important mediator of several auto-immune and chronic inflammatory diseases [2]. Interruption of CD40-CD40L signaling not only reduces the occurrence and inhibits the progression of atherosclerotic lesions in hypercholesterolemic mice [3,4], but also modulates the plaque architecture *in vivo* [5,6]. As a key regulator of the pathological process of atherosclerosis, CD40-CD40L has been recognized as an important potential target for atherosclerotic therapy.

Fenofibrate, the peroxisome proliferator-activated receptor (PPAR) activator, is an effective lipid-lowering agent widely used in clinical practice. *In vitro* and *in vivo* studies have indicated that PPAR activators modulate vascular inflammatory responses besides their lipid-lowering effects [7]. Currently, however, the exact mechanisms underlying the anti-inflammatory effect of fenofibrate are not well understood, nor are reports available in regard of its role in regulating CD40 and matrix metalloproteinase (MMP) expressions in the human vascular endothelial cells.

Therefore, in the present study, we investigated the effect of fenofibrate on tumor-necrosis factor-α-induced CD40 and MMP activity changes in human umbilical vein endothelial cells (HUVECs) to explore the mechanism of its anti-inflammatory effect.

MATERIALS AND METHODS

Materials

Dulbecco modified Eagle medium (DMEM) and fetal bovine serum (FCS) were produced by Gibco RBL. Fenofibrate and diethyl pyrocarbonate (DEPC) were from Sigma Chemical Co. Mouse anti-human CD40 monoclonal antibody (mAb), goat anti-mouse FITC-conjugated IgG and alkaline phosphatase-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology. TRIzol reagent was purchased from Invitrogen. RevertAid first strand cDNA synthesis kit was purchased from Fermentas (USA). TaqMan reverse transcription reagents was purchased from ABI.

Cell culture

HUVECs were isolated routinely from fresh umbilical cords obtained at normal deliveries in accordance with the ethical standards formulated in the Helsinki Declaration, and transferred to dishes in DMEM containing 20% FCS, penicillin (100 kU/L), streptomycin (100 g/L), and 25 μg/ml endothelial cell

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growth supplements at 37 ℃ in 5 % CO_2. The cultured cell monolayer was identified with phase-contrast microscope. When reaching confluency, the cells were trypsinized, counted and seeded into 50-ml culture flask at the density of 1×10^6 cells/cm^2. The culture medium was changed 24 later and the HUVECs were washed by D-Hank's solution for three times, and further cultured in FBS-free DMEM. In all the following experiments, HUVECs of the second or third passage were used. For each experiment, 4 different donor cell samples were used.

Flow cytometry for CD40

After culture for 24 h in FCS-free DMEM, the cells in each well were treated with different concentrations of fenofibrate (0, 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/L) and incubated for 24 h, followed by further incubation in the presence of 50 μg/L tumor necrosis factor-α (TNF-α) for 24 h. In the control group, FCS-free DMEM was used instead of TNF-α for treatment. For detection of CD40 expression, the HUVECs (1×10^6/ ml) harvested by trypsinization were incubated with 10 μl (200 μg/ml) of mouse anti-human CD40 mAb for 60 min at 4 ℃, washed twice with PBS, and centrifuged at 1000 r/min for 3 min before incubation with goat anti-mouse FITC-conjugated IgG 30 μl (1.4 μg/ml) for 60 min at 4 ℃. Finally, the cells were washed with PBS, fixed with 4% paraformaldehyde and analyzed with a Becton Dickinson FACScan flow cytometer using CellQuest software (Becton Dickinson). At least 5000 viable cells were analyzed for each experiment.

Total RNA extraction

The cells cultured in the above condition were lysed in 1 ml of TRIzol reagent and the total cellular RNA was extracted according to the manufacturer's instruction. The content of the total RNA was quantified by ultraviolet spectrophotometry (Beckman DU640, USA), and the average RNA yield was approximately 1 μg RNA/mg cells.

Reverse transcription

Reverse transcription reactions were carried out for each RNA sample in Eppendorf tubes using the first strand cDNA synthesis kit. Briefly, 5 μg of the total RNA and 1 μl of oligo (dT)18 primers were mixed in a reaction tube, in which DEPC-treated water was added to the final total volume of 12 μl. The above mixture was incubated at 70 ℃ for 5 min before addition of 4 μl of 5×reaction buffer, 1 μl of ribonuclease inhibitor (20 U/μl), 2 μl of dNTP mixture (10 mmol/L), and 1 μl of RevertAid M-MuLV reverse transcriptase (200 U/μl). Reverse transcription was carried out at 37 ℃ for 5 min, 42 ℃ for 60 min and 70 ℃ for 10 min. The reaction mixture was then cooled at 4 ℃ for immediate PCR amplification or stored at −20 ℃ for later use.

TaqMan probe and primers

The PCR primers and TaqMan probe for CD40 were designed using Primer Express 1.0 Software (Perkin-Elmer). CD40forwardprimer: 5'-TAATAAAC A GTCAGTGCTGTTCTTTGTG-3' CD40 reverse primer: 5'-GAACTCTGTGCAGTCACT CACCA-3, and the TaqMan probe for CD40: 5'-FAM- CAGCCAGGACA GAAA-TAMRA-3'. Human CD40 cDNA sequence was available in GenBank (accession number NT-011362). The TaqMan probes were labeled with a reporter fluorescent dye FAM (6-carboxyfluo- rescein) at the 5' end and a fluorescent dye quencher TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end. The specificity of the PCR primers was tested under normal PCR conditions in a thermal cycler prior to TaqMan PCR quantification.

Construction of CD40 standard curve and CD40 expression analysis by real-time PCR

The TaqMan real-time detection is based on fluorogenic 5' nuclease assay [8]. To determine the absolute copy number of the target transcript, a cloned plasmid DNA for CD40 was used to generate a standard curve. A CD40 plasmid containing 431 bp cDNA was inserted into a pGT vector (3000 bp). The copy number of the plasmid DNA template was calculated according to the relative molecular weight of the plasmid. The absolute copy number of CD40 mRNA in each sample was calculated based on the standard curve of the plasmid DNA. All reaction was carried out under the following amplification conditions: 50 ℃ for 2 min, 95 ℃ for 10 min followed by 45 cycles of 95 ℃ for 30 s and 60 ℃ for 1 min.

Gelatinolytic activity assay of MMP-2 and MMP-9

After culture in FBS-free DMEM for 24 h, the HUVECs were treated with fenofibrate at different concentrations (0, 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/L) for 24 h and then with anti-CD40 mAb (5 μg/ml) for another 24 h, followed by exposure to 50 μg/L TNF-α. The cells were collected subsequently and stored at −20 ℃ for assay of MMP-2 and MMP-9 activities with

zymography. The samples were fractionated in an 8% polyacrylamide gel (SDS-PAGE) containing gelatin (1 mg/ml) by electrophoresis at 100 V for 90 min at 4 ℃. The molecular weight standard proteins (BioRad) were run simultaneously. The gels were immersed in 2.5% Triton X-100 for 30 min at room temperature to remove SDS, and incubated in a digestion buffer (containing 50 mmol/L Tris-HCl, pH7.4, 150 mmol/L NaCl, 10 mmol/L CaCl2, 2 μmol/L $ZnSO_4$ and 0.01% Triton X-100) at 37 ℃ overnight to allow proteinase digestion of its substrate. The gels were rinsed again in distilled water, stained with 0.25% Coomassie brilliant blue R-250 in the mixture of methanol, acetic acid and water (volume ratio of 30∶10∶60) for 2 h, and destained with the mixture of methanol, acetic acid, and water (10∶10∶80). Gelatinolytic activities were represented as clear bands of digested gelatin against a dark blue background of stained gelatin. Densitometric analysis of the bands seen on the gels was performed using the Gel Doc 2000 and the results were expressed as arbitrary units of optical density.

Statistical analysis

The results are expressed as *Mean*±*SD*, and the differences in the value between the groups were examined by one-way AVOVA using Sigma STAT software. A *P* value less than 0.05 was considered to indicate a significant difference.

RESULTS

Fenofibrate reduced TNF-α-induced cell surface CD40 expression in HUVECs

The results from flow cytometry revealed that the unstimulated cells expressed CD40 at rather low levels, and the expression increased significantly from (12.01±2.64)% to (54.50 ±5.30)% in response to TNF-α stimulation ($P<0.01$). Treatment with fenofibrate at the concentrations of 1×10^{-4} and 2×10^{-4} mol/L significantly reduced TNF-α-induced CD40 expression to (33.65±4.70)% and (35.76±4.16)%, respectively ($P<0.01$), and the maximal inhibition was achieved at the concentration of 1×10^{-4} mol/L, whereas fenofibrate at 2×10^{-4} mol/L did not further decrease CD40 expression induced by TNF-α. The results suggested that fenofibrate lowered cell surface CD40 expression only within a limited concentration range (Fig.1).

Fenofibrate reduced TNF-α-induced CD40 mRNA expression in HUVECs

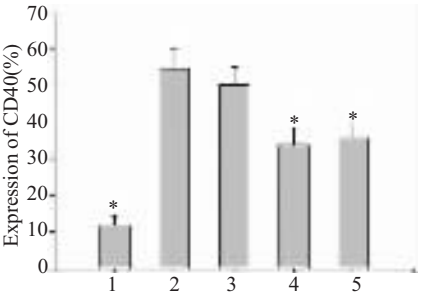

Fig.1 Fenofibrate reduced the cell surface expression of CD40 induced by TNF-α in HUVECs

1: Control; 2: TNF-α 50μg/L; 3: Fenofibrate 5×10^{-5} mol/L+TNF-α 50 μg/L; 4: Fenofibrate 1×10^{-4} mol/L+TNF-α 50 μg./L; 5: Fenofibrate 2×10^{-4} mol/L+TNF-α 50 μg/L. *$P<0.01$ *vs* TNF-α group (n=4)

The results of real-time PCR, similarly, revealed very low levels of CD40 mRNA expression in unstimulated HUVECs, and TNF-α-stimulated cells showed significantly increased CD40 mRNA expression (50.86±8.31 *vs* 350.78±23.72, $P<0.01$). Fenofibrate at the concentrations of 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/L significantly reduced TNF-α-induced CD40 mRNA expression to 280.20 ±19.96, 143.13 ±17.54, and 152.12±31.39, respectively ($P<0.01$), with the maximal inhibition achieved at 1×10^{-4} mol/L. Fenofibrate at 2×10^{-4} mol/L did not further decrease the induced CD40 mRNA expression, suggesting that fenofibrate lowered CD40 mRNA expression within a limited concentration range (Fig.2).

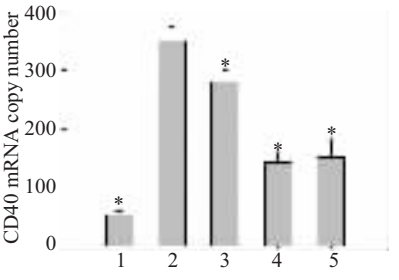

Fig.2 Fenofibrate reduced TNF-α-induced CD40 mRNA expression in HUVECs

1: Control; 2: TNF-α 50μg/L; 3: Fenofibrate 5×10^{-5} mol/.L+TNF-α 50 μg/L; 4: Fenofibrate 1×10^{-4} mol/L+TNF-α 50 μg/L; 5: Fenofibrate 2×10^{-4} mol/L+TNF-α 50 μg/L. *$P<0.01$ *vs* TNF-α group (n=4)

Effects of fenofibrate on MMP-2 and MMP-9 in TNF-α-stimulated HUVECs

Gelatin zymography for determining the relative enzyme activities of MMP-2 and MMP-9 showed that regardless of different treatments, all the gels presented a distinct gelatinase band at 92 kD corresponding to MMP-9. Without TNF stimulation, the cells yielded

only blurry bands at 72 kD and 66 kD to represent pro-MMP-2 and activated MMP-2, respectively. Compared with unstimulated cells, TNF-α stimulation resulted in a significant increase in gelatinase activities (P<0.01), as shown by the appearance of more distinct bands at 92 kD (MMP-9) and at 72 kD and 66 kDa (pro-MMP-2 and activated MMP-2, respectively). Addition of anti-CD40 mAb blocked the gelatinolytic activities of MMP-2 and MMP-9 induced by TNF-α. Fenofibrate at 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/L markedly inhibited MMP-2 and MMP-9 activities induced by TNF-α (P<0.01), indicating that fenofibrate significantly inhibited the stimulatory effect of TNF-α on MMP-2 and MMP-9 activities in HUVECs (Fig.3).

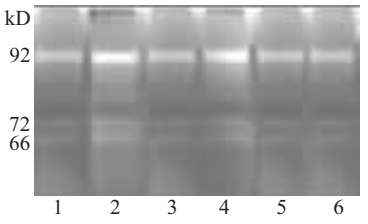

Fig.3 Effect of fenofibrate on gelatinolytic activities of MMP-2 and MMP-9 induced by TNF-α in HUVECs

Lane 1: Control; Lane 2: TNF-α 50 μg/L; Lane 3: Anti-CD40 antibody + TNF-α 50 μg/L; Lane 4: Fenofibrate 5×10^{-5} mol/L + TNF-α 50 μg/L; Lane 5: Fenofibrate 1×10^{-4} mol/L + TNF-α 50 μg/L ; Lane 6: Fenofibrate 2×10^{-4} mol/L + TNF-α 50 μg/L

DISCUSSION

Recent studies have demonstrated that CD40-CD40L inflammatory signaling pathway is a potent activator of endothelial cells and promoter of atherosclerosis [9]. Increasing evidences support the central role of the CD40-CD40L signaling pathway in atherosclerosis, and blocking CD40-CD40L interaction significantly prevents the development of atherosclerotic plaques and reduces the already existing lesions. Some drugs, such as statins and PPAR activators, possess anti-inflammatory properties besides their lipid-lowering effects, which shed light on a new pathway for atherosclerosis therapy[7].

To assess the localization of CD40 transcripts at the cellular level, we quantified CD40 mRNA level in TNF-α -stimulated HUVECs treated with fenofibrate using real-time quantitative PCR, which is one of the most sensitive methods currently known for specific detection of mRNA in isolated cells or small tissue samples. TaqMan real-time PCR exceeds other established methods for RNA quantization such as Northern blot analysis and semi-quantitative RT-PCR for not only its sensitivity but also its rapidity and accuracy [10, 11]. In the present study, we demonstrated that fenofibrate within the concentration range of 1×10^{-5} to 2×10^{-4} mol/L significantly decreased the cell surface expression and mRNA level of CD40 induced by TNF-α (P<0.01). Activation of the vascular cells via the CD40-CD40L signaling pathway interactions has shown to induce inflammatory responses and expression of the adhesion molecules and secretion of the pro-inflammatory cytokines, MMPs, tissue factors and chemokines [12], which are considered as the crucial players in atherogenesis. CD40-CD40L blockade has shown to prevent atherosclerotic plaque progression, promote plaque stability and prevent transplantation-associated vasculopathy, an accelerated form of atherosclerosis [13]. However, studies indicate that PPAR activators have such anti-inflammatory actions *in vitro* and *in vivo* as inhibition of cytokine-induced vascular cell adhesion molecule-1 expression in humam edothehelial cells, and may have beneficial effects against the progression of atherosclerosis in animal models [14]. Recently aspirin and PPAR-alpha activators were reported to inhibit monocyte chemoattractant protein-1 expression induced by high glucose concentration in human endothelial cells [15]. Notably, we found that fenofibrate at the concentration of 2×10^{-4} mol/L did not further decrease the expression of CD40 induced by TNF-α, the underlying mechanism of which needs further exploration.

We also found that the culture supernatants from TNF-α-stimulated cells had a significant increase in gelatinase activities (MMP-2 and MMP-9) in comparison with unstimulated cells. The MMP family probably plays a crucial role in undermining the tissue integrity in an atherosclerotic lesion, favoring plaque rupture and precipitation of unstable coronary syndromes [16]. Human vascular cells *in vitro* express MMP-1, gelatinases A and B (MMP-2, MMP-9) and MMP-3 upon stimulation with soluble mediators. IL-1 and TNF-α induce matrix MMP-3 expression via c-Jun N-terminal kinase in trabecular meshwork and a rheumatoid synovial cell line [17, 18]. Our results showed that preincubation with anti-CD40 antibodies

significantly reversed the upregulated activities of MMP-2 and MMP-9 induced by TNF-α. We also found that fenofibrate significantly diminished the gelatinase activities induced by TNF-α in HUVECs. We therefore conclude that fenofibrate reduces inflammatory response by inhibiting inflammatory signaling pathway of CD40-CD40L expression, and thus stabilizes atherosclerotic plaques, features that are believed to account for the beneficial effects of fenofibrate on cardiovascular morbidity and mortality.

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非诺贝特对 TNF-α 诱导的人脐静脉内皮细胞 CD40 表达和基质金属蛋白酶活性的抑制作用

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摘要:目的 研究非诺贝特对肿瘤坏死因子 -α(TNF-α)诱导的人脐静脉内皮细胞(HUVECs)CD40 表达和基质金属蛋白酶(MMP)活性的作用。方法 应用 RT-PCR 和流式细胞仪分别检测非诺贝特对 TNF-α 诱导的 HUVECs 的 CD40 mRNA 和细胞表面 CD40 表达的影响;用明胶酶谱法测定 TNF-α 对 HUVECs 的 MMP-2、MMP-9 活性的影响以及非诺贝特对它们的作用。结果 非诺贝特在 5×10^{-5}, 1×10^{-4} 和 2×10^{-4} mol/L 的浓度范围内能显著降低 CD40 mRNA 和细胞表面 CD40 的表达 ($P<0.01$),以 1×10^{-4} mol/L 的非诺贝特的效果最为明显;浓度为 2×10^{-4} mol/L 时,非诺贝特并没有进一步降低 CD40 mRNA 和细胞表面 CD40 的表达。非诺贝特能抑制 TNF-α 诱导的 HUVECs 中 MMP-2 和 MMP-9 活性的增加。结论 非诺贝特能降低 TNF-α 诱导的 HUVECs 的 CD40 表达,并且能抑制 TNF-α 诱导的 HUVECs 中 MMP-2 和 MMP-9 活性的增加。

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