Effects of Fibrate Compounds on Expression of Plasminogen Activator Inhibitor-1 by Cultured Endothelial Cells

Lennart Nilsson, Toshiya Takemura, Per Eriksson, Anders Hamsten

Abstract—The consistent positive correlation between triglyceride and plasminogen activator inhibitor-1 (PAI-1) levels in plasma and the fact that very low density lipoprotein (VLDL) induces secretion of PAI-1 from cultured human umbilical vein endothelial cells (HUVECs) and human hepatoblastoma cells have raised the question of whether fibrate treatment, the main effect of which is a profound lowering of plasma concentrations of VLDL, might improve fibrinolytic function by reducing the plasma levels of PAI-1. However, the findings of controlled clinical trials using various fibrate compounds have been discrepant. ECs express PAI-1 under normal conditions in humans. We therefore examined the effects of several fibrate compounds on PAI-1 expression and secretion by cultured HUVECs and the HUVEC-derived cell line EA.hy926. All fibrate compounds examined had significant effects on PAI-1 gene transcription in the EA.hy926 cells. Low concentrations of clofibrate acid and bezafibrate increased PAI-1 transcription and secretion, whereas Wy-14643 increased PAI-1 synthesis in a dose-dependent way. In contrast, both fenofibric acid and gemfibrozil markedly decreased PAI-1 transcription and secretion from HUVECs and EA.hy926 cells. Thus, stimulation of the transcriptional activity of the PAI-1 gene by some fibrates is linked to increased secretion of PAI-1 protein by the cells, whereas the opposite effects occur with other fibrate compounds. Whether the different effects on PAI-1 transcription and secretion by ECs in vitro also reflect differences in treatment effects on the regulation of plasma PAI-1 activity in vivo will have to be determined in larger-scale, controlled clinical trials. (Arterioscler Thromb Vasc Biol. 1999;19:1577-1581.)

Key Words: PAI-1 ■ fibrates ■ endothelial cells ■ transcriptional activity

Fibrates are widely used in the treatment of some forms of diet-resistant hyperlipidemia. The main effect consists of a profound lowering of the plasma concentrations of VLDL lipids and a moderate rise in HDL cholesterol.1 In addition, fibrates have favorable effects on blood coagulation and global fibrinolytic function, which may be at least partly mediated by the lowering of plasma concentrations of triglyceride-rich lipoproteins.2 The consistent positive correlation between triglyceride and plasminogen activator inhibitor-1 (PAI-1) levels in plasma and the fact that VLDL induces secretion of PAI-1 from cultured human umbilical vein endothelial cells (HUVECs)3–5 and human hepatoblastoma cells have raised the question of whether fibrate treatment, the main effect of which is a profound lowering of plasma concentrations of VLDL, might improve fibrinolytic function by reducing the plasma levels of PAI-1. However, the findings of controlled clinical trials using various fibrate compounds have been discrepant. ECs express PAI-1 under normal conditions in humans. We therefore examined the effects of several fibrate compounds on PAI-1 expression and secretion by cultured HUVECs and the HUVEC-derived cell line EA.hy926. All fibrate compounds examined had significant effects on PAI-1 gene transcription in the EA.hy926 cells. Low concentrations of clofibrate acid and bezafibrate increased PAI-1 transcription and secretion, whereas Wy-14643 increased PAI-1 synthesis in a dose-dependent way. In contrast, both fenofibric acid and gemfibrozil markedly decreased PAI-1 transcription and secretion from HUVECs and EA.hy926 cells. Thus, stimulation of the transcriptional activity of the PAI-1 gene by some fibrates is linked to increased secretion of PAI-1 protein by the cells, whereas the opposite effects occur with other fibrate compounds. Whether the different effects on PAI-1 transcription and secretion by ECs in vitro also reflect differences in treatment effects on the regulation of plasma PAI-1 activity in vivo will have to be determined in larger-scale, controlled clinical trials. (Arterioscler Thromb Vasc Biol. 1999;19:1577-1581.)

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individuals, whereas ECs and adipose tissue express PAI-1 under normal conditions in humans. The relative contribution of different cells and tissues to the PAI-1 contained in plasma may also differ between individuals, depending on the presence of obesity, hyperlipidemia, inflammation, and the degree of insulin resistance. This, in turn, could account for the heterogeneous PAI-1 responses in clinical trials. Against this background, we examined the effects of several fibrate compounds on PAI-1 expression and secretion by cultured ECs.

Methods

Materials

Wy-14643 and BRL-49653 were kind gifts from Dr Björn Dahllöf (Astra-Hässle, Möndal, Sweden), bezafibrate from Dr F. Hammerstein (Boehringer Mannheim GmbH, Mannheim, Germany), fenofibric acid from Dr Alain Mnoz (Laboratoires Fournier, Diac, France), and gemfibrozil from Dr Carol Germain (Parke-Davis, Ann Arbor, Mich). Clofibrate acid was purchased from Sigma Chemical Co. All fibrates and BRL-49653 were dissolved in dimethyl sulfoxide (DMSO).

Cell Culture

HUVECs were isolated from umbilical cords obtained at normal deliveries with permission from the local ethics committee. The umbilical vein was then cannulated and perfused with 50 mL PBS to remove any blood, after which the vein was filled with 20 mL of 0.1% collagenase dissolved in PBS and incubated for 15 minutes at 37°C. The collagenase solution was drained from the cord and collected, and the cord was gently flushed with 20 mL PBS, which was added to the collagenase solution. The cells in these pooled solutions were recovered by centrifugation at 200g for 5 minutes and seeded out on 9-cm culture dishes in M199 medium with 20% FCS, antibiotics/antimycotics (Sigma A-9909), and 25 µg/mL EC growth supplement (Sigma E-2759). The cells were subcultured when confluent onto 0.2% gelatin (in PBS)-coated dishes. Cells from pooled, multiple cords were used for experiments until the fourth passage. The endothelium-derived cell line EA.hy926 (a kind gift from Dr C.-J.S. Edgell, University of North Carolina, Chapel Hill) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, supplemented with 10% FCS, 100 µmol/L hypoxanthine, 0.4 mmol/L aminopterin, 16 mmol/L thymidine, penicillin, and streptomycin as described.

Determination of PAI-1 Protein Production

Semiconfluent cultures of HUVECs or EA.hy926 cells were incubated for 8 to 10 hours in M199 or DMEM medium, respectively, containing either 1% charcoal-treated FCS or 2% untreated FCS. This incubation was followed by a 14-hour incubation with fibrates added in the same type of medium. After the conditioned medium was collected and centrifuged at 10,000 rpm for 5 minutes, the PAI-1 protein concentration in the medium was quantified using an ELISA (TintELIZE PAI-1, Bioool), which detects active and inactive (latent) forms of PAI-1, as well as tissue plasminogen activator/PAI-1 complexes. The cells were trypsinized and counted. PAI-1 production was expressed as a percentage of control (vehicle treatment).

Statistical Methods

For each drug, differences in the effects of various concentrations of fibrate compounds on PAI-1 protein production and PAI-1 promoter activity were tested by ANOVA, with the Scheffe test used as a post hoc test.

Results

Effects of Fibrates on PAI-1 Production and mRNA Levels in ECs

Fibrates were incubated with HUVECs or with the HUVEC-derived cell line EA.hy926, and PAI-1 that accumulated in the culture medium was measured using an ELISA. Because the nuclear hormone receptor, peroxisome proliferator-activated receptor-α (PPARα), is activated by fibrates, we also studied the effects of the thiazolidinedione compound BRL-49653. Thiazolidinediones activate PPARγ, and BRL-49653 was used to further substantiate the notion that any suppression of PAI-1 production obtained with fibrates in ECs could be linked to PPAR activation. Influences on PAI-1 accumulation are summarized in Table 1. Because identical results were obtained when cells were incubated with medium containing either 1% charcoal-treated or 2% untreated FCS, experiments were pooled. Clofibrate acid, bezafibrate, and Wy-14643 increased the PAI-1 production from both cell types. The increase in PAI-1 production with clofibrate acid and bezafibrate occurred at fairly low concentrations and disappeared at higher concentrations of the compounds. Wy-14643, on the other hand, produced a graded increase in PAI-1 production from the ECs with increasing concentrations of the compound. In contrast, fenofibric acid, gemfibrozil, and BRL-49653 all produced a marked decrease in PAI-1 production. The MTT assay showed that cell viability was unaffected by the fibrates and BRL-49653 concentrations used in these experiments. However, higher fibrate concentrations markedly decreased cell viability. The basal production of PAI-1 as seen in the control (DMSO added) was 100 to 120 ng/10⁶ cells and 20 to 30 ng/10⁶ cells in HUVECs and EA.hy926 cells, respectively.
Effects of Fibrates on PAI-1 Transcription

A transfection assay was performed using a 804-bp fragment of the PAI-1 promoter coupled to the CAT gene. As summarized in Table 2, clofibrate acid, bezafibrate, and Wy-14643 significantly increased PAI-1 transcription in EA.hy926 cells. For clofibrate acid and bezafibrate, this effect occurred at a fairly low concentration of the compound and disappeared at higher concentrations. The opposite effect was obtained with fenofibric acid, gemfibrozil, and BRL-49653 (Table 2).

Discussion

This study shows that individual fibrate compounds have different effects on PAI-1 secretion by cultured ECs and that these differences are accounted for by different effects on PAI-1 transcription.

The diverse effects of fibrate compounds on EC expression of PAI-1 observed in the present study contrast with the uniform, dose-dependent suppressive effect of fibrates on PAI-1 synthesis seen in cultured cynomolgus monkey hepatocytes. The molecular mechanisms underlying these differences remain unknown. The regulatory mechanism by which the fibrate effect on PAI-1 expression is exerted in the hepatocytes is indicated to involve activation of PPARα, retinoid X receptor-α. PPAR expression in HUVECs and EA.hy926 cells is unknown. However, the diverse effects of fibrates indicate that PAI-1 regulation in ECs by fibrate compounds is not solely mediated by PPARα. Furthermore, the lowering effect of the thiazolidinedione BRL-49653 on PAI-1 transcription and secretion by HUVECs and EA.hy926 cells is unknown. However, the diverse effects of fibrates indicate that PAI-1 regulation in ECs by fibrate compounds is not solely mediated by PPARα.
TABLE 2. Effect of Different Fibrates and BRL-49653 on PAI-1 Promoter Activity (CAT Activity) in EA.hy926 Cells

<table>
<thead>
<tr>
<th>Fibrate</th>
<th>Concentration, μmol/L</th>
<th>CAT Activity, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofibrate</td>
<td>0</td>
<td>100.0±11.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>137.3±5.7*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>113.2±20.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>105.7±28.8</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>0</td>
<td>100.0±7.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>121.6±2.6†</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>101.9±1.3</td>
</tr>
<tr>
<td>Wy-14643</td>
<td>0</td>
<td>100.0±15.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>199.1±5.2‡</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>0</td>
<td>100.0±13.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>67.9±4.6*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>62.7±18.6*</td>
</tr>
<tr>
<td>BRL-49653</td>
<td>0</td>
<td>100.0±9.6</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>90.0±3.6</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>59.8±16.8*</td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>0</td>
<td>100.0±3.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>89.3±2.1†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>73.6±4.1†</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>69.7±2.9‡</td>
</tr>
</tbody>
</table>

Results (mean±SD) are given as % of control. Results are based on 3 experiments performed in triplicate. For each drug, differences in effects of varying concentrations were tested by ANOVA. *P<0.05, †P<0.01, ‡P<0.001 compared with control (after correction for β-galactosidase activity) using the Scheffé post hoc test.

HUVECs and EA.hy926 cells suggests that PPARγ is implicated in regulating PAI-1 expression in ECs. Some differences in experimental procedures also need to be considered. Higher fibrate concentrations, longer incubation times, and 10% bovine serum–supplemented medium (vol/vol) were used in the cynomolgus monkey hepatocyte studies by Arts et al. In contrast, we used cells that were incubated with medium containing 1% charcoal-treated FCS or 2% untreated FCS as well as lower fibrate concentrations to avoid the confounding effects of a range of serum substances known to induce PAI-1 and to optimize the conditions for demonstrating differential effects of individual fibrate compounds. It is notable in this context that opposite effects of gemfibrozil and bezafibrate on PAI-1 secretion have been demonstrated in HepG2 cells incubated in serum-free medium.

The concept that fibric acid derivatives could improve fibrinolytic function by lowering plasma PAI-1 activity originates from the fact that fibrates markedly lower the concentrations of VLDL and from the observation that a strong, positive correlation exists between triglyceride and PAI-1 levels in plasma. Furthermore, VLDL induces PAI-1 secretion from cultured HUVECs and human hepatoblastoma (HepG2) cells. However, clinical trials have demonstrated that fibrates producing a comparable triglyceride lowering have widely different effects on plasma PAI-1 activity. This finding in vivo along with previous cell biological studies and the present in vitro data from ECs strongly indicate that any fibrate effects on plasma PAI-1 activity in humans are at least partly a result of direct effects of the drug on PAI-1 synthesis in liver cells and/or ECs. The question then arises as to whether the diverse fibrate effects on PAI-1 expression in ECs observed in the present study could explain some of the discrepancies between the previous clinical trials. Clearly, inferences from cell culture studies to the situation in vivo should, for several reasons, be made with caution. PAI-1 synthesis occurs in a number of different cell types in culture, including ECs, hepatocytes, smooth muscle cells, and adipocytes, and is regulated by a large number of substances (reviewed in Reference 25). However, the behavior of cultured cells may not be identical to that of the same cell type in vivo. Furthermore, metabolic perturbations and the relative importance of different regulatory mechanisms may differ between participants in various clinical trials. These restrictions notwithstanding, some reflections can be made. Gemfibrozil has been indicated to reduce plasma PAI-1 activity in patients with primary hypertriglyceridemia and in survivors of myocardial infarction. This compound also markedly decreased PAI-1 transcription and secretion from HUVECs and EA.hy926 cells in the present study and has previously been shown to suppress PAI-1 synthesis in HepG2 cells and cultured cynomolgus monkey hepatocytes. Bezafibrate, on the other hand, seems not to lower PAI-1 and even tended to increase plasma PAI-1 activity in the BECAIT study (A.H. et al, unpublished data, 1999). In vitro in ECs, bezafibrate increased PAI-1 transcription and secretion at lower concentrations but had no effect at higher concentrations.

In summary, individual fibrate compounds have diverse effects on PAI-1 expression in ECs, the molecular mechanisms of which remain unknown. Whether the different effects on PAI-1 transcription and secretion by ECs in vitro also reflect differences in treatment effects on the regulation of plasma PAI-1 activity and global fibrinolytic function in vivo in humans will have to be determined in larger-scale, controlled clinical trials comparing different fibrates.

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