Inhibition by fibrates of plasminogen activator inhibitor type-1 expression in human adipocytes and preadipocytes

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Summary
Plasminogen activator inhibitor type-1 (PAI-1), an established marker and mediator of cardiovascular risk, is produced extensively in adipose tissue. Fibrates are hypolipidemic peroxisome proliferator activated receptor-alpha (PPARα) agonists. Recent laboratory and clinical observations indicate that they are also anti-atherosclerotic. Mechanisms responsible, however, remain to be fully understood. The present study was designed to elucidate modulation of PAI-1 expression in adipose cells by fibrates as a potential mechanism. Expression of PPARα was verified by PCR, immunohistochemistry, and Western blotting. In cultured preadipocytes and adipocytes gemfibrozil and fenofibrate significantly reduced PAI-1 protein expression by up to 55 ± 5% and 34 ± 4% under basal conditions and up to 56 ± 6% and 31 ± 6% under conditions of stimulation of the cells with 40 nM transforming growth factor (TGF)β, respectively. Quantification of mRNA showed that the gemfibrozil-induced effect was at least in part regulated at the transcriptional level. Incubations with non-fibrate PPARα agonists showed similar reductions in PAI-1 expression. The decrease in PAI-1 expression induced by gemfibrozil was inhibited by MK886, a PPARα inhibitor. Furthermore, preadipocytes isolated from PPARα-deficient mice produced significantly more PAI-1 than those from wild-type mice upon stimulation with TGFβ. Finally, fenofibrate reduced PAI-1 expression both in plasma and adipose tissue of hyperlipidemic mice. Our data support the view that PPARα activation down-regulates PAI-expression in adipose cells that may contribute in part to the reduction in cardiovascular mortality seen with fibrates in clinical trials.

Keywords
PAI-1, adipocyte, fibrate, metabolic syndrome, haemostasis

Introduction
Fibrates are lipid-lowering drugs employed in the treatment of various forms of dyslipidaemia (1). They are derivatives of fibric acid and agonists of the peroxisome proliferator activating receptor-alpha (PPARα) (2). Their main therapeutic effects are diminution of elevated concentrations of triglycerides (TGs) in blood and elevation of low concentrations of high-density (HDL) cholesterol. Both derangements are risk factors for cardiovascular disease (1). Low-density (LDL) cholesterol is reduced by fibrates but only modestly.

Results of several clinical trials indicate that fibrates can reduce cardiovascular morbidity and mortality (3–8). In the recent FIELD study, fibrates significantly reduced total vascular events (6). They appear to be particularly beneficial in patients with diabetes, a group with markedly high cardiovascular risk (1, 6). Potentially anti-atherosclerotic effects of fibrates include anti-inflammatory and anti-thrombotic effects (9–12). Thus, reduction of the incidence of cardiovascular events by fibrates may reflect factors in addition to their effects on lipids.

Plasminogen activator inhibitor type-1 (PAI-1) is a serine protease inhibitor (SERPIN) that inhibits plasminogen activators thereby attenuating fibrinolysis. Elevated concentrations of PAI-1 in blood have been implicated as a coronary disease risk factor and a determinant of thrombotic events (13, 14). Concentrations of PAI-1 are particularly high in patients with type 2 diabetes. It has been thought that PAI-1 may contribute to their significantly increased cardiovascular risk (15–17). In addition to liver and vascular endothelium, adipose cells appear to be an important source of PAI-1 circulating in blood (18–20). Thus, con-
centrations of PAI-1 in blood increase in parallel with body mass index (BMI) (21). Weight loss induced by dietary or surgical intervention lowers elevated concentrations of PAI-1 (22, 23). Two types of fat cells are seen in adipose tissue, adipocytes and preadipocytes. The latter produce even more PAI-1 than adipocytes (20). Potentially atherogenic cytokines such as interleukin-1beta (IL-1β), tumour necrosis factor-alpha (TNFα), and transforming growth factor-beta (TGFβ) stimulate PAI-1 expression in vascular elements and in adipose tissue (24). Conversely, these cytokines themselves are produced by adipose tissue. Thus, adipose tissue is a metabolically active autocrine and endocrine organ (25). The present study was performed to elucidate effects of fibrates on expression of PAI-1 in adipocytes and preadipocytes in view of its role as marker and mediator of cardiovascular risk.

Materials and methods

Fibrates

Fenofibrate and gemfibrozil were both purchased from Alexis (Plymouth Meeting, PA, USA). They were dissolved in dimethyl sulfoxide (DMSO). Care was taken not to exceed a final concentration of 0.1% DMSO in the cultures and all controls contained the same amount of DMSO, since DMSO itself can affect gene expression.

Isolation and culture of human and murine preadipocytes

Preadipocytes were isolated and cultured as described previously (26). In brief, human subcutaneous abdominal and mammary adipose tissue samples were obtained from patients at the time of elective plastic surgery. Donors of tissue were 35–to 65-year-old females with a BMI of 30 or greater and no known history of diabetes. All donors provided written informed consent. The protocol was approved by the Institutional Research Board of the University of Freiburg, Germany.

The tissue was digested in phosphate-buffered saline (PBS) containing 0.15% collagenase type I (Worthington) and 2% BSA (Sigma), pH 7.4 for 90 minutes (min) at 37°C. The digest was passed through a 100 µm nylon cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) to remove residual undigested tissue. After incubation with an erythrocyte lysing buffer (Dickinson, Franklin Lakes, NJ, USA) at a dilution of 1:150 for 1 h. Staining was visualized by the Vector stain ABC kit (Vector) and imaged under an inverted microscope (Olympus, Center Valley, PA, USA).

Culture of other cell types

Human umbilical endothelial cells (HUVEC) were cultured in M199 supplemented with 20% FCS and recombinant endothelial growth factor (Upstate Biotechnology, Lake Placid, NY, USA). Human monocytes were isolated from buffy coats by gradient density centrifugation and used after one night of rest in RPMI supplemented with 10% FCS. Human smooth muscle cells were cultured in RPMI supplemented with 10% FCS. HepG2 cells were cultured in MEM supplemented with 10% FCS, and 3T3-L1 cells in DMEM suplemented with 10% FCS. 3T3-L1 cells were either kept subconfluent to maintain preadipocyte phenotype or grown to confluence and adipogenesis was initiated with a standard adipogenic cocktail (see above). Prior to experiments all cell types with the exception of HUVECs were starved in serum-free media overnight.

Reverse transcription polymerase chain reaction (PCR) for PPARα, CD31, CD68, and α-actin

Total RNA was isolated from indicated cell types by chloroform phenol extraction. One µg of RNA was transcribed into cDNA with use of the 1st strand cDNA synthesis kit (Roche, Indianapolis, IN, USA). Subsequently, a standard PCR employing the following primers was performed as described previously (26, 27):

- PPARα: F-primer 5'-GAC GAA TGC CAA GAT CTG AGA AAG-3' and R-primer 5'- CGT CTC TTT TCT TTG AGT GTC T-3';
- CD31: F-primer 5'-TCC ACA TCA GCC CCA CCG GAAT-3' and R-primer 5'-TGG GCC ACA ATC GCC TTG TTC T-3';
- CD68: F-primer 5'-GAA CCC CAA CAA AAC CAA G-3' and R-primer 5'-TTG TAC TCC ACC GCC ATG TA-3';
- α-actin: F-primer 5'-TGC CCA TTA ATG AGG GCC TAC ATG-3' and R-primer 5'-GCC ATC TCG TCT TCG AAG TC-3'.

Products were visualised on a 2% agarose gel.

Immunohistochemistry for PPARα

Immunohistochemistry was performed as previously described (28). In brief, cells grown in chamber slides (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) were fixed in 4% paraformaldehyde and permeabilised in 0.5% TritonX 100 for 5 min each. After three washes with PBS and 30 min of blocking in 4% serum/PBS, cells were incubated with the primary anti-human PPARα antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:100 for 1 h and washed again and incubated with the corresponding biotin-labelled secondary antibody (Vector, Burlingame, CA, USA) at a dilution of 1:150 for 1 h. Staining was visualised by the Vector stain ABC kit (Vector) and imaged under an inverted microscope (Olympus, Center Valley, PA, USA).
tive staining was quantified using Image Pro as described previously (29).

**Western blotting**

Western Blotting was performed as described previously (29). Anti-human PPARα antibody was from Santa Cruz (Santa Cruz, CA, USA), anti-von Willbrand factor (vWF) from Zytomed (Berlin, Germany), anti-CD68 from Dako (Glostrup, Denmark), and anti-α-actin from Chemicon (Millipore, Billerica, MA, USA).

**Stimulation of cells**

After 16 h of serum-starvation, cells in 24-well plates (Nunc) were incubated with 700 µM of gemfibrozil or fenofibrate for 24 h in the absence or presence of 40 nM TGFβ (Roche). Concentrations of 700 µM were used based on results in initial dose response experiments that showed the greatest reductions in PAI-1 expression at this dose and persistent viability of cells (data not shown). Time course experiments employed 6, 12, 24, and 48 h incubations with 700 µM concentrations of gemfibrozil. Additional experiments employed 1 µM 8(S)-HETE (Alexis), a naturally occurring PPARα activator and MK886 (Alexis), a synthetic inhibitor of PPARα.

**Quantification of human PAI-1 mRNA**

Quantitative RT-PCR was performed to assay PAI-1 mRNA as described previously (26). In brief, total RNA was isolated by chloroform-phenol extraction. One µg RNA was transcribed into cDNA with use of the 1st strand cDNA synthesis kit (Roche). The cDNA obtained was subjected to quantitative real time-PCR with a Roche Light Cycler using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche). As endogenous control, GAPDH, an established housekeeping gene in adipocytes and preadipocytes, was employed (30). Conditions for quantification of PAI-1 mRNA were: F-primer 5’-gtg ttt cag cag gtg gcg c-3’, R-primer 5’-ccg gaa cag cct gaa gaa gtg -3’, 45 cycles of 15 seconds (s) at 95°C, 5 s at 65°C, and 12 s at 72°C. Conditions for
obtain total cell lysates, cells were scraped on ice in lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 0.1% Triton-X 100, proteinase inhibitors) and shock frozen repeatedly. LDH was measured with the use of the CytotoxOne kit (Promega). Total protein was assayed colorimetrically (Protein assay kit, Sigma). In addition, preadipocytes treated with 700 µM of gemfibrozil or fenofibrate in the presence or absence of 40 pM TGFβ for 24 h were trypsinised and diluted 1:1 in trypan blue (Sigma), and the ratio of viable cells / dead cells was assessed with the use of an inverted light microscope (Nikon).

Cytotoxicity assays
To test for cytotoxicity, total protein and lactate dehydrogenase (LDH) were quantified in supernatant fractions of media of cells treated with 700 µM of gemfibrozil or fenofibrate in the presence and absence of 40 pM TGFβ. Total concentrations of protein of the corresponding cell lysates were quantified as well. To obtain total cell lysates, cells were scraped on ice in lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 0.1% Triton-X 100, proteinase inhibitors) and shock frozen repeatedly. LDH was measured with the use of the CytotoxOne kit (Promega). Total protein was assayed colorimetrically (Protein assay kit, Sigma). In addition, preadipocytes treated with 700 µM of gemfibrozil or fenofibrate in the presence or absence of 40 pM TGFβ for 24 h were trypsinised and diluted 1:1 in trypan blue (Sigma), and the ratio of viable cells / dead cells was assessed with the use of an inverted light microscope (Nikon).

In-vivo mouse study
LDLR-deficient mice on a pure C57/BL6 background were obtained from Jackson at the age of six weeks and consumed a standard high-fat, high-cholesterol diet (HCD, 40 kcal% fat, 1.25% cholesterol, Sniff) for four weeks. Prior to HCD feeding blood was taken by retro-orbital bleeding. Subsequently, animals were

**Figure 3: Fibrates inhibit PAI-1 expression in human and murine preadipocytes.** A) Concentrations of PAI-1 protein were quantified by ELISA in supernatant fractions of conditioned media of human preadipocytes after incubation with 700 µM of gemfibrozil or fenofibrate under basal conditions and conditions of stimulation with 40 pM TGFβ. Results are expressed as % of control represent mean ± SEM of 12 experiments, each performed in triplicate. * signifies p<0.05. B) Concentrations of PAI-1 protein (ng/ml) were quantified by ELISA in conditioned media of human preadipocytes exposed to 700 µM gemfibrozil and 40 pM TGFβ for 6, 12, 24, and 48 h. Results represent mean ± SEM of four experiments, each performed in triplicate. All data points achieved statistical significance with a p-value below 0.05. C) Concentrations of PAI-1 protein (ng/ml) were quantified by ELISA in conditioned media of human preadipocytes exposed to 700 µM gemfibrozil and 40 pM TGFβ for 6, 12, 24, and 48 h. Results represent mean ± SEM of four experiments, each performed in triplicate. * signifies p<0.05. D) The preadipocyte cell line 3T3-L1 was incubated with 700 µM gemfibrozil for 24 h in the presence or absence of 40 pM TGFβ and PAI-1 protein was quantified by ELISA in the supernatants. Results are expressed as % of control and represent mean ± SEM of four experiments, each performed in triplicate. * signifies p<0.05.
randomised to two groups of six animals each, receiving either HCD or HCD supplemented with 0.05 %w/w of fenofibrate for another four weeks (formulated by Sniff, Soest, Germany). Finally, animals were anaesthetized, blood was taken by cardiac puncture and visceral fat (epididymal and peri-phrenic) depots as well as kidneys, heart, liver, and lungs were harvested in RNA-later (Qiagen, Hilden, Germany). Cholesterol, triglycerides (both WAK Chemie, Steinbach, Germany), and mouse PAI-1 (Innovative Research) were assayed before HCD feeding and at the end of the study. Mouse PAI-1 mRNA was quantified by quantitative real-time RT-PCR employing the following primers and conditions: For murine PAI-1: F-primer 5’-AGG GTT GCA CTA AAC ATG TCA G-3’ and R-primer 5’-GAC ACC CTC AGC ATG TTC ATC-3’, 45 cycles of 10 s at 95°C, 5 s at 59°C, and 7 s at 72°C. For murine GAPDH: F-primer 5’-TGC ACC ACC AAC TGC TTA G-3’ and R-primer 5’-GAT GCA GGG ATG ATG TTC-3’, same conditions as mPAI-1. Murine PAI-1 mRNA expression was analyzed employing the 2^ΔΔCt method (31).

**Statistical analysis**

Statistical analysis was performed with the use of Student’s two tailed t-test for paired data. Data from at least three different experiments and donors were analysed, and values were expressed as means ± standard errors of the means (SEM). Differences were considered to be significant for p-values <0.05.

**Results**

**Adipose cells express PPARG**

Total RNA isolated from preadipocytes contained PPARG mRNA as assessed by reverse transcript PCR (Fig. 1A). Immunohistochemical analysis demonstrated predominant nuclear expression of PPARG in these cells (Fig. 1B). Consequently, nuclear protein fractions contained more PPARG than cytosolic protein fractions as assessed by Western blotting (Fig. 1C). Our preadipocyte cultures contained less than 5% vWF- and CD68-positive cells, demonstrating the absence of relevant endothelial and monocyte/macrophage contamination (Fig. 2A). A total of 5–10% of alpha-actin-positive cells were detected as often seen in fibroblast-like cells such as preadipocytes (32). RT-PCR revealed transcripts of CD68 in both preadipocytes and adipocytes in line with previous reports (33, 34) and faint expression of CD31 in preadipocytes only (Fig. 2B).

**Fibrates inhibit expression of PAI-1 protein in human and murine preadipocytes**

Preadipocyte cultures constitutively expressed 56 ± 6ng/ml PAI-1 protein in 24 h. Stimulation of cells with 40 pM TGFβ increased basal expression of PAI-1 to 170 ± 16ng/ml (n=12, p=0.0002). Incubation with 700 µM of gemfibrozil and fenofibrate for 24 h resulted in a significant reduction of expression of PAI-1 protein by 36 ± 7 % (n=12, p=0.002) and 34 ± 4% (n=4, p=0.03) under basal conditions and by 44 ± 4% (n=12, p=0.006) and 29 ± 14% (n=4, p=n.s.) under conditions of stimulation with 40 pM TGFβ, respectively (Fig. 3A). These effects were concentration-dependent as illustrated for gemfibrozil in Figure 3B. A dose of 100 µM which is comparable to serum concentrations achieved in patients treated with gemfibrozil was sufficient to induce a significant reduction in PAI-1 protein expression (35). Additional time course experiments demonstrated that the decrease in expression of PAI-1-protein by gemfibrozil was apparent after as little as 6 h and maximal after 48 h (Fig. 3C). Incubation of undifferentiated 3T3-L1 cells, an established murine adipose cell line, with 700 µM of gemfibrozil significantly reduced PAI-1 protein expression by 24 ± 15% under basal conditions and by 32 ± 14% under conditions of stimulation with 40 pM TGFβ, corroborating our findings in human preadipocytes (Fig. 3D).

![Graph A](image1.png)

**Figure 4: Fibrates inhibit PAI-1 expression in human and murine adipocytes.** A) Concentrations of PAI-1 protein (% of control) were quantified by ELISA in supernatant fractions of conditioned media in vitro after incubation of the cells with 700 µM gemfibrozol or 700 µM fenofibrate for 24 h under basal and stimulated conditions (co-incubation with 40 pM TGFβ). Results represent mean ± SEM of four experiments, each performed in triplicate. * signifies p<0.05. B) Differentiated 3T3-L1 adipocytes were stimulated with 700 µM gemfibrozol in the presence of 40 pM TGFβ for 24 h and supernatants were assayed for PAI-1 protein (ng/ml) by ELISA. Results represent mean ± SEM of four experiments, each performed in triplicate. * signifies p<0.05.
Fibrates reduce release of PAI-1 protein from human and murine adipocytes

Differentially human adipocytes exhibited constitutive expression of 74 ± 12 ng/ml PAI-1 protein in the supernatant fraction of media from incubated cells (24 h). Under such conditions 700 µM gemfibrozil and fenofibrate attenuated expression of PAI-1 protein by 55 ± 5% (n=4, p=0.04) and 35 ± 4% (n=4, p=0.04). Stimulation with 40 pM TGFB increased expression of PAI-1 protein to 278 ± 33 ng/ml (n=4, p=0.009). Incubation of the cells with 700 µM gemfibrozil and fenofibrate under conditions of stimulation reduced synthesis of PAI-1 protein by 56 ± 5% (n=4, p=0.04) and 27 ± 6% (n=4, p=0.03) as shown in Figure 4A. Similar results were obtained when differentiated murine 3T3-L1 adipocytes were subjected to stimulation with 700 µM of gemfibrozil in the presence of 40 pM TGFB, suggesting that the effects observed are indeed adipocyte-derived (Fig. 4B).

Attenuation of expression of PAI-1 protein by gemfibrozil reflects decreased expression of PAI-1 mRNA

As illustrated in Figure 5A, PAI-1 mRNA constitutive expression was reflected by transcript levels of 3.5 ± 0.7 in preadipocytes (relative quotients of PAI-1 copy numbers to GAPDH copy numbers [n=3]). Incubation of cells with 700 µM gemfibrozil for 12 h resulted in a reduction of basal PAI-1 mRNA expression to 2.1 ± 0.8 (n=3, p=n.s.). Stimulation of preadipocytes with 40 pM TGFB for 12 h increased basal PAI-1 mRNA expression to 34.9 ± 1.9 (n=3, p=0.007). Under such conditions treatment with 700 µM gemfibrozil for 12 h resulted in a decrease of expression of PAI-1 mRNA to 9.9 ± 0.8 (n=3, p=0.05). Similar results were obtained in human adipocytes (Fig. 5B).

Lack of alteration of expression of t-PA protein in preadipocyte and adipocytes

t-PA was expressed constitutively by preadipocytes and adipocytes (range 2.1–3.7 ng/ml). Incubation with 700 µM gemfibrozil or fenofibrate did not alter its expression (data not shown).

Fibrates do not limit cell viability

Neither concentrations of total protein in cell lysates or supernatant fractions nor concentrations of LDH in supernatant fractions differed in cells treated with or without 40 pM TGFB alone or with 40 pM TGFB and 700 µM gemfibrozil. Thus, the decrease in expression of PAI-1 induced by fibrates was not reflective of cytotoxicity (n=3, each, data not shown). Staining with trypsin blue confirmed these findings. There was no increased fraction of stained cells to total cell numbers in cells incubated with 100 to 700 µM of gemfibrozil compared with fractions in untreated controls (n=3, data not shown).

PPARα mediates fibrate-induced decrease in PAI-1 production

Incubation of preadipocytes for 24 h with 1 µM of 8-(S)-HETE, a potent naturally occurring activator of PPARα, resulted in a decrease of expression of PAI-1 protein in the conditioned media by 29 ± 3% under basal and by 63 ± 6% under conditions of stimulation with 40 pM TGFB. Conversely, incubation of 24 h with 10 µM of MK886, a PPARα inhibitor, and 700 µM gemfibrozil abrogated the reduction of expression of PAI-1 protein induced by 700 µM gemfibrozil alone under basal and under conditions of stimulation (Fig. 6A).

To verify the negative involvement of PPARα in PAI-1 expression, we isolated preadipocytes from PPARα-deficient and -competent mice. PPARα-deficient preadipocytes showed a significantly higher induction of PAI-1 expression upon stimulation with TGFB (3.0 ± 0.1-fold vs. 1.6 ± 0.1-fold, p=0.02), corroborating the concept that PPARα inhibits PAI-1 expression (Fig. 6B). In accord with these data, gemfibrozil significantly reduced PAI-1 protein expression only in wild-type cells but not in PPARα-deficient preadipocytes.

Figure 5: Fibrates inhibit PAI-1 transcription in human preadipocytes and adipocytes. A) Effect of 700 µM gemfibrozil on expression of PAI-1 mRNA in human preadipocytes after 12 h of incubation. Results are expressed as ratios of PAI-1 copy numbers / GAPDH copy numbers. Cells were incubated with 700 µM gemfibrozil in the presence or absence of 40 pM TGFB. B) Effect of 700 µM gemfibrozil on expression of PAI-1 mRNA in in vitro differentiated human adipocytes after 12 h of incubation in the presence of 40 pM TGFB. Results are expressed as ratios of PAI-1 copy numbers / GAPDH copy numbers. Results represent mean ± SEM of three experiments, each performed in triplicate. * signifies p<0.05.
**Fenofibrate reduces PAI-1 in plasma and visceral adipose tissue in hyperlipidaemic mice**

To assess whether the effects observed in vitro also pertain to in vivo, LDLR-deficient mice consumed a high-cholesterol diet (HCD) for four weeks and were subsequently randomised into two groups receiving either HCD alone or HCD supplemented with 0.05 %w/w fenofibrate. Mice receiving fenofibrate had significantly lower levels of PAI-1 in plasma compared with respective controls (Table 1). Furthermore, adipose tissue specimens of these mice also contained lower amounts of PAI-1 mRNA (Table 2). In contrast, PAI-1 mRNA expression remained unchanged in cardiac tissue and even increased in renal and pulmonary tissue.

**Discussion**

The results obtained demonstrate that the fibrates gemfibrozil and fenofibrate are potent inhibitors of PAI-1 synthesis in primary cultures of human preadipocytes and adipocytes. As seen in the Helsinki Heart Study, the VA-HIT, the DAIS trial, and most recently in the FIELD study, fibrates are particularly effective in reducing cardiovascular risk in patients with a lipid profile characteristic of that in patients with type 2 diabetes and those with insulin resistance and the metabolic syndrome (3, 5–7). The classic profile is elevated concentrations of triglycerides in blood, normal to moderately elevated LDL cholesterol, increased small dense LDL, and decreased HDL-cholesterol. All these derangements are treatment targets of fibrates (1). Patients with the metabolic syndrome or frank type 2 diabetes are characterised also by markedly elevated concentrations in blood of PAI-1 suggesting that PAI-1 may contribute to a significantly increased cardiovascular risk (15, 16, 36–38). Reduction of expression of PAI-1 in preadipocytes and adipocytes may contribute to decreased concentrations of PAI-1 in blood, potentially accounting in part for the reduction in cardiovascular risk seen in clinical trials.

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**Figure 6: PPARα mediates fibrate-induced decrease in PAI-1 expression.**

A) Concentrations of PAI-1 protein (% of control) were quantified by ELISA in the supernatant fractions of human preadipocytes. Ten µM MK886, an inhibitor of PPARα, abrogates the decrease of expression of PAI-1 protein induced by 700 µM gemfibrozil under basal conditions and conditions of stimulation with 40 pM TGFβ. One µM 8(S)-HETE, a naturally occurring activator of PPARα, attenuates expression of PAI-1 protein in the conditioned media. Results represent mean ± SEM of four experiments, each performed in triplicate. * signifies p<0.05.

B) Preadipocytes were isolated from PPARα-deficient and -competent mice. Concentrations of PAI-1 protein were quantified by ELISA in supernatants from cells under basal conditions and conditions of stimulation with 40 pM TGFβ. Results represent mean ± SEM of three experiments, each quantified in triplicate. * signifies p<0.05.
The present findings are consistent with results in previous reports in studies of hepatic, endothelial, and vascular smooth muscle cells (39–42). In vitro, the effects of fibrates on expression of PAI-1 were obtained under conditions in which they were independent of triglycerides (40). Blood levels of PAI-1, however, correlate with blood levels of triglycerides in vivo, and very-low-density lipoprotein (VLDL)-bound triglycerides are powerful agonists of PAI-1 expression (43, 44). In rabbits, Zhao et al. demonstrated induction of PAI-1 expression in adipose tissue by high-fat diet accompanied by a significant increase in serum levels. Treatment with fenofibrate reduced PAI-1 expression in both locations (45). In line with these data, we provide direct evidence that fenofibrate reduces both plasma and adipose tissue PAI-1 in hyperlipidaemic mice. In some clinical studies fibrates led to a significant reduction of concentrations of PAI-1 in blood (46, 47). However, in others, no effect or an increase was observed (48–51). This disparity may reflect differences in the extent to which obesity, diabetes, insulin resistance, the type of hyperlipidaemia, and inflammation had contributed to baseline expression of PAI-1.

In the present study, both gemfibrozil and fenofibrate reduced expression of PAI-1 in adipocytes and preadipocytes. This is consistent with a class effect mediated by activation of PPARα. The fact that 8(S)-HETE, a strong naturally occurring inhibitor of PPARα, diminished expression of PAI-1 comparably to baseline expression of PAI-1.

Table 1: Fenofibrate reduces lipids and PAI-1 serum levels. LDLR-deficient C57/BL6 mice at the age of six weeks fed a high-cholesterol diet (HCD) for four weeks were randomised to two groups receiving either HCD or HCD supplemented with 0.05 %w/w of fenofibrate (FENO) for another four weeks. Plasma cholesterol, triglycerides and PAI-1 levels were measured. Values are given as mean ± SEM. * signifies a p-value ≤0.05 compared with baseline values. # signifies a p-value ≤0.05 comparing HCD and HCD-FENO groups with each other.

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<td>HCD</td>
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<tr>
<td>HCD</td>
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The present findings are consistent with results in previous reports in studies of hepatic, endothelial, and vascular smooth muscle cells (39–42). In vitro, the effects of fibrates on expression of PAI-1 were obtained under conditions in which they were independent of triglycerides (40). Blood levels of PAI-1, however, correlate with blood levels of triglycerides in vivo, and very-low-density lipoprotein (VLDL)-bound triglycerides are powerful agonists of PAI-1 expression (43, 44). In rabbits, Zhao et al. demonstrated induction of PAI-1 expression in adipose tissue by high-fat diet accompanied by a significant increase in serum levels. Treatment with fenofibrate reduced PAI-1 expression in both locations (45). In line with these data, we provide direct evidence that fenofibrate reduces both plasma and adipose tissue PAI-1 in hyperlipidaemic mice. In some clinical studies fibrates led to a significant reduction of concentrations of PAI-1 in blood (46, 47). However, in others, no effect or an increase was observed (48–51). This disparity may reflect differences in the extent to which obesity, diabetes, insulin resistance, the type of hyperlipidaemia, and inflammation had contributed to baseline expression of PAI-1.

In the present study, both gemfibrozil and fenofibrate reduced expression of PAI-1 in adipocytes and preadipocytes. This is consistent with a class effect mediated by activation of PPARα. The fact that 8(S)-HETE, a strong naturally occurring inhibitor of PPARα, diminished expression of PAI-1 comparably to baseline expression of PAI-1.

Table 2: Fenofibrate differentially modulates PAI-1 mRNA expression in different tissues. LDLR-deficient C57/BL6 mice at the age of six weeks fed a high-cholesterol diet (HCD) for four weeks were randomised to two groups receiving either HCD or HCD supplemented with 0.05 %w/w of fenofibrate (FENO) for another four weeks. Adipose tissue, liver, heart, kidney and lung were harvested at the end of the study and PAI-1 mRNA levels were quantified by real-time PCR. Values are given as 2-ΔΔCt ratios ± range. * signifies a p-value ≤0.05 comparing HCD and HCD-FENO groups with each other.

<table>
<thead>
<tr>
<th></th>
<th>2-ΔΔCt mPAI-1 Adipose tissue (ratio)</th>
<th>2-ΔΔCt mPAI-1 Liver (ratio)</th>
<th>2-ΔΔCt mPAI-1 Heart (ratio)</th>
<th>2-ΔΔCt mPAI-1 Kidney (ratio)</th>
<th>2-ΔΔCt mPAI-1 Lung (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCD</td>
<td>1.0 (0.8–1.2)</td>
<td>1.0 (0.72–1.38)</td>
<td>1.0 (0.55–1.79)</td>
<td>1.0 (0.45–2.22)</td>
<td>1.0 (0.29–3.34)</td>
</tr>
<tr>
<td>HCD+FENO</td>
<td>0.49 (0.46–0.53)*</td>
<td>0.69 (0.57–0.84)*</td>
<td>1.07 (0.50–1.23)</td>
<td>1.70 (0.91–3.18)</td>
<td>4.18 (3.16–5.52)*</td>
</tr>
</tbody>
</table>
What is known about this topic?
- Fibrates are lipid-lowering drugs.
- Fibrates exert pleiotropic, anti-inflammatory effects.
- Fibrates lower cardiovascular risk independently of changes in lipid profile.

What does this paper add?
- Fibrates decrease PAI-1 expression in adipose tissue in vitro and in vivo.
- This may in part explain their favourable effect on cardiovascular risk.

40 pM TGFβ in the absence or presence of 700 µM gemfibrozil suggest that the effects of fibrates on synthesis of PAI-1 are relatively specific and not attributable to cytotoxicity. Our data in vivo demonstrating a tissue-specific response to fibrate treatment are consistent with this interpretation.

Several reports suggest that cells other than preadipocytes such as macrophages and endothelial cells present in the stromal vascular fraction contribute to PAI-1 in blood. Consequently, one of the major limitations of this study is that in our cell culture model we cannot completely rule out a minor contamination by other cell types, particularly in the preadipocyte preparations. In fact, we detected the macrophage marker CD68 in our primary human preadipocyte and adipocyte cultures and the endothelial marker CD31 in preadipocyte cultures only by RT-PCR. However, whether this potential minor contamination affects our results, remains questionable since we were able to reproduce our findings in the cultures of undifferentiated and differentiated murine 3T3-L1 cells, a pure cell line. Also, several reports suggest that the specificity of markers such as CD68 and alpha-actin is much lower than generally anticipated, rendering the presence of a contamination questionable in the first place (32–34). Another limitation of our study stems from the fact that we only investigated adipose tissue from females. It is possible that there are inter-sex differences in response to fibrate treatment. In our animal study we only measured PAI-1 levels at the beginning of feeding and at the end of the study but not at randomisation. Theoretically, blood levels of PAI-1 could differ between both study groups at randomisation due to unequal food consumption. This is, however, extremely unlikely since weights and lipid profiles do not differ at this time point. Furthermore, the doses of fibrates employed in this study in vitro as well as in vivo are relatively high. Therefore, we cannot rule out that current dosing in humans is not high enough to observe a clinically relevant reduction of PAI-1 expression in adipose tissue. Also, data obtained in vitro and in murine studies do not always hold up in humans. There may not be such an effect in humans at all. Infact, the scarce data supporting a clinical reduction of PAI-1 blood levels by fibrates given their longstanding availability on the market could be seen supportive of this notion. Besides, as demonstrated in our animal study, regulation of PAI-1 expression by fibrates varies in different tissues. Thus, a reduction of PAI-1 expression in adipose tissue may not always translate into a reduction of PAI-1 levels in blood. However, since adipose tissue produces large amounts of PAI-1, it is likely that if there is a reduction of PAI-1 expression in human adipose tissue it will result in lower levels of PAI-1 in blood in patients suffering from obesity, potentially reducing cardiovascular risk. In addition a reduction of PAI-1 in adipose tissue may have local beneficial effects not investigated by this manuscript. These, and other issues require clarification by future clinical trials.

Diverse so-called pleiotropic anti-inflammatory effects have been attributed to fibrates recently, most of which have been associated with activation of PPARα. Fibrates inhibit TNFα-induced expression of vascular cell adhesion molecule-1 and thrombin-induced expression of endothelin-1 in endothelial cells (11, 56). They diminish expression of tissue factor in monocytes/macrophages (10). They prevent interleukin (IL)-1-induced secretion of IL-6 in smooth muscle cells (12). In animal studies, fibrates reduce atherogenesis and improve plaque stability in mice both in the presence and absence of diabetes and dyslipidaemia, providing direct evidence for an anti-atherosclerotic effect (57–61). In clinical trials, fibrates reduce concentrations in blood of fibrinogen, C-reactive protein, sCD40L, and IL-1β in addition to altering concentrations of lipids (12). Treatment with fibrates is associated with improved glycaemic control and lower concentrations in blood of leptin and insulin in patients with type-2 diabetes who have hypertriglyceridaemia, underscoring the potential value of treatment with fibrates of people with diabetes (62).

Our results suggest that, particularly in patients with the metabolic syndrome or type 2 diabetes, reduction of expression of PAI-1 in adipose tissue may be anticipated with treatment with fibrates. This could contribute to a cardiovascular risk reduction in this patient population.

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