Direct Effects of Gemfibrozil on the Fibrinolytic System

Diminution of Synthesis of Plasminogen Activator Inhibitor Type 1

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Background. Platelet-associated epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) can augment synthesis of plasminogen activator inhibitor type 1 (PAI-1). Accordingly, exacerbation of atherogenesis may accompany release of platelet-associated growth factors (or mitogens) occurring in association with occult, repetitive thrombosis and thrombolysis. In the Helsinki primary prevention trial, gemfibrozil decreased coronary events but did so essentially only in initially hypertriglyceridemic subjects. Such subjects are known to exhibit high concentrations of PAI-1 in plasma.

Methods and Results. To determine whether pharmacological concentrations of gemfibrozil directly affect PAI-1 synthesis, we characterized its effects on a human hepatoma cell line (Hep G2) in vitro. Gemfibrozil decreased basal PAI-1 secretion by 43% and attenuated the augmentation of PAI-1 synthesis over 24 hours induced by EGF and TGF-β by 37% and 39% without altering overall protein synthesis. Furthermore, it blocked the EGF and TGF-β-induced increases in PAI-1 mRNA over 6 hours by 65% and 60%. Increases in plasma PAI activity induced by infusion of purified growth factors or by autologous platelet lysates in rabbits were inhibited by gemfibrozil by more than 50%.

Conclusions. Beneficial effects of gemfibrozil in reducing coronary events in hypertriglyceridemic patients may depend, in part, on potentiation of fibrinolysis by direct diminution of synthesis of endogenous PAI-1. (Circulation 1992;85:1888–1893)

Key Words • thrombosis • atherogenesis • plasminogen activator inhibitor type 1 • gemfibrozil • Hep G2 cells

Growth factors (or mitogens) released from platelets can augment synthesis of plasminogen activator inhibitor type 1 (PAI-1) in Hep G2 cells (a human hepatoma cell line),1–6 human vascular endothelial cells,7 and in vivo.8 Furthermore, activation of platelets, degranulation, and release of platelet-associated growth factors in vivo can increase PAI-1 synthesis.8,9 In the Helsinki primary prevention trial,10–13 both coronary events and mortality were decreased by gemfibrozil, especially in subjects with initially low-density lipoprotein (HDL) cholesterol and hypertriglyceridemia.12 It is known that high concentrations of PAI-1 in plasma are associated with high concentrations of triglycerides, perhaps because both are influenced by hyperinsulinemia.14–16 We have shown that insulin and insulin-like growth factor stimulate production of PAI-1 in Hep G2 cells, suggesting that the liver may be one source of the insulin-induced increases in plasma PAI-1.17 Thus, it is not surprising that gemfibrozil, known to lower plasma triglycerides, has been found to lower plasma PAI-118 in survivors of acute myocardial infarction. Because reduction of mortality in the Helsinki study was most striking in patients in whom plasma PAI-1 activity was likely to have been particularly elevated in association with hypertriglyceridemia, the favorable effects of gemfibrozil may have been attributable to reduction of PAI-1 and potentiation of fibrinolysis with consequent diminished thrombosis and atherogenesis. Accordingly, we considered the possibility that gemfibrozil may decrease PAI-1 in a fashion independent of its lipid-lowering effects and that such direct effects may have contributed to benefit. In this study, we characterized direct effects of gemfibrozil on PAI-1 synthesis and mRNA levels independent of changes in concentrations of lipids in the media in Hep G2 cells in vitro and on PAI-1 synthesis in vivo in normal, normolipidemic animals in response to platelet-associated growth factors and to autologous platelet lysates.

Methods

Cell Culture and Labeling Procedures

Although diverse cell types produce PAI-1, hepatocytes have been implicated as one primary source of plasma PAI-1.19 In this study, Hep G2 cells, used as a model system, were cultured as previously described.5 Media were changed to serum-free medium (Dulbecco’s modified Eagle’s medium/Ham’s nutrient F-12 with

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HEPES) 24 hours before each experiment. Immediately before use, gemfibrozil was dissolved in culture medium containing 0.1% (vol/vol) N,N-dimethyl formamide (DMF) in final concentrations selected to be consistent with those in plasma in vivo after pharmacological administration. Metabolic labeling was performed with [35S]methionine and methionine-free medium (labeling medium). For pulse-chase experiments, cells were cultured in 35-mm wells to confluence, washed three times with labeling medium, incubated for 16 hours in 1.0 ml labeling medium containing 250 μCi [35S]methionine, washed twice with serum-free medium, and incubated for specified intervals in 1.0 ml serum-free medium with or without gemfibrozil. For assay of PAI-1 synthesis, cells that had been cultured to confluency were preincubated for up to 9 hours in 1.0 ml serum-free medium containing gemfibrozil, washed three times with labeling medium, and incubated for 20 minutes in labeling medium containing [35S]methionine and gemfibrozil. Control cells were labeled for 20 minutes in medium without gemfibrozil. PAI-1 was immunoprecipitated and quantified by sodium doceyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions with the use of a radioisotopic scanner as previously described.20

Assay of PAI-1 Antigen and Activity

PAI-1 antigen in conditioned media was assayed as previously described with an enzyme-linked immunosorbent assay (ELISA)21; PAI-1 activity in rabbit plasma was assayed spectrophotometrically22 with a kinetic, chromogenic substrate assay.23

Northern Blots

A 1.1-kb PAI-1 cDNA probe was generated by digestion of PAI-1 cDNA (kindly provided by Dr. T.-C. Wun) with EcoRI and AvaI.24 A gliceraldehyde-3-phosphate dehydrogenase (GAP, 0.6 kb) probe was obtained by Xba I/Hind III digestion of cDNA (ATCC#57091).25 A probe for factor X (FX, 1.1 kb) (kindly provided by Dr. Arnold Strauss) was obtained by EcoRI/PstI digestion of FX cDNA. cDNA probes were radiolabeled with [32P]dCTP by the random primer technique (Boehringer Mannheim, Indianapolis, Ind.). Total RNA was extracted and quantified spectrophotometrically as previ-
plastic syringes with a two-syringe technique and transferred immediately into 12.9 mM sodium citrate at 4°C. Plasma was separated immediately by centrifugation, and aliquots were frozen at −70°C until assay within 1 week.

Administration of Gemfibrozil and Cycloheximide

Selected animals were given intravenous infusions of gemfibrozil dissolved in 0.1% DMF PBS (8.75–20 mg/kg, 2.5 ml/kg) over 2 hours beginning 1 hour before infusion of purified platelet-associated growth factors or autologous platelet lysates prepared as previously described.2 In some, an additional infusion (8.75–20 mg/kg) was implemented over 2 hours beginning 2 hours after the initial infusion of growth factors or lysates. Some animals were given intravenous cycloheximide (2 mg/kg body wt) in PBS (2.5 ml/kg) over 10 minutes 1 hour before infusion of growth factors or lysates. In some, a second injection (1 mg/kg) of cycloheximide was given 2 hours after the infusion. Controls were given infusion of saline (2.5 ml/kg).

To characterize effects on protein and PAI-1 synthesis, [35S]methionine (80 μCi/kg) was administered intravenously 2 hours 40 minutes after infusion of gemfibrozil (8.75 mg/kg), cycloheximide (2 mg/kg), or saline. Twenty minutes later, 1-g samples of tissues of interest were obtained, minced thoroughly, and homogenized with a Polytron PTA-7. Trichloroacetic acid precipitable radioactivity was assayed as described previously.28 Pretreatment of rabbits with cycloheximide for 3 hours reduced protein synthesis (incorporation of [35S]methionine into trichloroacetic acid–insoluble protein in liver and diverse tissues) by 84% (n = 3). In contrast, gemfibrozil (8.75 mg/kg) did not affect incorporation (n = 3). Results were expressed as mean±SD.

Results

Elaboration of PAI-1 in Hep G2 Cells

Cultured Hep G2 cells exhibited basal secretion rates of PAI-1 (Figure 1) of 82±12 ng/10⁶ cells over 24 hours. Exposure of cells to epidermal (EGF) or transforming growth factor-β (TGFB) increased PAI-1 secretion as previously observed by 2.8-fold and 6.1-fold over 4 hours and 4.1-fold and 8.5-fold over 24 hours. Gemfibrozil attenuated basal PAI-1 secretion by 43±10%, and EGF and TGFB induced increases by 37±12% and 39±2% over 24 hours without altering overall protein synthesis (Figure 1). The increase of PAI-1 in the media accelerated when matrix had become relatively saturated.20 To determine whether inhibition of PAI-1 synthesis was secondary to changes in steady-state concentration of PAI-1 mRNA induced by gemfibrozil, Northern blots were performed. Within 6 hours after addition of gemfibrozil, PAI-1 mRNA had decreased significantly (Figure 2), with no change in coagulation factor X or GAP mRNA levels. Gemfibrozil blocked the 3.3±0.5-fold EGF-induced increase of PAI-1 mRNA by 65±2% and the 7.8±2.3-fold TGFB–induced increase by 60±8%.

The rapid decrease in PAI-1 mRNA in cells exposed to gemfibrozil could reflect an increased rate of degradation of PAI-1 transcripts. However, Northern blots after exposure of cells to actinomycin D showed that the decline of PAI-1 mRNA under basal conditions or when PAI-1 mRNA half-life was prolonged by stimulation with

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**Procedures in Experimental Animals**

New Zealand White rabbits (weight, 2.5–3.7 kg) were anesthetized with 20 mg/kg i.m. ketamine and 8 mg/kg i.m. xylazine. Agonists of PAI-1 synthesis were dissolved in phosphate-buffered saline (PBS) (1 ml/kg) and administered intravenously via a jugular venous catheter over 15 minutes.8 After specified intervals, blood samples were collected from a femoral arterial catheter into
TGF-β was not altered by gemfibrozil. Thus, the rapid decrease in steady-state concentrations of PAI-1 mRNA induced by gemfibrozil reflected decreased synthesis.

To determine whether the rate of release of PAI-1 protein from cells, independent of synthesis per se, was altered by gemfibrozil, cells were incubated for 16 hours with [35S]methionine, washed extensively with serum-free medium, and exposed to unlabelled media in the presence or absence of gemfibrozil for up to 24 hours. [35S]-PAI-1 was immunoprecipitated from cell lysates and assayed by SDS-PAGE. The rate of release of immunoprecipitable [35S]-PAI-1 was unchanged by gemfibrozil as judged from the time course of decline of labeled PAI-1 (half-life<6 hours) under basal conditions and after stimulation of the cells by TGF-β. To define effects of gemfibrozil on the rate of PAI-1 synthesis per se, cells were incubated for intervals of up to 9 hours in medium containing gemfibrozil and exposed to [35S]methionine only during the final 20 minutes of incubation. Thus, radiolabeled PAI-1 harvested represented newly synthesized protein.  Gemfibrozil reduced basal PAI-1 synthesis by 20% and attenuated the TGF-β-induced 4.5-fold increase over 6 hours by 49% (Figure 3). The decrease in steady-state concentrations of PAI-1 mRNA paralleled the decline in PAI-1 synthesis.

Effects of Gemfibrozil and Cycloheximide on Increases in Plasma PAI-1 Activity Induced In Vivo by Growth Factors and Autologous Platelet Lysates

Under control conditions, EGF (5 μg/kg) or autologous platelet lysates (2.8×10⁸/kg) increased plasma PAI-1 activity in vivo, as shown previously.  The 2-hour increase induced by EGF was attenuated by 43% and 55% by 8.75 mg/kg and 17.5 mg/kg gemfibrozil (Figure 4A), with no further attenuation with higher concentrations of gemfibrozil (40 mg/kg). Similar effects were noted with platelet lysates (Figure 4B), with attenuation of increments in PAI-1 after 4 and 5 hours by 33% and 53%. Cycloheximide (1 hour before and 2 hours after infusion of EGF) attenuated the PAI-1 increase over 2 hours by 95% (2 mg/kg) and 98% (3 mg/kg) (Figure 4C). No changes in t-PA activity or white cell or platelet counts were seen after gemfibrozil (8.75 mg/kg) or cycloheximide (2 mg/kg) (n=2 for each).

Discussion

Liver has been implicated as one primary source of plasma PAI-1 and plays a significant role in the metabolism of lipids and proteins in the fibrinolytic system. Accordingly, we used Hep G2 cells as a model system in this study. Our results indicate that gemfibrozil inhibits basal, EGF, and TGF-β-induced synthesis of PAI-1 in Hep G2 cells directly and attenuates the increases of plasma PAI-1 activity induced by platelet-associated growth factors and autologous platelet lysates in vivo. They indicate that some effects of gemfibrozil on PAI-1 synthesis are independent of lipid-lowering effects because concentrations of lipids were constant in Hep G2 cell media and the animals studied were normolipidemic. Attenuation of the growth factor–induced increases in PAI-1 mRNA is consistent with inhibition of transcription coupled with the intrinsic instability of PAI-1 mRNA. The rate and magnitude of changes in PAI-1 protein synthesis correlated closely with changes in PAI-1 mRNA. We have previously shown that infusion of platelet lysates in vivo, rich in EGF and TGF-β, increase steady-state levels of PAI-1 mRNA in liver and increase PAI activity in blood. In the present study, cycloheximide attenuated the increase in PAI activity in vivo, suggesting that it
reflects increased PAI-1 synthesis. In addition, we found that gemfibrozil directly diminished PAI-1 synthesis in vitro. Thus, attenuation of the induced increase in PAI activity in vivo seen with gemfibrozil is likely to reflect decreased synthesis.

**Pathogenetic and Clinical Implications**

High concentrations of PAI-1 in plasma have been associated with diverse conditions typified by thrombosis including sepsis, pregnancy, deep vein thrombosis, the postoperative state, and acute myocardial infarction. In animals, circulating and clot-bound PAI-1 inhibit fibrinolysis in vivo after acute pulmonary embolism and are prothrombotic. Furthermore, elevation of PAI-1 induced by administration of endotoxin inhibits clot lysis in vivo. Elevations in plasma PAI-1 may not only suppress endogenous fibrinolysis but also predispose to or exacerbate thrombosis. Under some circumstances, increases in plasma PAI-1 may be mediated by occult, ongoing thrombosis as judged from the effects of thrombin and growth factors released from activated platelets as well as those of platelet lysates on synthesis of PAI-1 in vitro. Changes in PAI-1 mRNA in vivo, and changes in plasma PAI-1 activity in vivo. As shown in the present study, gemfibrozil attenuates growth factor–inducible augmentation of PAI-1 synthesis. Despite the fact that correction of hypertriglyceridemia alone has not been universally effective in reducing mortality associated with coronary artery disease, in the Helsinki primary prevention trial, gemfibrozil decreased coronary mortality promptly, particularly among hypertriglyceridemic patients, who presumably had increased plasma PAI-1 activity as well. Elevations of plasma PAI-1 have been associated with both chronic coronary artery disease and acute myocardial infarction. Accordingly, the salutary effects of gemfibrozil on mortality in the Helsinki trial may be related, at least in part, to reduction in PAI-1 synthesis, restoration of a more favorable balance between thrombosis and thrombolysis, attenuation of thrombotic events leading to acute myocardial infarction and death secondary to coronary thrombosis, and possibly diminution of the rate of progression of coronary atherosclerosis driven by thrombin or platelet-associated mitogens. Thus, prevention or retardation of progression of coronary artery disease may be facilitated by measures designed to diminish synthesis of PAI-1 in addition to those designed to favorably modify plasma lipids and other known determinants of risk.

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S Fujii and B E Sobel

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