Fenofibrate Represses Matrix Metalloproteinase-2 in Endothelial Cells

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Abstract

Fenofibrate is a widely used to ameliorate hyperlipidemia and/or hypercholesterolemia in patients at risk of cardiovascular disease. To date, most of its favorable effects have been attributed to its activation of peroxisome proliferator activated receptor alpha (PPARα), which alters lipid metabolism, contributing to an improved lipid profile. Lines of evidence suggest pleotrophic effects of fenofibrate in cardiovascular system. Given that endothelium is the frontier of vasculature and its dysfunction is prelude of various vascular disorders, e.g. atherosclerosis and arterial stiffening, we investigated the effect of fenofibrate in human endothelial cell (EC) line (Eahy926). Fenofibrate treatment in ECs repressed matrix metalloproteinase-2 (MMP-2) expression in ECs as well as endothelial MMP-2 secretion, associated with decreased MMP-2 activity. In parallel, fenofibrate activated endothelial nitric oxide synthase (eNOS), demonstrated by Ser1177 phosphorylation. Inhibition of eNOS by L-NAME attenuated fenofibrate-suppressed MMP-2, indicating fenofibrate inhibits MMP-2 requires eNOS-derived NO. Collectively, these findings suggest that fenofibrate activates eNOS and increases NO-bioavailability, which in turn suppresses overactivation of MMP-2, contributing to maintenance of endothelial homeostasis. The present study provides a molecular rationale in which fenofibrate exerts vascular protective effects, facilitating the clinical application of its derived medicine. (J Intern Med Taiwan 2010; 21: 270-276)

Key Words ： **Fenofibrate, MMP-2, Endothelial Cell, eNOS, Nitric oxide**

Introduction

Fenofibrate is mainly used to reduce triglycerides levels in patients at risk of cardiovascular disease (CVD), including atheroclerosis and coronary heart disease. Several clinical trials, such as the Diabetes Atherosclerosis Intervention Study (DAIS) revealed that treatment with fenofibrate reduces the angiographic progression of coronary-artery disease in type 2 diabetes[1]. Similar to other fibric acid, it reduces low-density lipoprotein (LDL), very low density lipoprotein (VLDL) and triacylglycerides (TG) levels, whereas increases high-density lipoprotein (HDL) level. Endothelial cells (ECs), constituting the endothelium, the first

layer of blood vessels, provide the interface between the vessel wall and the blood circulation. Endothelial homeostasis exerts various vasoprotective effects, including vasodilation, anti-inflammation, as well as suppression of vascular smooth muscle cell (VSMC) proliferation and hypertrophy. The impaired endothelial homeostasis is an early marker for vascular pathogenesis, leading to atherosclerosis or arterial stiffening[2,3]. To prevent these pathophysiological alterations, two molecular components, i.e. endothelial nitric oxide synthase (eNOS)-derived NO bioavailability and matric metalloproteinases (MMPs), play major roles in maintaining the homeostasis of ECs, and extracellular matrix (ECM), the dynamic interactive milieu between ECs and VSMCs[4,5].

Vascular ECM is tightly regulated by MMPs, a family of zinc-dependent protease, which catalyze the degradation of collagen and other ECM components. Under stimulation such as oxidative stress and plasminogen activators, MMPs are upregulated or activated. i.e. the pro-MMPs converted to active MMPs[6,7]. Lines of evidence suggest a hierarchical pathway in which the eNOS-derived NO modulates MMP activity in the ECs and VSMCs[8,9].

In the present study, we investigated the effects of fenofibrate in ECs, in terms of MMP expression and activity. Due to the role of eNOS in MMP modulation throught its effector NO, we also examined the possible mechanism by which fenofibrate may inhibit overactivation of MMPs, i.e. through posttranslational regulation of eNOS on its activation site Ser-1177. Collectively, our study provides an additional explanation for the vascular protective effects exerted by fenofibrate.

Methods

Antibodies and reagents

N^G-Nitro-L-arginine Methyl Ester (L-NAME) and mouse anti-human MMP-2 monoclonal antibody were purchased from Calbiochem (San Diego, CA). eNOS, and phospho-eNOS (Ser-1177) were purchased from BD Bioscience Pharmingen (San Diego, CA). anti- pan actin Ab-5 antibody (ACTN05) was obtained from NeoMarkers (Fremont, CA). Fenofibrate was obtained from Synkem (France). Gelatin was purchased from Sigma (St Louis, MO).

Cell culture

Eahy926 cell line was obtained commercially from American Type Culture Collection (Rockville, MD) and cultured in medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco). Cells were seeded on 60 mm Petri dishes until ~90% confluence, and the culture medium was then changed to DMEM containing 0.1% BSA prior to experiments.

Western blot analysis

ECs were washed in PBS and lysed with RIPA buffer containing Nonidet P-40 (1%), SDS (0.5%) and protease inhibitor cocktail. Cells were disrupted by intermittent sonication. After centrifugation, the protein level of supernatant was measured using BSA as standard. Cell lysates were then subjected to SDS-PAGE followed by Western blotting. Antigen-antibody complexes were detected by horseradish peroxidase-labeled rabbit anti-mouse or goat anti-rabbit IgG with an ECL detection system (Pierce, Rockford, IL).

Zymographic analysis of MMP-2

Confluent ECs were cultured in serum free DMEM with or without fenofibrate (10 or 50 μl) for 24 hours. An equal amount (30 μl) of conditional culture medium was applied to gel. The activities of MMP-2 were analyzed by gelatin zymography. In brief, the conditional medium was applied to 10 % SDS-polyacrylamide gel containing 0.1 % gelatin. Commercially available MMP-2 was used as a reference. After electrophoresis, gels were incubated in Zymogram Renaturing Buffer (2.5 % triton X-100) for 1 hour, and then equilibrate the

gels in 1X Zymogram developing buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM CaCl₂, and 0.02% Brij-35) at room temperature with gentle agitation for 30minutes. Gels were transferred to fresh 1X Zymogram Developing Buffer and incubated at 37 °C for 24 hours. The gels were stained with 0.5 % coomassie brilliant blue and destained with destaining solution (methanol : acetic acid : water = 50 : 10 : 40). The gels were scanned using the UMAX 1220S scanner with a photo-mask (UMAX Corp.).

RNA isolation and real-time quantitative PCR

Total cellular RNA was extracted from ECs (~1.5 x 10^6 cells) using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's instruction. One microgram of total RNA was reverse-transcribed by M-MuLV reverse transcriptase (Finnzyme, Finland) in a total volume of 20 μl. The reverse transcriptase products were amplified with DyNAmo HS SYBR Green qPCR Kit (Finnzyme, Finland) in the reaction mix containing DyNAmo SYBR Green master mix and primers. Primers were designed for detection of human MMP-2 and GAPDH gene expression, as below:

> For MMP-2,
> forward: 5'-TGGCAAGTACGGCTTCTGTC-3';
> reverse: 5'-TTCTTGTCGCGGTCGTAGTC -3'.
> For GAPDH:
> forward: 5'-GAGTCAACGGATTTGGTCGT -3';
> reverse: 5'-GACAAGCTTCCCGTTCTCAG-3'.

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed by using an independent Student's t-test for two groups of data. P < 0.05 was considered statistically significant.

Results

Fenofibrate downregulates MMP-2 expression in ECs

Because MMP-2 is the predominant MMP
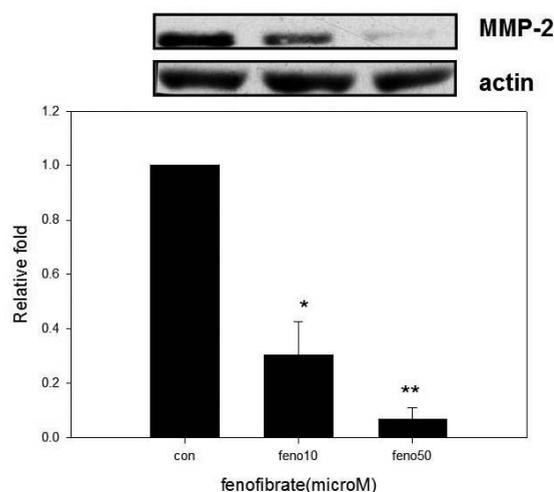
Fig. 1.Fenofibrate decreases MMP-2 protein expression in ECs.
Eahy926 cells were starved in 0.1% BSA DMEM for 16 hours before fenofibrate treatment. Eahy926 cells were cultured in the presence of fenofibrate (0, 10, 50 μM) for 24 hr, and cells were lysed. 50 μg cell lysate was loaded in each lane for Western blotting. The expression of MMP-2 was normalized to the expression of actin. Data are mean ± s.e.m. of three independent experiments.
(* : p value < 0.05   ** : p value < 0.01)

secreted from ECs[10], we examined the expression of MMP-2 in ECs with or withour fenofibrate treatment. As seen in Fig. 1, the MMP-2 protein level was significantly reduced by fenofibrate treatment in a dose-dependent manner. To further examine whether MMP-2 expression is regulated at transcriptional level, the quantitative realtime PCR revealed that MMP-2 mRNA level was also decreased by fenofibrate treatment (Fig. 2), consistent with the protein level change.

Fenofibrate suppresses MMP-2 secretion from ECs

To evaluate whether fenofibrate's inhibitory effect in endothelial MMP-2 expression is associated with decreased MMP-2 secretion as well as suppressed proteinase activity, we detected the MMP-2 released into EC culture medium, using gelatinase zymography. As expected, the activity
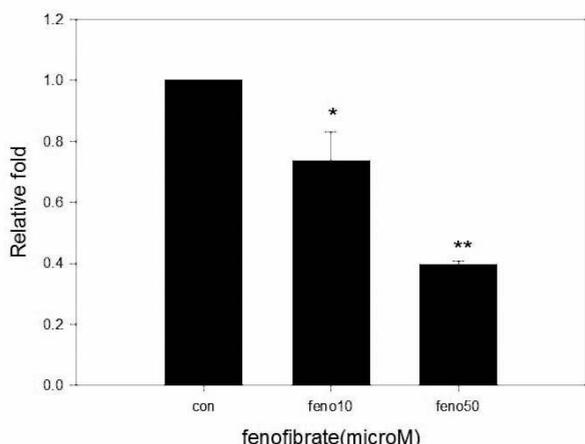
Fig. 2. Fenofibrate inhibits MMP-2 mRNA expression. Eahy926 cells were starved in serum free medium overnight prior to fenofibrate stimulation. Cells were then treated with fenofibrate (0, 10, 50 μM) for 24 hours. RNA was isolated and was converted to cDNA. The expression of MMP-2 mRNA was analyzed by real time PCR. GADPH mRNA expression was used for normalization in real time PCR. Data are mean ± s.e.m. of three independent experiments. (# : p value < 0.05   * : p value < 0.01)
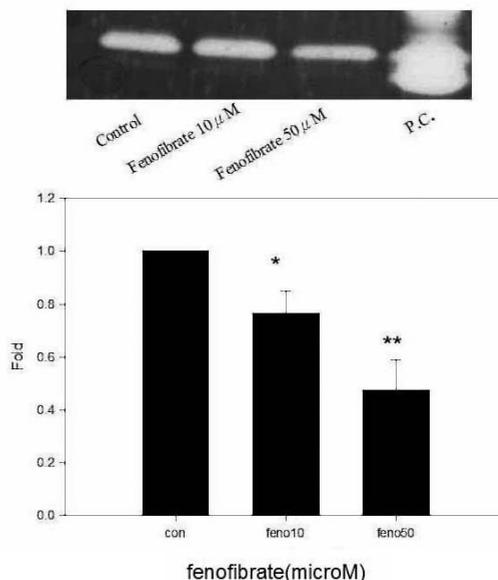
Fig. 3. Fenofibrate suppresses MMP-2 secretion in ECs. Eahy926 cells were starved in 0.1% BSA DMEM for 16 hours before fenofibrate treatment. After cells were treated with fenofibrate (10 or 50 μM) for 24 hr, culture medium was collected and 30 μl culture medium was used in each lane for zymography. Data are mean ± s.e.m. of four independent experiments. (# : p value < 0.05   * : p value < 0.01), P.C. : recombinant MMP-2 as positive control.

of secreted MMP-2 was also decreased in a dose-dependent manner by fenofibrate, as observed in (Fig. 3).

Fenofibrate repressed MMP-2 is requires eNOS-derived NO

To investigate the possible upstream signaling event for MMP-2 repression by fenofibrate, we asked whether NO bioavailability controlled by eNOS may play a central role. First, we detected the phosphorylation of eNOS Ser-1177, which was increased in both a dose-dependent(in a) as well as a time-dependent(in b) manner by fenofibrate treatment (Fig. 4). This increased eNOS phosphorylation indicates the activation of eNOS, which expectedly leads to an enhanced NO production in ECs[11]. Next, to find out whether eNOS-NO is a key component mediating fenofibrate-inhibited MMP-2, L-NAME was used to block the

activation of eNOS in ECs. As shown in (Fig. 5), L-NAME attenuated the fenofibrate-repressed MMP-2 secretion from ECs (in a); as well, L-NAME also reversed the fenofibrate-reduced MMP-2 expression in ECs (in b). Taken together, this finding suggests that fenofibrate requires eNOS-derived NO to repress MMP-2, both in its expression and its activation.

Discussion

In this study, we demonstrated that fenofibrate decreased MMP-2 level in human ECs, which process requires the activation of eNOS and hence enhanced NO bioavailability. Our observation that eNOS Ser-1177 phosphorylation is increased by fenofibrate is consistent with the previous finding that fenofibrate activates AMP-acitvated protein
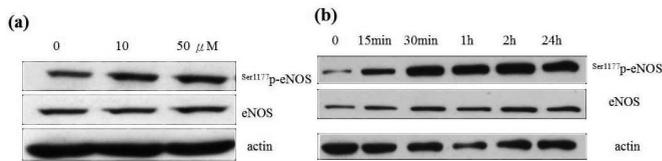
Fig. 4. Fenofibrate treatment increases phosphorylation of eNOS Ser1177:
(a) Eahy926 cells were cultured in serum-free condition for 16 hours before fenofibrate treatment. Eahy926 cells were treated with fenofibrate (0, 10, 50 μM) for 2 hours, and cell lysates were collected. 50 μg cell lysate was used in each lane for western blot analysis. (b) Eahy 926 cells were cultured in serum-free condition for 16 hours. Cells were treated with 50 μM fenofibrate for the time indicated in each lane. Cells were lysed and analyzed by Western blot.

Fig. 5. Fenofibrate mediated MMP-2 suppression is nitric oxide-dependent.
(a) Eahy 926 cells were pretreated with 500 μM L-NAME in serum free medium over night(16hr). Eahy 926 cells were then treated with fenofibrate (10, 50 μM), fenofibrate and L-NAME, or L-NAME for 24hr. Culture medium was collected and analyzed by zymograpghy. (b) Cells were lysed and cell lysate was used in western blotting.

kinase (AMPK) and eNOS phosphorylation in human umbilical vein ECs (HUVECs)[12]. Due to the multiple upstream kinases of eNOS activation, such as protein kinase B/Akt[13,14], it is conceivable that other protein kinases may also be activated by fenofibrate and mediate the activation of eNOS. Meanwhile, eNOS Ser-633 is another critical site, the phosphorylation of which has been suggested to contribute to the sustained activation of eNOS, associated with enhanced NO production[15]. Therefore, it is expectable that this site may also play a role in the repressive effect in MMP-2, due to the relatively slow effects of fenofibrate (24 hr treatment in ECs).

The interplay of NO and MMPs in various contexts have been intensively studied. Our finding that NO negatively regulates MMPs is in line with the previous notion that enhanced NO bioavailability prohibits the pathophysiological activation of MMPs, and thus inhibits ECM degradation and vascular remodeling[8]. Never-theless, there is also evidence implying a positive correlation of NO and MMP function[9]. Actually, NO/reactive nitrogen species can mediate activation and expression of MMPs by modulating cystein residues[16]. One

possible explanation for the seemingly paradox is that under physiological condition, NO is required for the normal function of MMPs and the NO-centered endothelial homeostasis keeps the MMP activation in check. The endothelial dysfunction occurring in metabolic disorder or CVDs is marked by an impaired NO bioavailability. The amelioration exerted by fenofibrate in enhancing NO could attenuate the detrimental effects downstream, such as overactivation of MMPs. However, under patho-physiological context, such as severe oxidative stress, the eNOS uncoupling may result in over-production of reactive nitrogen species, which becomes an undesirable events leading to MMP activation.

Previous study showed hypoxia significantly enhanced MMP-2 expression and activity in EC[17,18]. Studies of circulating level of MMPs and their inhibitors have been conducted previously during
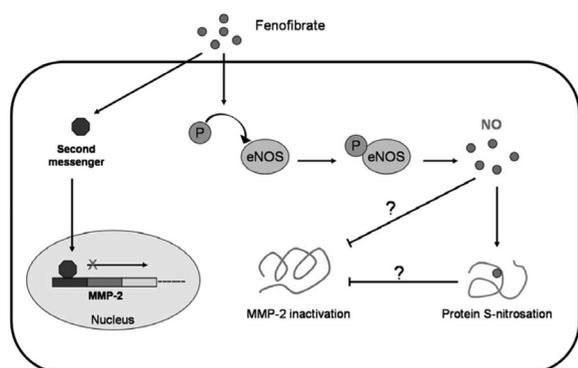
Fig. 6. The hypothetical scheme of fenofibrate su-
ppressed MMP-2 function.
Fenofibrate might triggers second messen-
ger and suppresses MMP-2 transcription.
Fenofibrate can also activates eNOS and
increases nitric oxide bioavailability. MMP-2
is then possibly be S-nitrosated directly and
loss its activity, or maybe, the other proteins
alter their function to inactivate MMP-2 via
S-nitrosation.

hemodialysis. Serum MMP-2 and increased oxidative stress are associated with carotid atherosclerosis in hemodialyzed patients[19]. Our study demonstrated that fenofibrate represses MMP-2 expression in EC and it is reasonable to propose that fenofibrate provides a protective role in EC under pathological conditions, eventhough the contrary effect was reported previously[20,21]. Therefore, the physiological function of MMP-2 raised by fenofibrate between normal and patient could be further discussed.

In this focused study, we provide one clue for the vascular beneficial effects of fenofibrate, in terms of its modulation in MMPs in ECs. The hypothetical scheme was demonstrated (Fig. 6). It is very likely that such effect may also be observed in VSMC, the other key constituent of vasculature. We would expect to observe inhibitory effects in SMC hypertrophy and proliferation, as well as overall preventive effects in atherogenesis, vascular remodeling and arterial stiffness.

Conclusion

In the present study, we demonstrated that fenofibrate, one of the most prominent fibrates, negatively regulates MMP-2 expression as well as activity in ECs. This regulation is largely, if not all, mediated by eNOS-derived NO. Taken together, these findings provide further understanding of beneficial effects provided by fenofibrate in ECs, which may contribute to clinical use of this medicine in reducing CVD risk in patients with metabolic symptoms.

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Fenofibrate抑制內皮細胞基質金屬蛋白酶-2
(Matrix Metalloproteinase-2)之表現

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摘　要

　　Fenofibrate 已廣泛使用於心血管疾病病患之高酯血症。現今之研究發現，fenofibrate經由活化過氧化體增生活化接受體α (Peroxisome Proliferator Activated Receptor type alpha, PPARα)，改變酯質代謝而達到血管保護作用，而多數証據亦證實fenofibrate經由多效作用 (Pleiotropic effect)對血管造成保護作用。以往研究顯示，血管內皮細胞失調對於動脈硬化之成因扮演極重要之角色，本實驗探討fenofibrate對於血管內皮細胞保護作用之機轉。血管內皮細胞經fenofibrate處理後可抑制基質金屬蛋白酶-2之表現、內皮細胞基質金屬蛋白酶-2之分泌與活性。Fenofibrate可經由磷酸化Ser1177造成活化，而經由L-NAME抑制內皮型一氧化氮合成酶(eNOS)可減緩fenofibrate造成抑制基質金屬蛋白酶-2之表現。本實驗證實了fenofibrate抑制基質金屬蛋白酶-2需要一氧化氮(NO)之參與。總之，本研究發現fenofibrate可活化eNOS，增加一氧化氮生物利用度，進而抑制基質金屬蛋白酶-2之表現。本研究提供fenofibrate造成血管保護作用之機轉，期望能衍伸為臨床之應用。