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# Research Article

# Fenofibrate inhibits angiogenesis in vitro and in vivo

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**Abstract.** Fenofibrate, a peroxisome proliferator-activated receptor (PPAR)-alpha activator, used as a normolipidemic agent, is thought to offer additional beneficial effects in atherosclerosis. Since angiogenesis is involved in plaque progression, hemorrhage, and instability, the main causes of ischemic events, this study was designed to evaluate the action of fenofibrate on angiogenesis. Our results show that fenofibrate (i) inhibits endothelial cell proliferation induced by angiogenic factors, followed at high concentrations by an increase in apoptosis, (ii) inhibits endothelial cell migration in a healing wound model, (iii) inhibits capillary tube formation in vitro, and (iv) inhibits angiogenesis in vivo. Concerning the mechanism of action, the inhibition of endothelial cell migration by fenofibrate can be explained by a disorganization of the actin cytoskeleton. At the molecular level, fenofibrate markedly decreased basic fibroblast growth factor-induced Akt activation and cyclooxygenase 2 gene expression. This inhibition of angiogenesis could participate in the beneficial effect of fenofibrate in atherosclerosis.

**Key words.** Angiogenesis; atherosclerosis; fenofibrate; Akt; cyclooxygenase 2; peroxisome proliferator activated receptor.

Angiogenesis is the formation of new blood capillaries from pre-existing vessels and involves endothelial cell proliferation, migration, and organization into capillary tubes. This is a crucial event in several pathologies, such as cancer and inflammatory diseases [1], and it also plays a significant role in the pathogenesis and progression of atherosclerosis. Indeed, angiogenesis, within the atheromatous plaque, has been associated with smooth muscle cell proliferation and is therefore involved in plaque progression [2]. In addition, reported data have consistently underlined a role for angiogenesis in plaque hemorrhage and instability [3, 4]. This instability is responsible for plaque rupture leading to thrombus formation and sub-sequent vascular occlusion responsible for an acute ischemic event (myocardial infarction or stroke) [5]. Therefore, inhibitors of angiogenesis may have potential therapeutic implications in patients with vascular risk, by reducing the progression and the acute complications of atherosclerosis. Vincent et al. [6, 7] and Weis et al. [8] have previously demonstrated that statins (inhibitors of HMG-CoA reductase, commonly used for lowering cholesterol synthesis) inhibit angiogenesis, which may explain, to a certain extent, why the vascular protection of statins is higher than that predicted by cholesterol lowering.

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Clinical studies have indicated that the administration of fibrates, also widely used in the treatment of diet-resistant hyperlipidemia, reduce the cardiovascular events and delay the progression of premature coronary atherosclerosis [9, 10]. The first rationale for the clinical use of fibrate derivatives, in atherosclerotic patients, is the decrease in elevated triglyceride level and the increase in high-density lipoprotein (HDL)-cholesterol level. The mechanism of fibrate action on lipoprotein metabolism involves the activation of peroxisome proliferator-activated receptor-alpha (PPAR-alpha), a member of the nuclear receptor superfamily. Three distinct PPARs exist (alpha, beta or delta, and gamma), which are selectively activated by ligands [11]. Activated PPARs heterodimerize with the retinoid X receptor, and then bind to peroxisome proliferator responsive elements, thereby regulating gene expression. In addition, PPARs can also modulate transcription by interfering negatively with signaling pathways probably by protein-protein interactions [12]. Furthermore, several studies suggest that fibrates might not only reduce atherosclerosis development through their normolipidemic properties, but also by reducing inflammation, at the level of the vascular wall [10, 13–16]. Since vascular endothelial cells express PPAR-alpha [15, 17, 18], we assessed the effect of fenofibrate on angiogenesis. This study demonstrated that fenofibrate decreases angiogenesis both in vitro and in vivo. The cellular mechanism involved in this effect was also investigated.

## Materials and methods

### Chemicals

Recombinant angiogenic factors, basic fibroblast growth factor (bFGF) and oncostatin M (OSM) were supplied by R&D Systems (Abington, UK). Fenofibrate was purchased from Sigma-Aldrich (St Quentin-Favallier, France), and WY-14,643 from Merck Eurolab (Fontenay-sous bois, France), LY-29402, a phosphatidylinositide 3-kinase (PI3K) inhibitor, was purchased from Sigma-Aldrich, and all these compounds were dissolved in ethanol. The final concentration of ethanol in the medium did not exceed 1%, and ethanol was routinely added to the control culture medium at the same concentration.

### Endothelial cell culture

The HMEC-1 cell line was kindly provided by Dr Ades (Center for Disease Control and Prevention, Atlanta, Ga.), who established this cell line by transfecting human dermal endothelial cells with SV40 A gene product and large T-antigen. These cells have properties similar to those of the original primary microvascular endothelial cells [19]. Moreover, HMEC-1 proliferation, migration, as well as capillary tube formation are greatly increased by angiogenic factors such as bFGF, vascular endothelial growth factor (VEGF), and OSM [6, 7, 20], while their proliferation is inhibited by the specific anti-angiogenic agent angiostatin [21]. HMEC-1 cells were used because they are representative of microvascular endothelial cells [22], and because angiogenesis occurs in the micro vasculature, but not in large blood vessels [23]. HMEC-1 cells were cultured in a complete medium containing MCDB131 medium (Sigma) supplemented with 15% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml epidermal growth factor (EGF) (Euromedex, Souffelweyersheim, France) and 1 µg/ml hydrocortisone (Pharmacia-Upjohn, St Quentin en Yvelines, France). HMEC-1 were used before the 15th passage, because after 25 passages, changes in the morphology and functions of endothelial cells would make them unsuitable to study angiogenesis.

### Endothelial cell proliferation assay

For the proliferation assay, EGF was omitted and a lower concentration of FCS (7.5%) was used. Briefly, after trypsinization (0.05% trypsin w/v; Sigma), the HMEC-1 were seeded at a concentration of $5 \times 10^4$ cells per well in a 24-well plate (Nunc, Roskilde, Denmark) and incubated with or without the cytokines (2.5 ng/ml OSM, 25 ng/ml bFGF). Fenofibrate and WY-14,643 were added at concentrations indicated in the results section. After incubation for 3 days, cells were detached by trypsin, and resuspended in Isoton II solution (Coulter, Villepinte, France) to count in a particle counter (Coulter Z1; Coultronics, Margency, France).

### Cytotoxicity

Cells were cultured and treated with fenofibrate or WY-14,643 at the higher concentration used (100 µM) for 3 days. Then cells were collected and cytotoxicity was tested by trypan blue dye exclusion.

### Apoptosis analysis

Endothelial cells, in a complete medium, were treated with fenofibrate or vehicle alone, for 24 h, and the number of apoptotic cells was determined according to Ormerod et al. [24]. Endothelial cells were stained with propidium iodide (PI)/phosphate-buffered saline (PBS) (5 µg/ml; Sigma), and Hoechst 33342 (1 µg/ml, Sigma) for 3 min, and then analyzed using a Coulter Epics V Flow Cytometer with UV laser excitation (100 mW) at 357 and 337 nm. Blue fluorescence (Hoechst 33342-DNA, between 430 and 530 nm) and red fluorescence (PI-DNA, above 630 nm) were measured for each endothelial cell. PI-positive cells were gated out when analyzing apoptosis.

### Cell cycle analysis

Cell cycle determination was performed according to Vindelov's technique [25]. Endothelial cells, in a com-

plete medium, were treated with fenofibrate or vehicle alone for 24 h. Endothelial cells were stained with 1 µg/ml Hoechst 33342 (Sigma) for 30 min at 37°C and the percentage of cells in each cell cycle phase was determined by flow cytometry (EPICS XL-MCL; Coulter).

**Endothelial cell repair of a wound**

Endothelial cells were cultured in a 24-well culture plate. When HMEC-1 cells were confluent, a wound was made under standard conditions. Then, after washing with PBS, cells were incubated for 48 h with MCDB131 containing 2% FCS (concentration of FCS which allows cell survival but not cell proliferation) with or without bFGF (25 ng/ml), and in the absence or presence of fenofibrate or WY-14,643 at different concentrations. After 48 h of incubation, cells were washed twice with PBS and then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were then stained with Giemsa. Cell repair of the wound was determined by measuring the number of cells, in two randomized 1400-µm$^2$ wounded areas, for each condition.

**Capillary tube formation**

1) In a three-dimensional fibrin gel

A microcarrier cell culture was devised according to the method of Nehls and Drenckhahn [26]. In brief, HMEC-1 were allowed to attach to the cytodex-3 microcarrier beads (Sigma). The beads were then embedded in a fibrin matrix, obtained by coagulation of a solution of purified fibrinogen at 8 mg/ml in M131 culture medium (Cascade Biologics, Portland, Me.) containing bFGF (25 ng/ ml) and 5% microvascular growth supplement (MVGS) (Cascade biologics) as source of growth factors, 10% FCS, 1% L-glutamine, and 0.2 mM aprotinin, in the presence or absence of fenofibrate or WY-14,643. After addition of thrombin (2 units/ml, final concentration), fibrin gel was formed, and then 500 µl of complete culture medium containing 10% FCS and 5% MVGS was added and changed every 3 days. Kinetic formation of capillary tubes arising from the periphery of microcarrier beads was observed. These capillaries were photographed with a camera on an inverted microscope.

2) on a Matrigel matrix

Matrigel matrix (11.7 mg/ml; Becton Dickinson, Le Pont-de-Claix, France), was kept in ice, in a liquid form. Then, 200 µl per well of this matrix solution was added to a 24-well culture plate. After gelation, at 37°C for 30 min, the gels were overlaid with 500 µl of complete medium containing $4 \times 10^4$ HMEC-1 cells. The day after, medium was replaced by medium used for the proliferation assay, and cells were incubated with bFGF (25 ng/ml) and with vehicle alone or with fenofibrate. After a 24-h incubation, capillary tubes were photographed.

**MMP-2 and MMP-9 secretion by SDS-polyacrylamide gel electrophoresis zymography**

The supernatants of HMEC-1 incubated for 24 or 48 h, in the absence or presence of fenofibrate with or without bFGF, were collected. Then, 10 µl of each supernatant was loaded on a 7.5% polyacrylamide gel containing 10% sodium dodecyl sulfate (SDS) and 1 mg/ml gelatin under non-reducing conditions and then subjected to electrophoresis. Gels were then washed in 2.5% Triton X-100 for 1 h to remove SDS. Gelatinase activity was revealed by its proteolytic activity after an overnight incubation at 37°C in developing buffer containing 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.6. The gel was then stained with Coomassie brilliant blue R-250 solution (0.25%). Proteolytic activity was detected as clear bands against the blue background of stained gelatin.

**Confocal microscopy analysis of actin filaments**

Confocal microscopy analysis of actin filaments was performed according to the protocol of Menager and colleagues [7, 27], on bFGF-stimulated HMEC-1, after a 48-h incubation with fenofibrate or vehicle alone. Actin filaments were detected by staining with tetramethyl rhodamine isothiocyanate (TRITC)-labeled phalloidin. Computer-assisted image analysis of fluorescence was performed using a confocal microscopy scanning laser microscope (Leica TCS, wavelength excitation 488 nm, emission 525 nm for FITC; 540/570 nm for TRITC).

**Cyclooxygenase 2 mRNA expression**

1) Total RNA extraction

Cells were seeded in a six-well plate (Nunc) until confluent and then incubated for 6 h in the presence or absence of bFGF (25 ng/ml), with fenofibrate or vehicle alone. Cells were then detached by scraping and washed twice in PBS. Total RNA extraction was performed using the SV Total RNA isolation system (Promega, Madison, Wis.) according to the manufacturer's instructions.

2) RT-PCR assay

RT-PCR of cyclocygenase 2 (cox-2) was performed as previously described [28] using oligonucleotide primers (which flank intron DNA) HSCOX2-S (5′-CCGGACAGGATTCTATGGAGA-3′ sense bases 232–252) and HSCOX2-AS (5′-CAATCATCAGGCACAGGAGG-3′ antisense bases 531–512) for the RT-PCR assay. RT-PCR amplification of $\beta$-actin (5′-ATCTGGCACCACACCTT CTA-CAATGAGCTGCG-3′ sense primer; 5′-CGTCATACTCCTGCTTGCTGATCCA-CATCTGC-3′ antisense primer) was performed as control. The cox-2 (300 bp) and the $\beta$-actin (838 bp) cDNA amplification products were analysed in agarose gel (1.5%) electrophoresis using ethidium bromide staining.

**Analysis of Akt activation by Western blot**

Endothelial cells were treated with 25 ng/ml bFGF, in the absence or presence of 100 μM fenofibrate, or 100 μM WY-14,643 or 20 μM LY-29402, for 15 h. Subconfluent cells were lysed with 200 μl of lysis buffer (triton 1%, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin). After sonication and centrifugation, the protein yield was quantified using the Bradford assay [29]. The cell lysates were mixed with sample buffer containing 2-mercaptoethanol and SDS and heated for 5 min at 95 °C. Equal amounts of protein (40 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Amersham, UK). Membranes were immunoblotted overnight with polyclonal anti-Akt or polyclonal anti-phosphoSer 473 Akt antibodies (1:1000, Ozyme, Saint Quentin Yvelines, France), and detected using horseradish peroxidase-conjugated secondary antibody (1:5000; Dako, Trappes, France), and the enhanced chemiluminescence (ECL) detection system (Amersham).

**In vivo angiogenesis assay**

To analyze the anti-angiogenic properties of fenofibrate, a Matrigel model described by Passaniti et al. [30] was used. Briefly, 300 μl of Matrigel (11.7 mg/ml, phenol red free, Becton Dickinson) in liquid form maintained at 4 °C was mixed with or without 1 μg bFGF, with vehicle alone or with fenofibrate (500 μM), and then injected subcutaneously in the dorsa of 7-week-old Swiss *nu/nu* female mice (Janvier, France) using a 24-gauge needle. Each group contained five mice. High concentrations of fenofibrate (5–50 times higher than the efficient in vitro doses) were used because of the large amount of bFGF used to induce a rich vascular network (more than 100 times higher than in the in vitro test), as described by Dellian et al. [31]. Ten days after injection, tissue containing the Matrigel plugs including the adjacent skin were removed, fixed overnight in absolute ethanol and embedded in paraffin. Matrigel sections, 5 μm thick, were prepared. The endogenous peroxidase activity was quenched by 3% $H_2O_2$ for 10 min. Neovessels were visualized by incubation of Matrigel sections with a rat antibody against mouse platelet-endothelial cell adhesion molecule 1 (PECAM-1; Pharmingen, Le Pont-de-Claix, France) and then with a biotinylated goat anti-rat IgG antibody. After washing, sections were incubated with streptavidin-peroxidase and the vessels were revealed by the peroxidase substrate diaminobenzidin. Meyer's hematoxylin was used for counterstaining. The vascularization level around the Matrigel plugs was evaluated as described previously by Weidner et al. [32].

**Statistical analysis**

Significant values were determined using a two-tailed non-parametric Mann-Whitney test using the InStat software (Sigma). The results are expressed as mean value ± SEM. $p < 0.05$ was considered significant.

## Results

**Fenofibrate reduces endothelial cell net growth rate**

Fenofibrate induced a dose-dependant decrease in growth rate of endothelial cells stimulated by 2.5 ng/ml OSM or 25 ng/ml bFGF. From 0.1 to 10 μM fenofibrate, this decrease was significant but slight, whereas at 50 and 100 μM a major inhibitory activity was observed (fig. 1A). In contrast, when cells were not stimulated, the inhibitory effect of fenofibrate on cell growth rate only began from 50 μM fenofibrate. Moreover, when low concentrations of fenofibrate (0.1 and 1 μM) were added to unstimulated endothelial cells, a slight increase in cell growth was observed but it did not remain significant. Interestingly, for 50 μM fenofibrate, the growth of endothelial cells was similar in unstimulated and stimulated cells, indicating that at this concentration, fenofibrate can inhibit the activity of angiogenic factors. This effect of fenofibrate on endothelial cells was associated with cell rounding. Absence of toxicity was assessed by trypan blue dye exclusion. This effect of fenofibrate on endothelial cell net growth rate was then compared to that of another PPAR-alpha activator, WY-14,643. Similar results were obtained with WY-14,643, but it required higher concentrations to inhibit endothelial cell proliferation significantly, as shown in figure 1B. However, WY-14,643 at 100 μM inhibited both unstimulated and bFGF-stimulated endothelial cell proliferation.

**Fenofibrate induces apoptosis and blocks the G1/S transition of the cell cycle**

When HMEC-1 cells were incubated for 24 h with fenofibrate, in a complete medium, a dose-dependent increase in cell apoptosis was observed, which became statistically significant at 50 μM fenofibrate, as shown in figure 1C. In contrast, cell distribution in the different phases of the cell cycle was not affected by concentrations of fenofibrate lower than 50 μM (data not shown), whereas 100 μM fenofibrate significantly blocked the cell cycle in the G0/G1 phase (53 ± 2.1% versus 41.1 ± 1.1% in G0/G1 phase for fenofibrate-treated cells and untreated cells respectively, $p < 0.01$, $n = 4$).

**Fenofibrate inhibits endothelial cell repair of a wound**

After a 48-h incubation, fenofibrate at 10 μM significantly inhibited cell migration in the wound area, in a dose dependent manner, in both bFGF-stimulated and unstimulated endothelial cells (table 1). As already noted for endothelial cell proliferation, 50 μM fenofibrate totally inhibited the effect of growth factors. The PPAR-alpha activator WY-14,643 also significantly inhibited the repair at 50 μM (table 1).

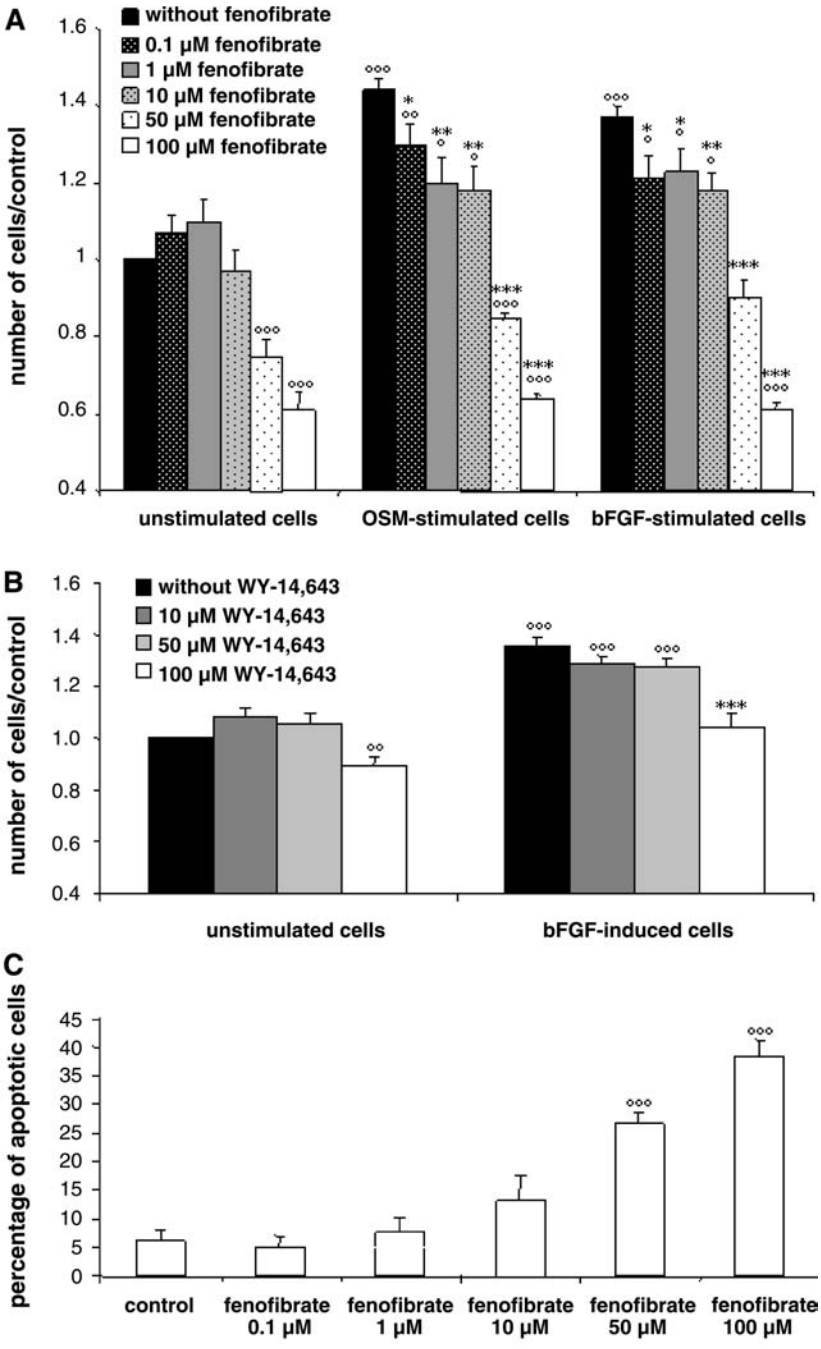


Figure 1. Effects of fenofibrate and WY-14,643 on HMEC-1 net growth rate in the presence or absence of growth factors, and of fenofibrate on apoptosis. (*A*) A-Fenofibrate decreases endothelial cell net growth rate. $5 \times 10^4$ HMEC-1 cells were seeded, then incubated at the indicated concentrations of fenofibrate, in the absence or presence of OSM (2.5 ng/ml) or bFGF (25 ng/ml). Results of six experiments in duplicate are expressed as the ratio of the number of cells counted over the number of cells counted in the control ± SEM after a 3-day incubation (°$p<0.05$, °°$p<0.01$, °°°$p<0.001$ compared with untreated cells; *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with OSM- or bFGF-stimulated cells; n = 12). (*B*) WY-14,643 decreases endothelial cell net growth rate. $5 \times 10^4$ HMEC-1 cells were seeded, then incubated at the indicated concentrations of WY-14,643, in the absence or presence of bFGF (25 ng/ml). Results of six experiments in duplicate are expressed as the ratio of the number of cells counted over the number of cells counted in the control ± SEM after a 3-day incubation (°°$p<0.01$, °°°$p<0.001$ compared with untreated cells, ***$p<0.001$ compared with bFGF-stimulated cells; n = 12). (*C*) Fenofibrate induces apoptosis of endothelial cells. HMEC-1 cells, in a complete medium, were treated for 24 h with fenofibrate (0.1, 1, 10, 50, and 100 µM). The number of apoptotic cells was determined by flow cytometry. Autofluorescence of endothelial cells was systematically corrected for the percentage of Hoechst 33342-stained cells. Results of six experiments in duplicate are expressed as the percentage of apoptotic cells in each well ± SEM (°°°$p<0.001$ compared with untreated cells; n = 12).

Table 1. Effect of fenofibrate and WY-14,643 on endothelial cell migration, in a wound-healing model, after a 48-h incubation.

| | Number of migrated cells | |
|---|---|---|
| | unstimulates cells | bFGF-stimulated cells |
| Control | 228 ± 11 | 465 ± 67°°° |
| Fenofibrate 10 μM | 143 ± 36° | 308 ± 32* |
| Fenofibrate 50 μM | 155 ± 19°° | 168 ± 25°, *** |
| Fenofibrate 100 μM | 105 ± 5°°° | 127 ± 14°°°, *** |
| WY-14,643 10 μM | 232 ± 38 | 458 ± 13°°° |
| WY-14,643 50 μM | 188 ± 10° | 188 ± 18*** |
| WY-14,643 100 μM | 137 ± 28°° | 187 ± 15°, *** |

A wound was performed on confluent endothelial cells which were then incubated with or without bFGF (25 ng/ml) in the absence or presence of fenofibrate or WY-14,643. Results of three experiments in duplicate show counted cells (mean ± SE) that migrated in a 1400-$\mu m^2$ wounding area after a 48-h incubation. (°$p<0.05$, °°$p<0.01$, °°°$p<0.001$ compared with untreated cells; *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with bFGF-stimulated cells).

### Fenofibrate inhibits capillary tube formation in a fibrin matrix and on Matrigel

The presence of fenofibrate, at 50 μM, significantly reduced the formation and length of capillary tubes, and at 100 μM, the formation of capillary tubes was totally inhibited (fig. 2, panels 3, 4). No obvious inhibition was observed at lower concentrations (fig. 2, panel 2). In a similar manner, WY-14,463 also significantly inhibited capillary tube formation, in a fibrin matrix, at 50 μM, and totally at 100 μM (fig. 2, panels 5, 6). The inhibition of capillary tube formation by fenofibrate at 50 and 100 μM, in a fibrin gel, was also observed with another microvascular endothelial cell lines (human bone marrow microvascular endothelial cells, data not shown).
Similarly, fenofibrate at 50 and 100 μM significantly inhibited capillary tube formation on a basement membrane Matrigel (data not shown).

### Fenofibrate does not modify MMP-2 and MMP-9 secretion by endothelial cells

The incubation of endothelial cells with fenofibrate for 24 or 48 h did not modify the proteolytic activity of supernatants of endothelial cells (data not shown).

### Fenofibrate induces actin depolymerization

In the absence of fenofibrate, actin filaments were organized in stress fibers, extending over the cytoplasm of endothelial cells, and cell spreading with focal adhesion sites was observed (fig. 3A). The presence of 50 μM and 100 μM fenofibrate for 48 h inhibited the formation of actin filaments (fig. 3C, D). This might also explain the cell rounding and the decrease in cell spreading on culture plates that were observed when HMEC-1 were incubated with 50 and 100 μM fenofibrate.

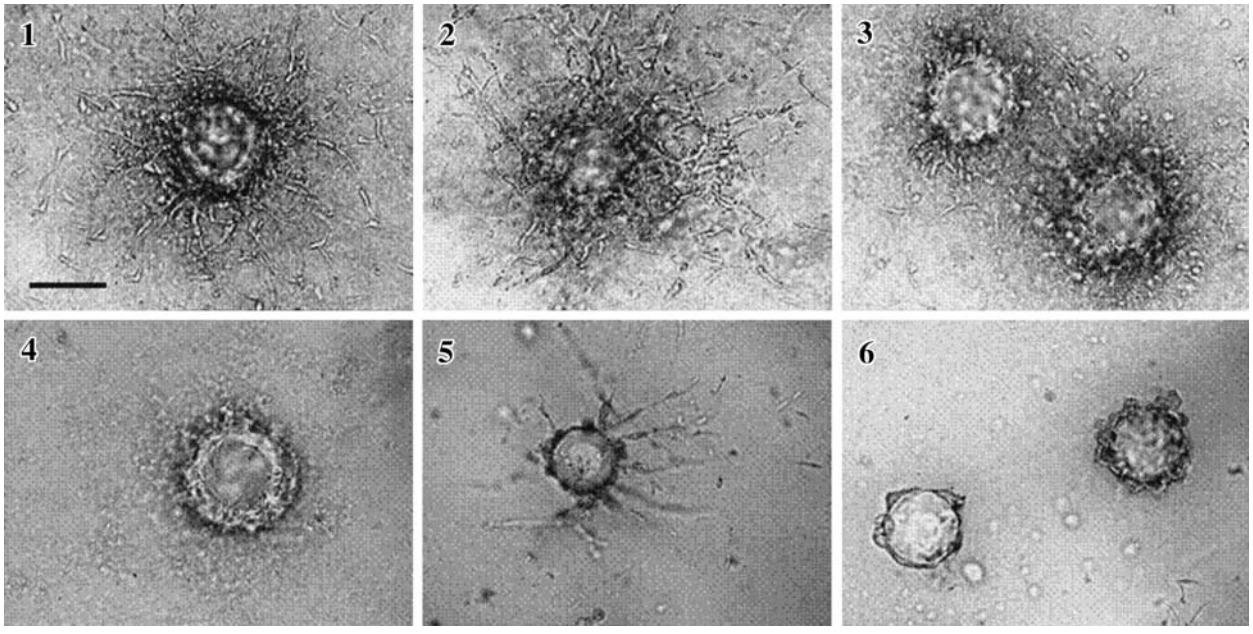


Figure 2. Effects of fenofibrate and WY-14,643 on capillary tube formation in a three-dimensional fibrin gel. Fenofibrate inhibits capillary tube formation, in a three-dimensional gel of fibrin. Comparison with the effect induced by WY-14,643. Formation of capillary tubes arising from the periphery of microcarrier beads, in a fibrin gel, was observed in the presence of different concentrations of fenofibrate or WY-14,643. Panel 1, control; panel 2, fenofibrate 10 μM; panel 3, fenofibrate 50 μM; panel 4, fenofibrate 100 μM; panel 5, WY-14,643 50 μM; panel 6, WY-14,643 100 μM (scale bar, 150 μm).

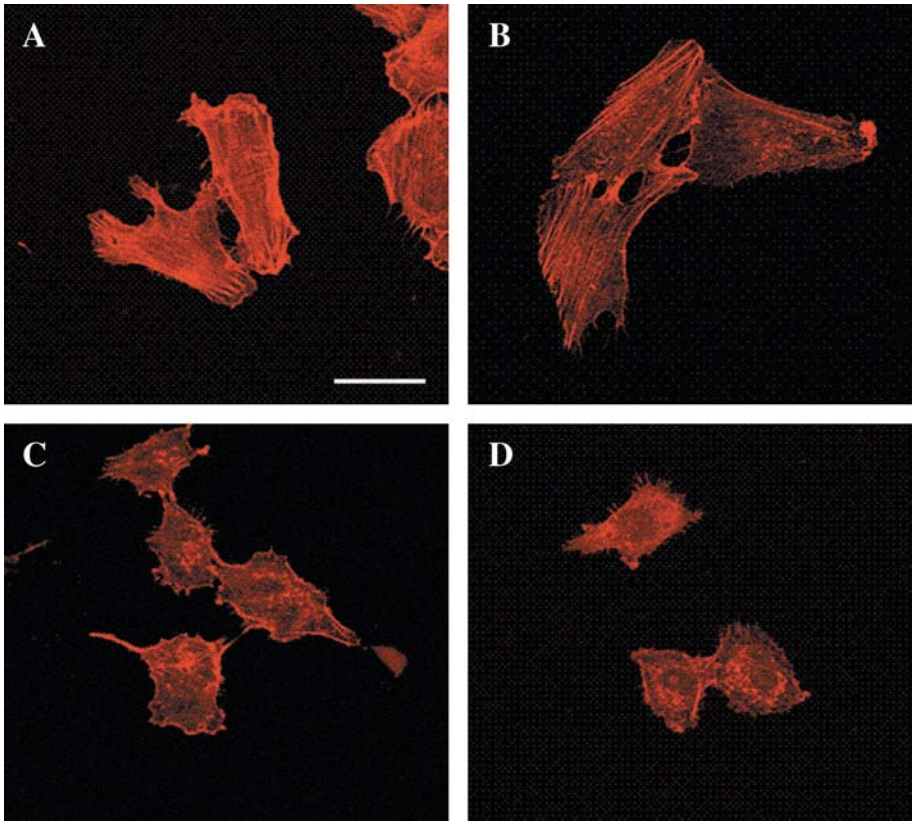


Figure 3. Effect of fenofibrate on the actin stress fibers. The organization of actin cytoskeleton of bFGF-stimulated endothelial cells was analyzed by staining actin fibers with TRITC-labeled phalloidin and subsequent confocal microscopy observation, after a 48-h incubation with fenofibrate (scale bar, 100 μm). (*A*) Control. (*B*) Fenofibrate 10 μM. (*C*) Fenofibrate 50 μM. (*D*) Fenofibrate 100 μM.

**Fenofibrate inhibits cox-2 mRNA expression**

In unstimulated endothelial cells, the cox-2 basal transcript was very low and not influenced by fenofibrate (fig. 4A). In contrast, when cells were stimulated by bFGF, a significant increase in cox-2 mRNA expression was observed. This bFGF-induced cox-2 expression was dose dependently inhibited by fenofibrate (from 10 μM). The effect of bFGF was totally inhibited at 100 μM fenofibrate (fig. 4A).

**Fenofibrate decreases the phosphorylations of Akt (phosphoSer 473)**

Fenofibrate at 100 μM induced a major decrease in Akt phosphorylation, without modification of the total amount of Akt, as shown by Western blot analysis (fig. 4B). WY-14,643 at 100 μM also reduced Akt phosphorylation, without affecting the total amount of Akt (fig. 4B). As a control, LY-29402, an inhibitor of PI3K, also inhibited Akt phosphorylation, at Ser 473.

**Fenofibrate inhibits bFGF-induced angiogenesis in vivo, in the Matrigel mouse model**

Matrigel containing 1 μg bFGF exhibited an abundant and continuous vascular network in the connective tissue around the Matrigel plugs (table 2). In contrast, mice which received Matrigel containing bFGF and 500 μM fenofibrate developed a limited number of discontinuous vessel spots around the Matrigel plugs (table 2). In the control and fenofibrate groups, in the absence of bFGF, only few vessels were detected, as shown in table 2.

**Discussion**

Fenofibrate, a PPAR-alpha activator, has demonstrated ability to slow atherosclerosis progression and to prevent the occurrence of cardiovascular events [10, 33]. Recent evidence suggests that beneficial effects of fenofibrate may extend beyond its effects on lipoprotein metabolism. As endothelial cells express PPAR-alpha, we have tested the effect of fenofibrate on angiogenesis, because it contributes to both plaque progression and complications. Fenofibrate was tested at concentrations found in the plasma of patients treated with fenofibrate at therapeutic doses [34]. Moreover, the same ranges of fenofibrate concentration have been used in the literature to analyze its effect on cell function [15, 35, 36].

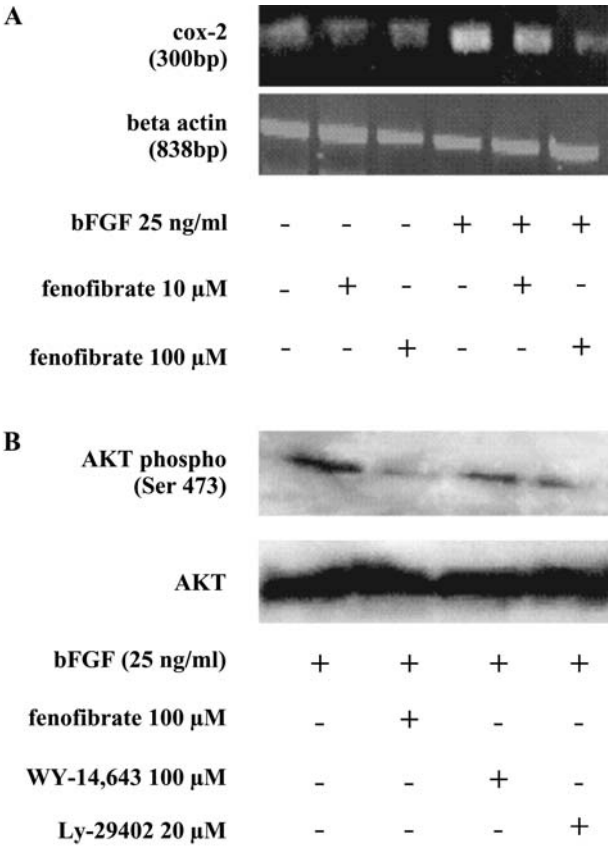


Figure 4. Effects of fenofibrate on cox-2 mRNA expression and Akt-Ser 473 activation. (*A*) Fenofibrate inhibits cox-2 mRNA expression. Endothelial cells were seeded in a six-well plate, and incubated for 6 h in the absence or presence of bFGF (25 ng/ml), at different concentrations of fenofibrate. Total RNA extraction was followed by a RT-PCR of cox-2 and $\beta$-actin. (*B*) Fenofibrate inhibits Akt-Ser 473 activation. Cellular extracts of HMEC-1 were analyzed by Western blot using polyclonal anti-Akt and polyclonal anti-phosphoSer 473 Akt antibodies. Blots were developed with the ECL reagent. The effect of fenofibrate (100 µM), WY-14643 (100 µM), LY-29402 (20 µM) on Akt phosphorylation was studied after a 12-h incubation in the presence of bFGF (25 ng/ml).

Table 2. Effect of fenofibrate on angiogenesis, in vivo, in a matrigel model.

| | Angiogenic index | |
|---|---|---|
| | without bFGF | with bFGF (1 µg) |
| Control | 2.33 ± 0.931 | 14.00 ± 0.447°°° |
| Fenofibrate (500 µM) | 2.67 ± 0.516 | 5.67 ± 0.683°, *** |

Matrigel, kept on ice, was mixed with or without bFGF (1 µg per 300 µl), with vehicle alone or containing fenofibrate (with a final concentration of fenofibrate in Matrigel of 500 µM). Three hundred microliters of these mixtures was subcutaneously injected into mice, each group containing five 7-week-old Swiss *nu/nu* female mice. After 10 days, matrigel plugs were removed, and an immunohistochemical analysis using an antibody against mouse PECAM-1 was performed. Angiogenesis was then quantified according to Weidner's method, which defines an angiogenic index corresponding to the mean of vessels counted in each areas studied (three areas per mouse). Results are expressed as the mean of the angiogenic index of each group ± SEM (°$p<0.05$, °°°$p<0.001$ compared with the control group without bFGF and without fenofibrate; ***$p<0.001$ compared with bFGF-enriched matrigel plugs).

Our results demonstrate that at pharmacological concentrations, fenofibrate is an inhibitor of angiogenesis. This inhibition was first attributed to a significant and dose-dependent inhibition of endothelial cell net growth rate. Its effect was more obvious when endothelial cells were stimulated by angiogenic growth factors (bFGF or OSM [20, 37]). From 50 µM fenofibrate, similar cell growth rates were observed in both unstimulated and stimulated endothelial cells. This was due to a dose-dependent increase in apoptosis, and also from 100 µM, to an arrest in the cell cycle in the G0/G1 phase. Secondly, fenofibrate also induced a dose-dependent inhibition of repair of a wound made in confluent endothelial cells. This inhibition was mostly related to an inhibition of cell migration, as in the experimental conditions used (low concentration of FCS), the contribution of proliferation to wound repair is very slight. However, participation of the apoptotic action of fenofibrate, mostly at concentrations higher than 50 µM, cannot be excluded. This inhibitory action on endothelial cell migration was analyzed. It did not affect MMP-2 and MMP-9 secretion, proteases which are critically involved in this process. The fenofibrate-decreased wound repair was explained by an inhibition of actin polymerization. Indeed, actin polymerization generates protusions and contractile forces required for cell migration.

This inhibition of endothelial cell migration by fenofibrate is consistent with the recent work of Goetze et al. [38], who observed a similar activity on human umbilical vein endothelial cells, representative of the macrovasculature. However, in our study, microvascular endothelial cells were used, because angiogenesis occurs in the microvasculature and not in large blood vessel [23].

These inhibitions of endothelial cell proliferation and migration are relevant to explain the inhibition of angiogenesis in in vitro models (capillary tube formation), and in vivo models (bFGF-enriched Matrigel plugs).

The molecular mechanism by which fenofibrate inhibits angiogenesis was then investigated. As the inhibitory effect mainly occurred when endothelial cells were stimulated by angiogenic factors, fenofibrate might act by preventing the growth factor stimulatory effect. The effect of fenofibrate was therefore tested on cox-2 gene expression and Akt activation, which are both involved in angiogenic factor-induced angiogenesis [28, 39–44]. Cox-2, an immediate early response gene, induced by a variety of growth factors, is inhibited by fenofibrate in bFGF-stimulated HMEC-1. This inhibition of cox-2 transcription was not restricted to endothelial cells as it was also previously reported for PPAR-alpha activator in smooth muscle cells [45], and macrophages [46]. Our study also in-

dicated that fenofibrate inhibited the bFGF-induced Akt activation, as shown by the decrease in the phosphorylated form at Ser 473. This inhibition could also contribute to the anti-angiogenic activity of fenofibrate because activated Akt has been reported to contribute to endothelial cell growth, prevention of apoptosis, and endothelial cell migration [47–49]. Indeed, Akt action on cell migration was reported as due to a remodeling of the actin cytoskeleton into stress fibers [50]. Therefore, the inhibition of Akt activation may also explain the disorganization of actin stress fibers by fenofibrate. This inhibition of Akt activation by PPAR-alpha activators was also reported by Goetze et al., who used stimulated human umbilical vein endothelial cells [38].

As the inhibition of endothelial cell proliferation, migration, and capillary tube formation by fenofibrate was also observed with WY-14,643, another PPAR-alpha activator, the activation of PPAR-alpha by fenofibrate may be involved in its anti-angiogenic activity. However, a molecular mechanism independent of PPAR-alpha has also to be considered. Further investigations are required to determine the precise role of PPAR-alpha in the effects of fenofibrate.

In conclusion, this study demonstrated that fenofibrate induces an anti-angiogenic effect both in vitro and in vivo. This inhibition was associated with actin stress fiber depolymerization, inhibition of Akt activation, and inhibition of cox-2 mRNA expression. As angiogenesis is critically involved in atherosclerotic plaque growth and instability, this anti-angiogenic activity could account for the greater than expected beneficial effects of fenofibrate for atherosclerosis treatment and prevention of vascular ischemic events.

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