# REGULATORY ASPECTS OF FIBRINOGEN AND PLASMINOGEN ACTIVATOR INHIBITOR-1 GENE REGULATION

## *ROLE OF THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR*

Proefschrift
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College van Promoties
te verdedigen op donderdag 28 oktober 1999
te klokke 16.15 uur

door

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geboren te Hengelo
in 1969

**Promotiecommissie:**

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ISBN 90-5412-056-8

The studies presented in this thesis were performed at the Gaubius Laboratory TNO-PG, Leiden and were financially supported by the Netherlands Organisation for Scientific Research (NWO) project 902-23-181

Financial support by the Gaubius Laboratory TNO-PG and the Netherlands Organisation for Scientific Research (NWO) for the publication of this thesis is gratefully acknowledged.

The printing of this thesis was further financially supported by the J.E. Jurriaanse Stichting, the Nederlandse Vereniging voor Hepatologie, Laboratoires Fournier, the Dr. Saal van Zwanenbergstichting, the Dr. Ir. J.H. van der Laar Stichting, and Parke-Davis B.V.

THELA · THESIS

## Stellingen

1 Het verlagend effect van fibraten op plasma fibrinogeen concentraties wordt gemedieerd door de peroxisoom proliferator-geactiveerde receptor-α
*dit proefschrift*

2 Via welk mechanisme fibraten PAI-1 gen expressie beïnvloeden blijft vooralsnog onduidelijk
*dit proefschrift*

3 PPARα is de verbindende factor tussen het lipiden-metabolisme en de stolling
*dit proefschrift*

4 De plasma PAI-1 concentratie in obese mannen en vrouwen wordt niet bepaald door de hoeveelheid visceraal vet
*dit proefschrift*

5 Het gunstige effect van visolie op hart- en vaatziekten kan verklaard worden door de sterke PPARα-activerende capaciteit van N-3 vetzuren
*Daviglius et al., N Engl J Med, 1997; 336:1046; Krey et al., Mol Endocrinol, 1997; 11:779*

6 De herkomst van plasma PAI-1 is nog altijd onbekend
*Loskutoff and Samad, Arterioscler Thromb Vasc Biol. 1998; 18:1*

7 Een orphan-receptor is maar een tijdelijke wees

8 Obesitas is een ziekte
*Spiegelman and Flier, Cell. 1996; 87:377; Perusse et al, Obes Res. 1999; 7:111*

9 De huidige diermodellen voor obesitas onderzoek zijn ontoereikend voor een verklaring naar het ontstaan van obesitas bij de mens

10 Liposuctie is meer dan het afzuigen van overtollig vet

11 Sterkere botten door regelmatig sporten zijn geen garantie voor het niet breken van die botten

12 Hoewel onbetrouwbaar, is e-mail een uitstekend communicatie middel

*Wat niemand zoekt wordt zelden gevonden*
*Pestalozzi*

*Aan mijn ouders*

## Contents

# *CHAPTER 1*

## General introduction

## Introduction

The blood coagulation and the fibrinolytic (or plasminogen/plasmin) systems are critical for haemostasis, because the former prevents bleeding by the formation of fibrin clots, whereas the latter guarantees maintenance of vascular patency by the removal of fibrin clots. When deregulated, both systems may contribute to thrombotic or haemorrhagic disorders. The two systems have also been implicated in tissue remodelling and cellular migration, which are crucial mechanisms for the repair of blood vessels. In addition, they participate in a variety of other processes such as embryonic development, reproduction, wound healing, cancer, and brain function (for a review, see Carmeliet and Collen[1] and references cited therein). During the last few years, the importance of the coagulation and plasminogen/plasmin systems in these processes is highlighted by the generation of transgenic mice lacking or overexpressing factors involved in these two systems (reviewed by Carmeliet and Collen[2]).

The coagulation and the plasminogen/plasmin systems are multicomponent enzyme cascades. In short, initiation of the plasma coagulation system is triggered by tissue factor, which functions as a cellular receptor and cofactor for conversion of the serine proteinase factor VII to active factor VIIa. This complex activates factor X directly or indirectly via activation of factor IX, resulting in the generation of thrombin, which in turn mediates conversion of fibrinogen (Fbg) to fibrin[3] (see scheme depicted in Fig 1). The fibrin formed during, for example, haemostasis, inflammation or tissue repair, plays a temporary role and must be removed when normal tissue structure and function is restored.

Dissolution of fibrin clots is mainly mediated by the plasminogen/plasmin system.[4] In this system, a proenzyme, plasminogen, is converted to the active enzyme plasmin by plasminogen activators (PAs), primarily tissue-type PA (t-PA) and urokinase-type PA (u-PA). The plasmin formed is a potent, broad-spectrum proteinase that degrades fibrin and other extracellular matrix proteins, and activates latent matrix metalloproteinases (MMPs) and growth factors.[5] Two inhibitors control the activity of plasmin: $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin. The activity of t-PA and u-PA is fine-tuned at many levels, including the interaction with specific inhibitors, of which PA inhibitor 1 (PAI-1) is considered to be the physiological relevant one[6] (Fig 1).

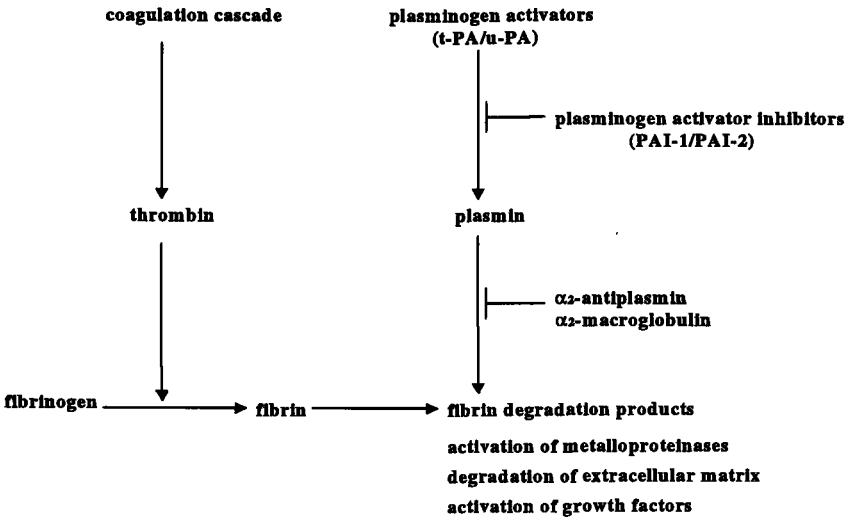


**Figure 1. Schematic representation of the coagulation and plasminogen/plasmin systems.**
t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor.

Consistent with the importance for so many biological processes, the synthesis of the key components of the coagulation and plasminogen/plasmin systems is tightly regulated, and disturbances in (plasma) levels of such factors have pathophysiological consequences. In humans, many prospective and cross-sectional studies have consistently shown that elevated plasma Fbg levels are associated with an increased risk of atherothrombotic disease.[7-10] On the other hand, humans with acquired or congenital low plasma levels of Fbg are suffering from bleeding tendencies.[11] Transgenic mice deficient of Fbg show similar bleeding patterns,[1,12] stressing the important role of Fbg in haemostasis. Numerous clinical studies have also pointed to a relationship between increased PAI-1 levels and an increased risk of cardiovascular events.[10,13,14] In contrast, bleeding tendency is observed in humans with low or undetectable plasma PAI-1 levels.[15-17] The physiological role of PAI-1 in haemostasis as deduced from clinical studies has been confirmed using transgenic mice. Mice overexpressing (human) PAI-1 displayed fibrin-rich venous occlusions in the tail and hind legs,[18] whereas PAI-1 deficient mice are largely protected from developing venous thrombosis following injection of endotoxin.[1]

In this thesis, emphasis is on regulatory aspects of Fbg and PAI-1 expression, with particular reference to the regulatory role of fibrates. Fibrates are hypolipidaemic drugs and are among the few compounds able to effectively lower Fbg and PAI-1 plasma levels in humans *in vivo*. However, the precise mechanism(s) by which fibrates exert their effects on

Fbg and PAI-1 levels is unknown. In the following paragraphs reported effects of fibrates on plasma Fbg and PAI-1 levels in humans will be briefly summarized. Subsequently, possible mechanism(s) by which fibrates could affect gene expression as well as relevant aspects of Fbg and PAI-1 regulation will be described. Finally, the outline and aims of this thesis are given.

## Effect of fibrates on plasma Fbg and PAI-1

Fibrates are widely used in the treatment of diet-resistant hyperlipidaemia. These drugs effectively lower elevated plasma triglyceride and low-density lipoprotein (LDL)-cholesterol levels, and enhance high-density lipoprotein (HDL)-cholesterol levels.[19,20] Although the lipid-lowering effects of different fibrates are comparable, variable results have been found with respect to changes in plasma levels of Fbg and PAI-1 in various clinical studies (see Table 1 for data on Fbg and Table 2 for data on PAI-1). This variability in effects may reflect different actions of different fibrates. On the other hand, different effects have been reported for the same fibrate. Inasmuch differences in study group, study design or assays employed contribute to the variability in reported results is uncertain. Studies involving gemfibrozil, for example, were performed in hyperlipidaemia patient groups as different as hypertriglyceridaemic and/or hypercholesterolaemic patients, survivors of myocardial infarction, patients with a history of thrombosis and patients with severe atherosclerosis. With regard to the assays, different methods exist for determination of Fbg or PAI-1 each of which has been shown to yield different results. For Fbg, the following assay principles exist: assays measuring functional Fbg (e.g. Clauss method),[21] assays measuring the amount of clottable protein (e.g. gravimetric),[22] and assays measuring antigen (e.g. radial immunodiffusion[23] and enzyme immune assay [EIA][24]. The assay principles were shown to yield different Fbg values.[25-29] PAI-1 can occur in different molecular forms (active, latent, or in complex with t-PA or u-PA), which are recognized with different efficiency in different assay systems available, resulting in different antigen values.[30, 31]

## Fibrates and the modulation of gene expression; role of peroxisome proliferator-activated receptor (PPAR)

Before the identification of the peroxisome proliferator-activated receptor (PPAR) as an important mediator of fibrate-modulated gene expression (see below), a variety of mechanisms of action have been proposed by which fibrates could modulate gene expression.

Fibrates may modulate gene expression by interference with cellular signal transduction pathways[32]: fibrates have been shown to increase intracellular calcium levels and to modulate protein kinase C (PKC) activity, factors known to be part of many signalling cascades. Also,

Table 1: Summary of fibrate studies with regard to effects on plasma Fbg levels

| Fibrate | Type of patient | Duration of treatment | Effect on plasma Fbg | Method | Reference |
|---|---|---|---|---|---|
| bezafibrate | type IIb and type IV† | 8 weeks | - 21 % | Clauss | [95] |
| bezafibrate | type IV† | 6 weeks | -17 % | synerese | [96] |
| gemfibrozil | type IV† | 12 weeks | -24 % | RID | [97] |
| clofibrate | hypertriglyceraemic | 6 months | - 9 % | Clot weight | [98] |
| ciprofibrate | hypercholesterolaemic | 12 weeks | - 18 % | Clauss | [99] |
| bezafibrate | hypercholesterolaemic | 4 months | - 10 % | Clauss | [100] |
| fenofibrate | hypercholesterolaemic | 4 months | - 16 % | Clauss | [100] |
| gemfibrozil | hypercholesterolaemic | 4 months | + 20 % | Clauss | [100] |
| bezafibrate | diabetes + hypercholesterolaemic and/or hypertriglyceridaemic | 3 months | -29 % | nephelometric | [101] |
| bezafibrate | atherosclerosis + hyperfibrinogaemia | 15 days | -44 % | RID | [102] |
| gemfibrozil | atherosclerosis | 13 weeks | = | Clauss | [103] |
| gemfibrozil | previous myocardial infarction | 8 weeks | + 26 % | Clauss | [104] |
| gemfibrozil | patients with coronary heart disease | 2 months | + 11 % | gravimetric | [105] |

† , type according to Fredrickson. Fbg, fibrinogen; RID, radial immunodiffussion,

**Table 2: Summary of fibrate studies with regard to effects on plasma PAI-1 levels**

| Fibrate | Type of patient | Duration of treatment | Effect on plasma PAI-1 antigen | Effect on plasma PAI-1 activity | Assay used for PAI-1 antigen | Reference |
|---|---|---|---|---|---|---|
| gemfibrozil | type IIb† | 10-12 weeks | n.d. | = | - | 106 |
| bezafibrate | type IIb and type IV† | 8 weeks | = | = | Imulyse | 95 |
| gemfibrozil | type IV† | 12 weeks | n.d. | - 44 % | - | 97 |
| gemfibrozil | type IV and type V† | 6 weeks | = | = | TintElize | 107 |
| gemfibrozil | type IV and type V† normalization of triglycerides | 6 weeks | - 49 % | = | TintElize | 107 |
| fenofibrate | type IV and type V† | 6 weeks | = | = | TintElize | 107 |
| gemfibrozil | hypertriglyceridaemic | 4 months | n.d. | = | - | 108 |
| gemfibrozil | previous myocardial infarction | 8 weeks | -19 % | = | TintElize | 104 |
| gemfibrozil | deep vein thrombosis† | 1 month | n.d. | = | - | 109 |
| bezafibrate | hyperlipidaemic heart transplants | 8 weeks | -26 % | -18 % | n.m. | 110 |

†, type according to Fredrickson. PAI-1, plasminogen activator inhibitor-1; n.d, not determined, n.m., not mentioned.

specific interference of fibrates with growth factor responses has been reported.[33] For example, fibrates were found to trigger the phosphorylation of the epidermal-growth-factor receptor, thereby modulating its function.[33] Furthermore, studies have demonstrated that fibrates induce proto-oncogenes of the Jun family, genes whose expression is also affected by PKC and growth factor regulatory pathways.[34]

More recently and, as it turned out, more importantly, fibrates were found to modulate gene expression by activating a nuclear transcription factor, PPAR. PPAR is a member of a large family of ligand-inducible transcription factors that include receptors for retinoids, vitamin D, thyroid and steroid hormones (for a review see Evans[35]). PPAR, upon heterodimerization with the 9-*cis*-retinoic acid receptor (RXR), binds to specific response elements termed peroxisome proliferator-response elements (PPREs), thus regulating the expression of target genes. Most PPREs identified to date reside in genes involved in intra- and extracellular lipid metabolism (reviewed by Schoonjans et al.[36]).

The mammalian PPAR family is composed of at least three genetically and pharmacologically distinct subtypes, PPARα, PPARβ/δ and PPARγ.[37] PPARα is predominantly expressed in tissues exhibiting high catabolic rates of fatty acids (liver, heart, kidney and muscle), while PPARγ is more adipose tissue selective, where it triggers adipocyte differentiation and lipid storage by regulating the expression of genes for adipogenesis. PPAR β/δ shows a ubiquitous expression pattern, its exact function being still unknown.

The term PPAR was introduced by its virtue of being activated by peroxisome proliferators, a diverse group of chemicals that include hypolipidemic drugs, herbicides and industrial plasticizers.[38] Administration of these chemicals to rodents results in a dramatic proliferation of hepatic peroxisomes as well as liver hyperplasia.[39] PPARs were cloned based on sequence homology with members of the steroid hormone superfamily and since the physiological ligands for PPARs were initially unknown, they were considered orphan receptors. Activation of PPARs was thought to occur via an unknown ligand that was induced as a result of a perturbation in lipid metabolism.[40] More, recently, sensitive ligand-binding assays showed that fatty acids, eicosanoids and fibrates are *bona fide* PPAR ligands that directly activate PPAR.[41-43] Different ligands were found to bind the different PPAR isoforms with different specificity.[41-43] Fibrates are assumed to mainly activate PPARα.[41,42] However, some fibrates also activate PPARγ, albeit to a much lesser extent.[41-43]

## Fibrinogen and PPAR

Fbg is a plasma glycoprotein synthesized by liver parenchymal cells. The protein is secreted as a dimer of two Aα-, Bβ- and γ-polypeptides linked by disulphide bonds.[44,45] The three Fbg polypeptides are encoded by three separate, closely linked genes situated on the same chromosome and located in the sequence γ, Aα and Bβ, with the gene for the Bβ-chain

transcribed in the opposite direction.[46] In the last 20 years, several studies concerning the transcriptional regulation of the three Fbg genes in different species have contributed to the identification of regulatory promoter-elements and their interacting factors involved in expression of the three Fbg chains. Key regulatory regions of the human Fbg Aα-, Bβ- and γ-chain genes are summarized in Fig 2, and include binding sites for hepatic nuclear factor-1 (HNF-1), upstream stimulatory factor (USF), CAAT/binding protein (C/EBP), glucocorticoid receptor elements.[47-52] Interestingly, the same regulatory sites are present in the promoters of all three Fbg chain genes of humans as well as other species, resulting in co-ordinate regulation of the three chain genes at the transcriptional level.[53,54]

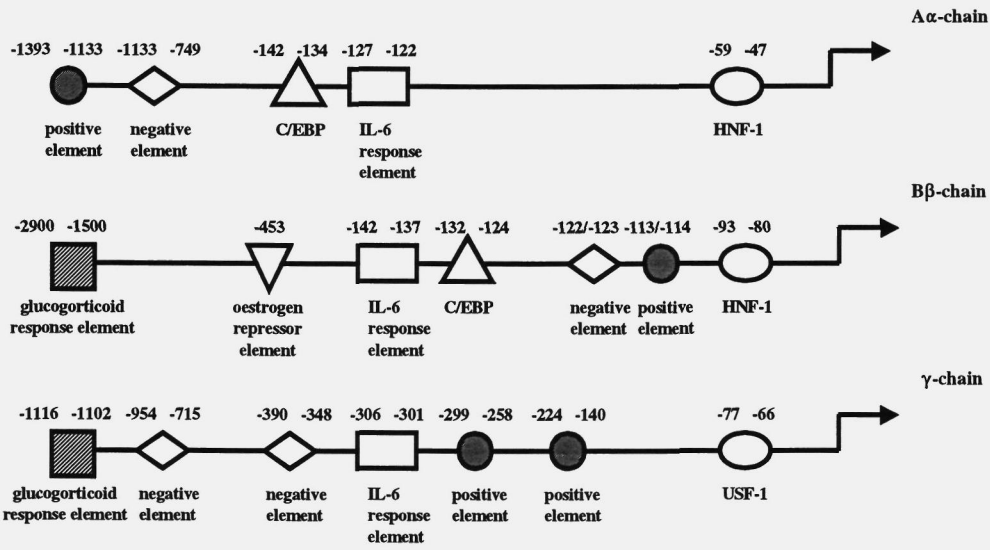


**Figure 2. Schematic representation of the regulatory elements in the human Fbg A α- Bβ- and γ-chain gene promoters.** Location of the regulatory sequences are taken from Hu et al, [47] Anderson et al,[48] Dalmon et al,[49] Mizuguchi et al,[51] Asselta et al,[52] and Humphries et al.[111] C/EBP, CAAT binding protein; IL-6, interleukin-6, HNF-1, hepatic nuclear factor-1; USF-1, upstream stimulatory factor-1. Positive and negative elements refer to promoter regions found to up- or downregulate basal and IL-6 induced expression in HepG2 cells.

Little is known about the mechanism by which fibrates affect Fbg synthesis. In the human hepatoma cell line HepG2, bezafibrate was shown to decrease the mRNA levels of the Fbg Aα-, Bβ and γ-chains, but a role of PPAR was not investigated.[55] Analysis of the promoter regions of the three human Fbg genes revealed putative PPREs in al three chains. Whether these elements bind PPAR/RXR heterodimers and are involved in fibrate-mediated modulation of Fbg expression remains to be determined. Fibrates may also change Fbg expression by

competition of PPAR for binding of other factors necessary for Fbg transcription. For example, fibrates have been shown to repress transcription of the apolipoprotein CIII gene in liver by down-regulating the expression of the strong positive transcription factor HNF-4 as well as by displacement of HNF-4 binding by non-productive PPAR/RXR heterodimers.[56] A similar mechanism could be applicable to HNF-1 and USF-1, factors that have been found limiting for basal expression of the three Fbg chains.

An important aspect of Fbg expression is that Fbg is an acute phase protein, induced by the cytokine, interleukin-6 (IL-6). Recent evidence indicates that activated PPARα can interfere negatively with cytokine-induced signalling pathways.[57-59] Among these is the Signal Transducer and Activator of Transcription protein 3 (STAT3) pathway, which pathway is generally assumed to play a role in IL-6-mediated transcriptional regulation.[60,61] An involvement of STAT3 in the regulation of the human Fbg B β-chain and the rat γ-chain has been reported.[62,63] It is conceivable that in the same manner PPARα also plays an important role in down-regulating cytokine-increased fibrinogen gene expression.

## PAI-1 and PPAR

Many cell types, including human hepatocytes, monocytes, fibroblasts, endothelial cells and smooth muscle cells, have been found to express PAI-1 *in vitro*.[6,64,65] However, the exact contribution of these cell types to plasma PAI-1 levels *in vivo* is still a matter of debate. Northern blotting analysis showed PAI-1 mRNA expression in many rodent and human tissues *in vivo*, including liver, heart, kidney, aorta, lung and placenta.[66-69] *In situ* hybridisation analysis revealed expression of PAI-1 in hepatic parenchymal, Kupffer, endothelial cells and vascular endothelial and smooth muscles cells.[70,71] Brommer et al,[72] provided evidence that circulating PAI-1 in man indeed originates (partly) from hepatic cells.

Recently, adipose tissue, in particular the visceral fat area, has also been considered to be an important source of PAI-1 in the circulation.[73-76] Adipocytes are a major target of PPARγ activators.[77] In addition, these cells contain significant amounts of PPARα and PPARβ.[78]

*In vitro* gene regulation studies have identified many factors and promoter elements that are important in the regulation of basal and stimulated PAI-1 expression.[79-81] Some of these findings may also be of relevance for the fibrate-modulated PAI-1 synthesis. In hepatocytes, PAI-1 synthesis is shown to be stimulated by PKC activators, such as phorbol myristate acetate (PMA).[82] This is of interest because fibrates and other PPARα activators have been reported to modulate the activity of PKC.[32,83] Secondly, as shown in cultured endothelial and hepatoma cells, PAI-1 synthesis can be stimulated by growth factors, such as epidermal growth factor (EGF) and transforming growth factor β (TGFβ).[84,84] Studies in rat hepatocytes indicate that

fibrates may interfere with the EGF signal transduction pathway through phosphorylation of the EGF-receptor via activation of PKC.[33]

Both PMA and growth factor stimulation of PAI-1 expression were shown to involve the transcription factor activator protein-1 (AP-1).[60,85] AP-1 is a collection of homodimeric and/or heterodimeric complexes composed of Jun and Fos gene products.[86] These complexes interact with a common DNA binding site, the PMA responsive element (TRE) and activate gene transcription. Several TREs have been identified in the PAI-1 promoter.[87] PMA-stimulated PAI-1 expression was shown to depend on binding of c-jun homodimers to a proximal TRE,[88] whereas TGFβ induction of PAI-1 is mediated by two distal TREs.[89] Interestingly, Sakai et al[90] showed that PPARα can downregulate transcription of the glutathione transferase-P gene in rat hepatocytes through squelching of c-jun. Furthermore, PPARα activation was shown to affect the expression of the proto-oncogene Jun family.[34] Whether fibrates can modulate PAI-1 expression through interference with different aspects of PKC or growth factor signalling pathways remains to be established.

Fibrates may also modulate PAI-1 expression by direct interaction of (activated) PPARα with the PAI-1 promoter. Several *in vitro* studies indicate that PAI-1 expression is induced by triglyceride-rich emulsions and fatty acids derived from very low density lipoprotein (VLDL).[91,92] Recently, a VLDL response element (VLDLRE) was identified in the promoter region of the PAI-1 gene, mediating VLDL- and fatty acid-induced PAI-1 transcription.[93,94] Analysis of the VLDLRE showed that there is some homology with the PPRE consensus sequence. As fatty acids are natural ligands of PPARs, and fibrates are known to affect fatty acid composition and also activate PPAR directly, it has been suggested that PPAR may be directly involved in the regulation of PAI-1 gene expression.[93]

## Outline and aims of this thesis

The work presented in this thesis was directed primarily at establishing the effects of hypolipidaemic fibrates on plasma levels of Fbg and PAI-1 and at elucidating the gene regulatory mechanism(s) involved, with particular emphasis on the role of PPARα. Most of the work concentrated on liver hepatocytes as a source of Fbg and PAI-1, but, on basis of recent publications, adipose tissue was also evaluated as a contributor of plasma PAI-1.

In **Chapter 2**, we sought to learn more about the variability in the reported effects of fibrates on haemostatic parameters by carefully studying the effects of fibrates *in vivo* in humans under well-defined conditions using well-characterized assays. The effects of two fibrates, gemfibrozil and ciprofibrate, on plasma concentrations of Fbg and PAI-1 were studied in primary hyperlipidaemic patients after six and twelve weeks of treatment using different

assay systems for Fbg and PAI-1.

In subsequent studies the mechanism(s) by which fibrates change the expression of Fbg and PAI-1 were investigated *in vitro*. The studies described in **Chapter 3** are directed at elucidating the regulatory mechanism(s) by which fibrates suppress PAI-1 synthesis in cultures of primary hepatocytes from cynomolgus monkey (*Macaca fascicularis*). The potency of four different fibrates to decrease PAI-1 synthesis was determined and the possible interference of the fibrates with protein kinase C activity, and EGF and TGFβ signalling in the fibrate-inhibited PAI-1 expression was evaluated. In addition, a first attempt was undertaken to investigate the role of the nuclear hormone receptors PPARα/RXRα.

In **Chapter 4**, a possible involvement of PPARα/RXRα in mediating the effects of fibrates on PAI-1 expression was further studied. In addition, a role of PPARα/RXRα in fibrate-modulated Fbg expression was studied. Because PPARα activation cannot be directly determined in monkey hepatocytes, we used a stably-transfected Chinese hamster ovary cell-line system, containing a reporter gene under control of several PPREs, to determine the PPARα-activating capacity of the various test compounds. We compared the efficacy of several fibrates, an RXR specific ligand (9-*cis* retinoic acid) and two specific PPARα-activators (Wy14,643 and 5,8,11,12-eicosatetraynoic acid [ETYA]) to alter Fbg and PAI-1 synthesis with the capacity of the compounds to activate PPARα. Apolipoprotein A-I (apo A-I), a gene under control of PPARα, was included as a positive control.

**Chapter 5** describes a study in which the role of PPARα in the regulation of Fbg plasma levels was investigated *in vivo*. First, the kinetics of the effect of fibrates on the expression of the three Fbg chains genes in rats was studied, after which the question was addressed whether PPARα is involved in this regulation. For this aspect of the study we made use of PPARα-deficient mice.

In **Chapter 6**, the contribution of the amount of visceral fat to plasma PAI-1 levels was investigated. Moderately obese men and women were subjected to an energy-deficient diet for 13 weeks. Visceral and subcutaneous fat depots were assessed by Magnetic Resonance Imaging (MRI). Before and after weight loss relationships between anthropometric variables, fat distribution and fibrinolytic variables were determined in men and women. We reasoned that if the visceral fat area is to contribute directly to plasma PAI-1 levels, proportional changes in visceral fat should correlate with proportional changes in PAI-1 levels. Therefore, the relationship between diet-induced changes in visceral fat area and plasma PAI-1 levels in both sexes was studied.

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*CHAPTER 2*

# Effects of gemfibrozil and ciprofibrate on plasma levels of tissue-type plasminogen activator, plasminogen activator inhibitor-1 and fibrinogen in hyperlipidaemic patients

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*Thrombosis and Haemostasis 1997; 78:1167-1172*

## Abstract

Evaluation of fibrate treatment in humans has focused primarily on its anti-lipidaemic effects. A potentially favourable haemostasis-modulating activity of fibrates has also been recognized but the data are not consistent. We sought to learn more about this variability by examining the effects of gemfibrozil and ciprofibrate on plasma levels of tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) and fibrinogen in primary hyperlipidaemic patients after six and twelve weeks of treatment using different assay systems for PAI-1 and fibrinogen. Although both fibrates effectively lowered triglyceride and cholesterol levels, no effect on the elevated baseline antigen levels of t-PA and PAI-1 was observed after fibrate treatment. However, both fibrates influenced plasma fibrinogen levels, albeit in a different way. Fibrinogen antigen levels were elevated by 17.6% ($p<0.05$) and 24.3% ($p<0.001$) with gemfibrozil after six and twelve weeks, respectively, whereas with ciprofibrate there was no effect. Using a Clauss functional assay with either a mechanical end point or a turbidity-based end point, no significant change in fibrinogen levels was seen after six weeks of gemfibrozil treatment. However, after twelve weeks, gemfibrozil enhanced functional fibrinogen levels by 7.2% ($p<0.05$) as assessed by the Clauss mechanical assay, but decreased functional fibrinogen levels by 12.5 % ($p<0.0001$) when a Clauss assay based on turbidity was used. After six or twelve weeks of ciprofibrate treatment, functional fibrinogen levels were decreased by 10.1% ($p<0.001$) and 10.5% ($p<0.0001$), respectively on the basis of Clauss mechanical and by 14.2% ($p<0.001$) and 28.2% ($p<0.0001$), respectively with the Clauss turbidimetric assay. A remarkable and consistent finding with both fibrates was the decrease in functionality of fibrinogen as assessed by the ratio of functional fibrinogen (determined by either of the two Clauss assays) to fibrinogen antigen.
Taken together, our results indicate that at least part of the variability in the effects of fibrates on haemostatic parameters can be explained by intrinsic differences between various fibrates, by differences in treatment period and/or by the different outcomes of various assay systems. Interestingly, the two fibrates tested both reduced the functionality of fibrinogen.

## Introduction

Fibrates are a class of hypolipidaemic drugs used in the treatment of diet-resistant dyslipidaemia. Fibrates effectively lower elevated plasma triglyceride and low-density lipoprotein (LDL) cholesterol levels, and enhance high-density lipoprotein (HDL) cholesterol levels.[1-4] In addition, fibrates have been reported to modulate plasma levels of non-lipid cardiovascular risk factors such as tissue-type plasminogen activator (t-PA), its main physiological inhibitor, plasminogen activator inhibitor-1 (PAI-1) and fibrinogen.[3-4] This latter action may contribute to their favourable action in the prevention of coronary artery

disease (CAD).[4-6]

Although the lipid-lowering effects of different fibrates are comparable, variable results have been found with respect to changes in plasma levels of t-PA, PAI-1 and fibrinogen in various clinical studies. This variability in effects may reflect the different actions of different fibrates. For example, gemfibrozil significantly lowered PAI-1 antigen levels, whereas, in the same study, no change in PAI-1 antigen levels was caused by fenofibrate.[7] In another study using fenofibrate, bezafibrate and gemfibrozil, plasma fibrinogen levels were increased by gemfibrozil and decreased by the two other fibrates.[8] On the other hand, different effects have been reported for the same fibrate. t-PA and PAI-1 antigen levels were lowered by gemfibrozil in several studies,[7,9] while in other studies no effect on these parameters was found.[10,11] Similarly, gemfibrozil was shown to decrease as well as to increase plasma fibrinogen levels.[12,13] These discrepancies may either derive from differences in study group, study design or from differences in the assays employed. Studies involving gemfibrozil, for example, were performed in hyperlipidaemic patient groups as different as hypertriglyceridaemic and/or hypercholesterolaemic patients,[7,12] survivors of myocardial infarction,[9] patients with a history of thrombosis[10] and patients with severe atherosclerosis.[14] The duration of these studies varied from several weeks to several months.[7,8,12] With regard to the assays, it is known that different methods can yield different results. For example, PAI-1 can occur in different molecular forms: latent PAI-1, active PAI-1 and PAI-1 in complex with t-PA or urokinase-type PA (u-PA). Different commercially available PAI-1 assays recognize these different forms with differing efficiency, resulting in different antigen values.[15,16] For fibrinogen, different assay principles also exist: assays based on clotting rate, amount of clottable protein or immunological assays.[17,18]

From the above, it is obvious that no clear picture exists of the effects of fibrates on t-PA, PAI-1 and fibrinogen plasma levels. In an attempt to gain more insight into their action, we compared the effects of two fibrates on these haemostatic parameters in a well-defined group of patients over two treatment periods, applying well-characterized assays. Gemfibrozil is the most widely used and investigated fibrate. Ciprofibrate is a more recently developed second generation fibrate that has been reported to have a similar safety profile but is used in a lower dosage than gemfibrozil.[19] We examined the influence of gemfibrozil and ciprofibrate on t-PA, PAI-1 and fibrinogen levels after six and twelve weeks of treatment in patients with primary hyperlipidaemia. t-PA and PAI-1 levels were measured by enzyme immunoassay (EIA). For PAI-1, two different EIAs were used, one detecting all molecular forms of PAI-1 with the same efficiency and one that is sensitive to latent and active PAI-1, but is relatively insensitive to complexed and degraded forms of PAI-1.[15,20] Fibrinogen was determined by three different assays, measuring either fibrinogen protein (by an EIA) or functional fibrinogen (by two variations of the Clauss method, one with a turbidity-based end point, the other with a mechanical end point).

## Materials and methods

**Patients and study design**

In this study, t-PA, PAI-1 and fibrinogen were assessed in patients with hyperlipidaemia, participating in an intervention trial on the effects of gemfibrozil versus ciprofibrate on lipid and lipoprotein parameters. [21] Briefly, patients with primary hyperlipidaemia (type IIa and IIb according to the Fredrickson classification) and with a total cholesterol level equal to or higher than 6.5 mmol/l were randomly allocated to treatment with 100 mg/day ciprofibrate (n=51) or two times 600 mg/day gemfibrozil (n=48). Among the patients three types of hyperlipidaemia existed: 14 patients with familial hypercholesterolaemia (FH), 58 patients with familial combined hyperlipidaemia (FCH) and 27 patients with polygenetic hypercholesterolaemia (PH). Mean triglyceride levels at study entry were 1.8 ± 0.1, 3.8 ± 0.7, and 3.1± 1.2 mmol/l for the FH, FCH and PH group, respectively. Age, body mass index and gender of the gemfibrozil and ciprofibrate group were comparable, being 52.4 ± 12.5 and 51.4 ± 11.4 years, 25.6 ± 3.4 and 27.1 ± 4.4 kg/(m) $^2$, and 56% and 62% male, respectively. In the gemfibrozil group 32 out of 42 patients were smokers versus 23 out of 40 patients in the ciprofibrate group. All the patients in this study gave their written consent in accordance with the Helsinki Declaration. The study was approved by the Medical Ethical Committee of the institute where it was carried out (Slotervaart Hospital, Amsterdam).

The study consisted of a four-week dietary wash-out period in which the patients were placed on or continued on a standard hypocholesterolaemic diet followed by a baseline placebo period of four weeks. After the placebo period a double-blind active twelve-week treatment period was started. Five visits took place, the first visit prior to the four-week dietary period and the second visit prior to the placebo period. The other three visits were immediately before and after six and twelve weeks of active treatment. At visits two, three, four and five, blood samples were collected from fasting patients in trisodium citrate according to the protocol of the Leiden Fibrinolysis Working Party. [22] The blood samples were immediately put into melting ice. After centrifugation (30 min, 3000g at 4 °C) plasma was collected and stored at -80 °C. The overall compliance to the study medication was > 95% in both treatment groups. Adverse events were rare, mild and equally distributed between the gemfibrozil- and ciprofibrate-treated patients. Laboratory safety parameters did not show any significant changes between the two treatment groups.

**Assays**

Human t-PA and PAI-1 antigen were determined using commercially available enzyme immunoassay (EIA) kits (Thrombonostika t-PA from Organon Teknika, Turnhout, Belgium; Innotest PAI-1 from Innogenetics, Zwijndrecht, Belgium; and Imulyse PAI-1 from Biopool AB, Umeå, Sweden). The Innotest PAI-1 assay detects all molecular forms of PAI-1 to a similar degree. [15] The Imulyse Biopool PAI-1 assay detects latent and active PAI-1 with similar efficiency, but is relatively insensitive to complexed forms of PAI-1. [20]

Fibrinogen antigen was determined using the Fibrinostika-Intact Fibrinogen EIA from Organon Teknika, Turnhout, Belgium. In this assay the high and low molecular weight (HMW + LMW) forms of fibrinogen are measured specifically using a monoclonal antibody against the intact carboxyl-terminal end of the fibrinogen Aα chain as the capture antibody and a monoclonal antibody against the amino-terminal end of the A α-chain, including fibrinopeptide A, as the tagging antibody. [23] Functional fibrinogen was determined by the Clauss assay[24] with two end point detection methods: in one type of assay a change in turbidity was used to assess the end point (MLA Electra 1000c, Baxter; reagents from Dade, Utrecht, The Netherlands); in a second one a mechanical end point was used (KC10, Amelung; reagents from BioMerieux, Marcy-l'Etoile, France). These two methods will be referred to as Clauss turbidimetric and Clauss mechanical, respectively.

In the ciprofibrate group two highly aberrant fibrinogen measurements (7.81 and 8.03 g/l) were observed using the Clauss mechanical assay, while in the Clauss turbidimetric assay 2.09 and 2.71 g/l and in the antigen assay values of 2.01 and 2.67 g/l were found. Measurement of functional fibrinogen levels in these patients at the other three visits exhibited values between 1.90 and 2.40 g/l. As the values of the two Clauss assays are generally correlated, the two aberrant fibrinogen values were considered outliers and were not included in the analysis.

**Electrophoresis**

Molecular weight forms of fibrinogen were examined using the PhastGel Gradient system (Pharmacia Biotech B.V, Woerden, The Netherlands), with gels containing a gradient of 4-15% (w/v) acrylamide. After staining of the bands with Coomassie blue, the bands were scanned and quantified using a LKB 2222-020 Ultrascan XL Laser Densitometer.

**Statistics**

Since the distribution of the t-PA and PAI-1 levels in the baseline and treatment groups was not normal, we used non-parametric tests for statistical analysis. The data are presented as median and central 90% range values. Fibrinogen values (Clauss and antigen) were normally distributed and presented as means (±SEM).

The significance of the effects of gemfibrozil and ciprofibrate treatment was assessed by Student's paired *t*-test for fibrinogen and the Wilcoxon Signed Ranks test for t-PA and PAI-1 levels. For baseline values, the levels after the placebo period were taken. P-values lower than 0.05 were considered significant. Correlations between the different variables were determined by Spearman's rank correlation analysis ($r_s$).

## Results

### Lipid parameters

During the four-week dietary wash-out period, significant changes in triglyceride (from 2.43 ± 1.70 to 3.06 ± 0.32 mmol/l; $p<0.01$), cholesterol (from 7.14 ± 1.29 to 9.00 ± 1.87 mmol/l; $p<0.00001$), HDL-cholesterol (from 1.23 ± 0.37 to 1.19 ± 0.36 mmol/l; $p<0.05$), and LDL-cholesterol (from 4.84 ± 1.40 to 6.53 ± 2.03 mmol/l; $p<0.00001$) were observed in the total patient group. Total cholesterol levels further increased during the subsequent placebo period to 9.30 ± 2.16 mmol/l ($p<0.001$), whereas triglyceride, HDL-cholesterol and LDL-cholesterol levels remained constant at 3.29 ± 0.42 mmol/l ($p=0.30$), 1.15 ± 0.37 mmol/l ($p=0.10$) and 6.65 ± 2.58 mmol/l ($p=0.43$), respectively.

The effects of gemfibrozil and ciprofibrate on the lipid parameters for the two treatment groups are summarized in Table 1. With both fibrates significant ($p<0.0001$) and comparable changes in plasma cholesterol (on average -15%), triglyceride levels (-42%), HDL-cholesterol levels (+7%) and LDL-cholesterol levels (-11%) were found after 6 weeks of treatment. These values did not significantly change during the next 6 weeks of treatment.

**Table 1: Effect of gemfibrozil and ciprofibrate on plasma cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol levels**

| | gemfibrozil (n=48) | | | | ciprofibrate (n=51) | | | |
|---|---|---|---|---|---|---|---|---|
| | total cholesterol (mmol/l) | triglycerides (mmol/l) | HDL-cholesterol (mmol/l) | LDL-cholesterol (mmol/l) | total cholesterol (mmol/l) | triglycerides (mmol/l) | HDL-cholesterol (mmol/l) | LDL-cholesterol (mmol/l) |
| 4 wk placebo | 9.03±1.22 | 2.25±1.95 | 1.19±0.36 | 6.78±2.30 | 9.03±1.22 | 2.25±1.95 | 1.19±0.36 | 6.78±2.30 |
| 6 wk treatment | 7.46±1.27* | 1.17±1.99* | 1.29±0.39* | 5.80±2.08* | 7.90±1.23* | 1.65±1.84* | 1.21±0.20* | 5.98±1.77* |
| 12 wk treatment | 7.54±1.27* | 1.14±2.25* | 1.39±0.43* | 5.63±2.19* | 7.84±1.23* | 1.48±1.77* | 1.25±0.46* | 5.96±1.73* |

Values are means ± SD; * $p<0.0001$ from baseline levels using a paired Student's *t*-test

**Table 2: Effect of gemfibrozil and ciprofibrate on plasma t-PA and PAI-1 antigen levels.**

| | gemfibrozil | | | ciprofibrate | | |
|---|---|---|---|---|---|---|
| | t-PA (ng/ml) | PAI-1 (ng/ml) | | t-PA (ng/mL) | PAI-1 (ng/mL) | |
| | | Innotest | Imulyse | | Innotest | Imulyse |
| 4 wk placebo | 16.3 | 79.7 | 41.1 | 14.8 | 77.7 | 45.4 |
| | (8.7-24.8) | (26-177) | (10-86) | (8.3-22.2) | (26-181) | (6-94) |
| 6 wk treatment | 16.1 | 80.4 | 37.2 | 14.8 | 91.5 | 47.0 |
| | (8.8-23.5) | (23-150) | (9-95) | (8.5-22.8) | (25-189) | (13-115) |
| 12 wk treatment | 15.7 | 82.6 | 34.1 | 15.5 | 77.5 | 43.0 |
| | (9.5-24.5) | (29-178) | (9-119) | (6.8-23.1) | (20-188) | (15-104) |

Values are median (central 90% range)

**Effects of fibrates on plasma t-PA and PAI-1 levels**

The effects of gemfibrozil and ciprofibrate on plasma t-PA and PAI-1 antigen levels are shown in Table 2. Before and after fibrate treatment, a broad variation between individual plasma t-PA and PAI-1 antigen levels was observed. t-PA levels ranged from 8 to 25 ng/ml and PAI-1 levels from 20 to 189 ng/ml (Innotest) or from 6 to 119 ng/ml (Imulyse). A similar broad variation was observed in normolipidaemic men and women by others (25,26). The higher PAI-1 values obtained with the Innotest assay as compared with the Imulyse assay are similar as previously observed (15) and are due to different specificities and/or different calibrators of the two assay systems. Comparison of plasma PAI-1 values measured by the two assay systems showed a strong correlation ($r_s$=0.8).

No significant changes in t-PA or PAI-1 levels (using either the Innotest or the Imulyse test) were observed during the placebo treatment or after treatment with gemfibrozil or ciprofibrate for six or twelve weeks (data not shown). Also, analysis of the three subgroups of hyperlipoproteinaemia patients (see Methods section) showed no significant difference in t-PA or PAI-1 levels before or after the various treatments (data not shown).

A correlation between baseline t-PA and PAI-1 plasma levels ($r_s$=0.5 for both PAI-1 assays) and between plasma triglyceride and PAI-1 levels ($r_s$=0.5 and $r_s$=0.4 for Innotest and Imulyse PAI-1, respectively) was found. These correlations remained after fibrate treatment, even when PAI-1 levels were not lowered concomitantly with the triglyceride levels ($r_s$=0.5 and $r_s$=0.4 for Innotest and Imulyse PAI-1, respectively).

**Effects of fibrates on plasma fibrinogen levels**

Treatment with gemfibrozil resulted in a significant increase (0.53 ± 0.22 g/l; $p<0.05$) in fibrinogen (HMW + LMW) antigen concentrations after six weeks, and an even higher increase (0.73 ± 0.20 g/l; $p<0.001$) after twelve weeks. Treatment with ciprofibrate had no significant effect on the plasma levels of fibrinogen antigen after six or twelve weeks (Fig. 1). Measurement of functional fibrinogen by the two Clauss variants showed a more complicated picture. Using the Clauss turbidimetric method, gemfibrozil treatment caused a reduction (0.43 ± 0.10 g/l; $p<0.0001$) only after twelve weeks, whereas the Clauss mechanical showed a slight increase in functional fibrinogen levels (0.24 ± 0.10 g/l; $p<0.05$).
With both the Clauss turbidimetric and the Clauss mechanical assays, ciprofibrate treatment reduced functional fibrinogen levels: by 0.48 ± 0.13 and 0.32 ± 0.11 g/l ($p<0.001$), respectively after six weeks and by 0.95 ± 0.10 and 0.31 ± 0.13 g/l ($p<0.0001$), respectively after twelve weeks. Remarkably, the ratio of functional fibrinogen to fibrinogen antigen was decreased with both fibrates (Fig. 1C).

In a first attempt to investigate why different results were obtained using the different assays for measuring fibrinogen concentrations, we examined whether fibrates, VLDL-

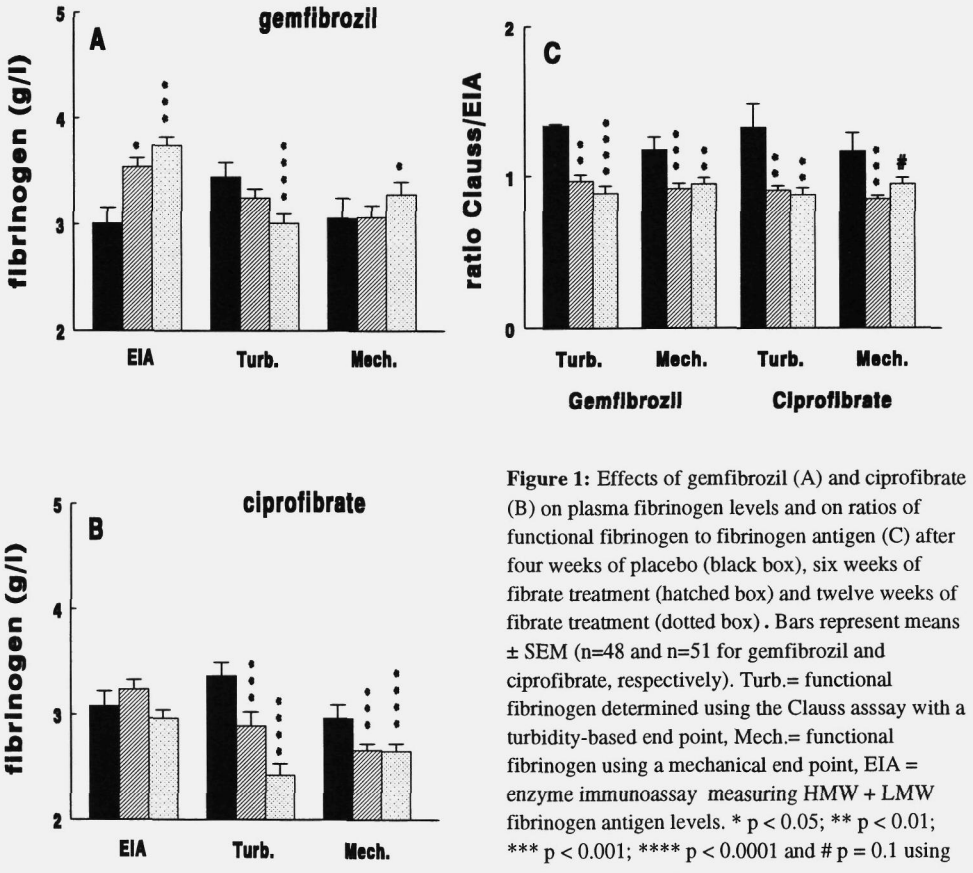


**Figure 1:** Effects of gemfibrozil (A) and ciprofibrate (B) on plasma fibrinogen levels and on ratios of functional fibrinogen to fibrinogen antigen (C) after four weeks of placebo (black box), six weeks of fibrate treatment (hatched box) and twelve weeks of fibrate treatment (dotted box). Bars represent means ± SEM (n=48 and n=51 for gemfibrozil and ciprofibrate, respectively). Turb.= functional fibrinogen determined using the Clauss asssay with a turbidity-based end point, Mech.= functional fibrinogen using a mechanical end point, EIA = enzyme immunoassay measuring HMW + LMW fibrinogen antigen levels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ and # $p = 0.1$ using the Student's paired *t*-test.

triglyceride or LDL-cholesterol were directly interfering with the fibrinogen antigen or Clauss assays. Concentrations of gemfibrozil and ciprofibrate up to 1 mmol/l or concentrations of VLDL-triglyceride or LDL-cholesterol up to 10 mmol/l (maximal concentrations of VLDL-triglyceride and LDL-cholesterol levels observed in the patients) did not influence the outcome of the various assays (data not shown). To further evaluate a role of "environmental" factors in the plasma, recovery experiments of fibrinogen for the three fibrinogen assays were performed by adding 1.35 mg/ml (final concentration) fibrinogen (KabiVitrum, Stockholm, Sweden) to patient plasma samples before and after 12 weeks of fibrate treatment. Recovery of fibrinogen by EIA in baseline samples was 88 ± 9% (n=10),

after gemfibrozil treatment 86 ± 9% (n=5), and after ciprofibrate treatment 86 ± 4% (n=5). Recovery of fibrinogen by Clauss turbidimetric and Clauss mechanical in baseline samples was 99 ± 13% (n=10) and 100 ± 13% (n=10), after gemfibrozil treatment 108 ± 5% (n=5) and 90 ± 15% (n=5), and after ciprofibrate treatment 83 ± 9% (n=5) and 93 ± 6% (n=5), respectively. These data are consistent with a change in fibrinogen rather than with a role of "environmental" factors in the plasma of fibrate-treated patients to explain the observed changes in ratio. Since it has been suggested that changes in the distribution of the molecular-weight forms of fibrinogen may account for changes in functionality of fibrinogen (27), we analysed twenty selected plasma samples of the patients by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) before and after fibrate treatment. No changes in the distribution of the molecular weight forms were observed after treatment (data not shown).

## Discussion

Our results obtained with gemfibrozil and ciprofibrate in a group of 99 primary hyperlipidaemic patients confirm the well-established lipid-regulating effects of fibrates, and indicate that the variability in effects on haemostatic factors may be due at least partly to intrinsic differences between the various fibrates, but also to different outcomes of the various assays and differences in treatment period. Both fibrates effectively lowered elevated plasma triglyceride and cholesterol levels, but had no effect on plasma t-PA or PAI-1 antigen levels, irrespective of the PAI-1 assay system used. The two fibrates affected plasma fibrinogen levels differently. Fibrinogen antigen levels were increased by treatment with gemfibrozil after six and twelve weeks, but not with ciprofibrate. In contrast, a decrease in functional fibrinogen levels was found with ciprofibrate after six and twelve weeks of treatment. In accordance with the antigen data, gemfibrozil increased functional fibrinogen after twelve weeks using a Clauss assay with a mechanical end point, but surprisingly, fibrinogen levels were decreased after twelve weeks using a Clauss assay in which turbidity was used to assess the end point. These findings may explain in part the diversity and inconsistency of the previously reported effects of fibrates on plasma fibrinogen levels, but they also hamper a proper comparison and evaluation of different studies.

One remarkable finding of this study is that with both fibrates the ratio of functional fibrinogen to fibrinogen protein ("functionality" of fibrinogen) was decreased. Changes in the functionality of circulating fibrinogen have also been observed during acute myocardial infarction and subsequent thrombolytic therapy.[27-29] Seifried et al.[27] ascribed these changes in functionality to an altered distribution of the three molecular weight forms of fibrinogen in plasma, forms which have different clotting behaviour.[30,31] During the initial phase of acute myocardial infarction the relative amount of the fast-clotting HMW fibrinogen in the total fibrinogen content is increased, explaining at least in part the relatively high functional

fibrinogen levels in these patients. Similarly, there was a fall in functional fibrinogen when HMW fibrinogen was converted to partly degraded fibrinogen during t-PA infusion.[27] However, limited proteolysis is unlikely to be true for the observed changes in functionality of fibrinogen as observed in this study after fibrate treatment: an initial comparison of the molecular weight distribution of plasma fibrinogen before and after fibrate treatment using SDS/PAGE showed a very similar distribution pattern. Apparently, more subtle changes in the fibrinogen molecule should be considered, since we found no evidence for an "environmental" factor in the plasma albeit VLDL-triglyceride, LDL-cholesterol or a fibrate-induced factor, to interfere with the functionality of fibrinogen.

The question arises whether the changes observed in fibrinogen levels after fibrate treatment as documented here should be considered beneficial or not. Prospective studies like the Northwick Park Heart Study[32] or the ECAT study[33] have pointed to the importance of plasma fibrinogen levels as an independent risk indicator in cardiovascular disease (CVD). The association between fibrinogen and risk was independent of the assay used (viz. gravimetric, Clauss or nephelometric). However, a straightforward interpretation of the fibrinogen findings in the present study is complicated, because different assays show different, sometimes conflicting, results. Since the protein mass of a thrombus would not alter dramatically by the relatively small increase (20-30%) in fibrinogen levels associated with an increased risk of CVD, it is suggested that the clot structure rather than the mass of deposited fibrin turns fibrinogen into a risk factor, as discussed by Blombäck.[34] Clots formed at higher fibrinogen concentrations are much tighter and much more rigid, less prone to fibrinolysis and considered to be more thrombogenic than clots formed at low fibrinogen concentrations.[34] Support for this concept comes from studies of several dysfibrinogenaemias associated with thrombotic diseases[35,36] and from the observation that fibrin gel structures formed in vitro from plasma samples from young (<45 years) male post-infarction patients were tighter than those formed from plasma from age-matched control men.[37] Bröijersén et al.[38] recently reported that gemfibrozil did not influence the fibrin gel structure in hyperlipidaemic patients, but in their study no effect by gemfibrozil on fibrinogen levels was found either. Whether the observed changes in plasma fibrinogen levels as found in the present study after fibrate treatment will result in less tight and rigid fibrin gel structures needs to be examined in future studies.

In accordance with other investigations,[39,40] in our study at baseline hyperlipidaemia plasma PAI-1 antigen levels were elevated and a correlation between plasma PAI-1 and triglyceride levels and between PAI-1 and t-PA levels was observed. Although lowering triglyceride levels by diet or drugs has been shown to be associated with a decrease in PAI-1 levels,[40] thus suggesting a direct and causal relationship, we found no concomitant lowering of triglyceride levels and PAI-1 levels by gemfibrozil and ciprofibrate. This uncoupling between triglyceride and PAI-1 levels was also seen with users of oral contraceptives, women

taking fish or olive oil and after triglyceride infusion in patients with non-insulin dependent diabetes mellitus.[41-43] A lack of effect of gemfibrozil on PAI-1 has also been reported by others,[10,11,44] but in some studies a decreasing effect of gemfibrozil on PAI-1 levels has been shown.[7,9,12] Our findings indicate that this variation between studies is unlikely to be due to differences in assay methodology, since we demonstrated that two assays detecting different types of PAI-1 with differing efficiency, showed quantitative but not qualitative differences. We suggest therefore, that other factors such as differences in patient groups or baseline levels may account for the variability observed.

In conclusion, our results show that although gemfibrozil and ciprofibrate exerted comparable effects on plasma triglyceride and cholesterol levels in a group of hyperlipidaemic patients, they influenced plasma fibrinogen levels differently. Baseline plasma PAI-1 and t-PA levels were elevated but not affected by the fibrate treatment. An important finding is that the two fibrates decreased the functionality of plasma fibrinogen. We showed that different fibrinogen assay methods and differences in treatment period influence the outcome of the fibrate treatment which explains in part the variability observed in the literature.

## Acknowledgements

This study was supported by a grant from the Netherlands Organisation for Scientific Research (NWO) (project 900-523-181).

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*CHAPTER 3*

# Studies on the mechanism of fibrate-inhibited expression of plasminogen activator inhibitor-1 in cultured hepatocytes from cynomolgus monkey

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*Arteriosclerosis Thrombosis and Vascular Biology 1997; 17:26-32*

## Abstract

Fibrates are widely used drugs in hyperlipidemic disorders. In addition to lowering serum triglyceride levels, fibrates have also been shown to reduce elevated plasma plasminogen activator inhibitor 1 (PAI-1) levels *in vivo*. We demonstrate that fibrates suppress PAI-1 synthesis in cultured cynomolgus monkey hepatocytes in a concentration-dependent way (0.1 - 1.0 mmol/L) and independent of their lipid-lowering effect. Different fibrates showed different potency in suppressing PAI-1 production: gemfibrozil and clofibric acid, at a concentration of 1 mmol/L, reduced PAI-1 synthesis over 24 h to 52 ± 20 % and 60 ± 5 %, while clofibrate and bezafibrate lowered PAI-1 synthesis only to 86 ± 17 % and 84 ± 15 % of control values, respectively. These changes in PAI-1 production by fibrates correlated with changes in PAI-1 mRNA levels and were also visible at the level of gene transcription. Fibrates did not lower basal PAI-1 synthesis, but attenuated an acceleration of PAI-1 production during culture. The suppressing effect of fibrates on PAI-1 synthesis could not be mimicked with activators or inhibitors of protein kinase C. Furthermore, fibrates did not inhibit the increase in PAI-1 synthesis induced by epidermal growth factor or transforming growth factor-ß. These results make mechanisms involving PKC modulation or growth factor receptor inactivation as a mode of action of fibrates unlikely. The suppressing effect of fibrates on PAI-1 synthesis could involve the nuclear receptor peroxisome proliferator-activated receptor (PPAR), and its heterodimeric partner, the retinoid X receptor (RXR). The alpha forms of PPAR and RXR were both found to be expressed in cynomolgus monkey hepatocytes. The ligand for RXRα, 9-*cis* retinoic acid, suppressed PAI-1 synthesis to the same extent as gemfibrozil, while a combination of gemfibrozil and 9-*cis* retinoic acid had no more effect on PAI-1 synthesis than any of these compounds alone at optimal concentrations. In conclusion, fibrates downregulate an induced PAI-1 production in cynomolgus monkey hepatocytes independent of a decrease in triglyceride levels. A possible involvement of PPARα/RXRα in this downregulation is discussed.

## Introduction

Fibrates are a class of hypolipidemic drugs widely used in the treatment of diet-resistant hyperlipidemia. In humans, fibrates effectively lower elevated serum triglycerides and increase high-density lipoprotein cholesterol. Fibrates also moderately lower low-density lipoprotein cholesterol levels in patients with hypercholesterolemia.[1] In addition to these lipoprotein profile-altering effects, some fibrates also exert a favourable influence on plasma levels of hemostatic risk factors, such as plasminogen activator inhibitor 1 (PAI-1). These combined actions of fibrates may be beneficial in reducing the risk of coronary heart disease.[1]

The mechanism by which fibrates reduce plasma PAI-1 levels is unknown. Several

reports have documented a correlation between plasma triglyceride levels and PAI-1 levels.[2] Also, lowering triglyceride levels by diet or drugs has been shown to be associated with a decrease in PAI-1 levels[2-5], thus suggesting a relationship between triglyceride and PAI-1 levels.[2,6] On the other hand, several lines of evidence suggest a mechanism of action of fibrates independent of their triglyceride lowering effect. For example, the fibrate gemfibrozil was shown to significantly lower PAI-1 antigen levels, while in the same study no change in PAI-1 antigen levels by fenofibrate was observed, although both fibrates were equipotent in lowering triglyceride levels.[7] Furthermore, it was shown that gemfibrozil reduces PAI-1 secretion in vitro in the human hepatoma cell line Hep G2, suggesting a direct effect of this drug on PAI-1 expression.[8]

In the present report we demonstrate that fibrates directly, i.e. independent of lowering triglyceride levels, suppress PAI-1 expression in primary cultures of hepatocytes from cynomolgus monkey. We have further used this in vitro model of cultured monkey hepatocytes to compare the efficacy of various fibrates to lower PAI-1 production and to study the mechanism(s) by which fibrates exert their action. Studies were designed to evaluate the role of a number of signal transduction pathways reported to be affected by fibrates and/or to be involved in PAI-1 gene expression. First, we tested the role of protein kinase C (PKC), the activity of which has been shown to be modulated by fibrates[9,10] and to be important for PAI-1 expression in Hep G2.[11] Secondly, fibrates have been reported to affect the phosphorylation of growth factor receptors and thereby their signal transduction activity.[12] We have evaluated whether fibrates interfere with the response of hepatocytes to epidermal growth factor (EGF) and transforming growth factor-ß (TGF-ß), growth factors which have been found to be inducers of PAI-1 expression in Hep G2.[13,14] Thirdly, we examined a possible role of the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR), which is activated by fibrates.[15] PPAR has been reported to downregulate gene expression through squelching of c-Jun[16], a transcription factor which we found to be critical in PAI-1 gene expression in the human hepatoma cell line Hep G2 (Arts et al, unpublished data)

## Materials and Methods

### Materials

Clofibric acid, clofibrate, dexamethasone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Gemfibrozil was a gift of Dr.B. Bierman, Warner-Lambert (Hoofddorp, The Netherlands). Bezafibrate was obtained from Boehringer Mannheim B.V. (Almere, The Netherlands). Stock solutions of fibrates (1 mol/L) and phorbol 12-myristate 13-acetate (PMA) (100 µmol/L) were prepared in dimethyl sulfoxide (DMSO) and ethanol, respectively and kept at -20 °C. Before use, fibrate stocks were diluted in incubation medium and kept for 2 h at 37 °C to ensure complete dissolution of the fibrates. Human epidermal growth factor (EGF) was obtained from Campro (Veenendaal, The Netherlands), transforming growth factor-ß (TGF-ß) from Harbor Bioproducts (Norwood, MA), tyrphostin

from Brunschwig (Amsterdam, The Netherlands) and insulin (Actrapid Penfill 1.5) from Novo Nordisk Farma B.V. (Zoeterwoude, The Netherlands). 9-*cis* retinoic acid was a gift of Drs. M. Klaus and C. Apfel, Hoffmann-LaRoche Ltd. (Basle, Switzerland). The specific protein kinase C inhibitor Ro 31-8220 was a gift of Dr. G. Lawton, Hoffmann-LaRoche (Welwyn Garden City, U.K.). Deoxycytidine 5[ $\alpha$-$^{32}$P]triphosphate (3 Ci/mol), [$^{35}$S]methionine (>1000 Ci/mmol) and the Megaprime-kit were obtained from Amersham Nederland BV ('s Hertogenbosch, The Netherlands). The Tintelize enzyme immunoassay kit for determination of PAI-1 antigen was from Biopool (Umeå, Sweden). Bradford protein reagent was from Biorad (Veenendaal, The Netherlands). Other materials used in the methods described below have been specified in detail in relating references or were purchased from standard commercial sources.

**Isolation and culture of cynomolgus monkey primary hepatocytes**

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys ( *Macaca fascicularis*, 1.5-3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at that institute. The isolation procedure was essentially as described for human hepatocytes [17,18] with a few modifications as described by Kaptein et al.[19] Total cell yields varied from 0.5 to 1.5x10$^9$ viable cells. Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%, w/v), was at least 65%. The cells were seeded in culture dishes at a density of 2x10$^5$ viable cells per cm$^2$ and were maintained in Williams E medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 135 nmol/L insulin, 50 nmol/L dexamethasone, 2 mmol/L L-glutamine (Flow Laboratories, Irvine, U.K.), 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml kanamycin, at 37 °C in a 5% $CO_2$/ 95% air atmosphere. After 16 hours, the non-adherent cells were washed from the plates and the remaining cells were refreshed with the same medium as described above. After 8 h, the medium was changed to incubation medium in which the amount of insulin was lowered to 10 nmol/L. Experiments were started 24 h after hepatocyte isolation.

Conditioned media were obtained by incubating cells at 37 °C for various times with incubation medium containing the appropriate concentration of the test compound or stock solvent (DMSO or ethanol; final concentration 0.1% (v/v)) as control. For prolonged incubations, the media were refreshed every 24 h. Conditioned media were centrifuged for 4 min at 5000 g in a Beckman Microfuge centrifuge to remove cells and cellular debris, and the samples were kept at -20 °C until use. The cells were washed twice with ice-cold phosphate buffered saline, and were used for isolation of RNA or nuclei.

**Northern blot analysis**

Total RNA was isolated from at least 2x10$^6$ simian hepatocytes according to Chomczynski and Sacchi. [20] RNA was fractionated by electrophoresis in a 1% (w/v) agarose gel under denaturing conditions using 1 mol/L formaldehyde[21], and blotted to Hybond-N filter according to the manufacturers instructions. The filters were hybridized overnight at 63 °C (with the PAI-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PPAR $\alpha$ and RXR$\alpha$ probe) or at 42 °C (with the acyl-CoA oxidase (ACO) probe) in hybridization mix, being: 7% (w/v) sodium dodecylsulfate (SDS), 0.5 mol/L $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.2, 1 mmol/L EDTA, containing 3 ng of [$\alpha$-$^{32}$P]CTP-labeled probe/ml. After hybridization with GAPDH or PAI-1 probe, the filters were washed twice with 2xSSC (1xSSC being: 0.15 mol/L NaCl, 0.015 mol/L $Na_3$citrate), 1% (w/v) SDS, and twice with 1xSSC, 1% (w/v) SDS for 20 min at 63 °C. In the case of hybridizations with PPAR $\alpha$ or RXR$\alpha$ probe the filters were washed four times for 20 min with 2xSSC, 1% (w/v) SDS at 63 °C, while blots hybridized with ACO probe were washed three times for 15 min with 2xSSC, 1% (w/v) SDS and one time for 15 min with 0.5xSSC,

1% (w/v) SDS at 42 °C. The filters were then exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80 °C. The relative intensities of the bands present were determined on a Fujix Bas 1000 phosphoimager.

**cDNA probes**

cDNA probes used are: a 2.5 kb EcoRI fragment of the human PAI-1 cDNA [22]; a 1.2 kb PstI fragment of the rat GAPDH cDNA provided by Dr.R. Offringa[23]; a 1.3 kb NruI/BamHI fragment of the human PPAR α cDNA provided by Dr.F.J. Gonzalez[24]; a 1.4 kb EcoRI/BglII fragment of the human RXR α cDNA provided by Dr.J. Grippo (Hoffmann-LaRoche, Nutley, USA); a 2.0 kb SacI fragment of the rat ACO cDNA, provided by Dr.T. Osumi[25]; and a 1.2 kb PstI fragment of the hamster actin cDNA provided by Dr.W. Quax. [26]

**Assays**

PAI-1 antigen levels in conditioned media of cynomolgus monkey hepatocyte cultures were determined with an adapted Tintelize PAI-1 assay from Biopool (Umeå, Sweden). This assay normally does not recognize cynomolgus monkey PAI-1 antigen, but when the coating antibody is replaced by a goat anti-human PAI-1 polyclonal antiserum (5µg/ml) (Biopool, Umeå, Sweden), both monkey and human PAI-1 antigen can be determined. Monkey PAI-1 antigen values were calibrated using the human PAI-1 calibration sample present in the kit. Overall protein synthesis was determined by measuring the incorporation of [ $^{35}$S]methionine into the 10% (w/v) trichloroacetic acid precipitable fraction of radiolabeled conditioned medium and cell extract. [27] Cell viability was assessed by the MTT assay [28] which is based on the cellular reduction of MTT by mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically at 545 nm. Protein was measured using the Bradford protein assay. Nuclear run on assays were performed according to Groudine et al[29] with some minor modifications as described by Twisk et al. [30]

**Statistical analysis**

Statistical significance of differences was calculated using Student's t-test for paired data with the level of significance selected to be $p < 0.05$ (*) and $p < 0.01$ (**). Values are expressed as means ± S.D.

## Results

**Dose-dependency and time-course of the effect of different fibrates on PAI-1 synthesis**

Cynomolgus monkey hepatocytes were incubated with three concentrations (0.1, 0.3 or 1 mmol/L) of four different fibrates (gemfibrozil, clofibric acid, clofibrate or bezafibrate) for two consecutive periods of 24 h. As shown in Figure 1, all four fibrates dose-dependently lowered PAI-1 levels, reaching 52±20% (gemfibrozil), 60±5% (clofibric acid), 86±17% (clofibrate) and 84±15% (bezafibrate) of control values after 24 h at a 1 mmol/L concentration. During the second 24 h incubation period, the fibrates did not lower PAI-1 levels markedly further than in the first 24 h incubation period (Fig. 1). Similar results were obtained when experiments were performed with medium containing 10% (v/v) lipoprotein-depleted serum or 1% (v/v) human serum albumin instead of 10% (v/v) fetal calf serum,

indicating that fibrates can suppress PAI-1 synthesis independent of changes in triglyceride levels (data not shown). The decreases in PAI-1 production by fibrates were not due to diminished cell viability (as tested with the MTT test) or changes in overall protein synthesis (as assessed by simultaneous measurement of the incorporation of [$^{35}$S]methionine into trichloroacetic acid-precipitable products). Fibrate concentrations higher than 1 mmol/L, however, were found to lower overall protein synthesis (data not shown). These results indicate that different fibrates possess different potency to reduce PAI-1 expression.

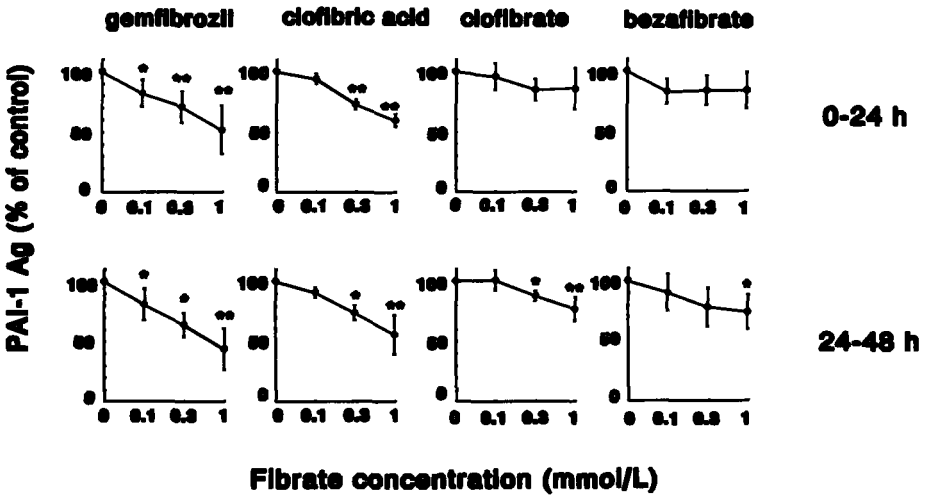


**Figure 1. Effect of fibrates on PAI-1 antigen production in cultured simian hepatocytes**
Cynomolgus monkey hepatocytes were incubated for two consecutive periods of 24 h with different concentrations of gemfibrozil, clofibric acid, clofibrate and bezafibrate, and the conditioned media were analyzed for PAI-1 antigen as described in the Methods section. Results are means ± S.D. of 3 to 7 independent experiments performed in duplicate; the data are expressed as percentage values of controls. Control values ranged between 171 and 1700 ng/ml over the first 24 h period and between 295 and 2501 ng/ml over the second 24 h period in the different experiments. Values significantly different from control values are indicated with asterisk (*:$p<0.05$ and **:$p<0.01$).

Figure 2 shows a representative time-course of the suppressive effect of gemfibrozil (1 mmol/L) on PAI-1 antigen accumulation. Both in the absence and presence of gemfibrozil, PAI-1 antigen levels increase linearly in time during the first 16 h. However, between 16 and 24 h of incubation, PAI-1 shows an accelerated increase under control conditions, whereas with gemfibrozil PAI-1 continues to accumulate at a constant rate. This results in an about 2-fold higher PAI-1 antigen level in conditioned medium of control cells than in the

conditioned medium of gemfibrozil-treated cells after 24 h. The accelerated increase in PAI-1 synthesis varied between 32% and 75% in five independent experiments and was in magnitude comparable to the observed inhibition of PAI-1 synthesis by gemfibrozil. Similar results were obtained with clofibric acid, and analysis of the attenuating effect of fibrates on PAI-1 synthesis in Hep G2 cells learned that these effects could be attributed also to a diminishing effect of fibrates on the induction of PAI-1 synthesis during culture of Hep G2 cells (data not shown). Apparently, fibrates do not inhibit basal PAI-1 synthesis, but prevent the accelerated production of PAI-1, as occurring during control incubation conditions.

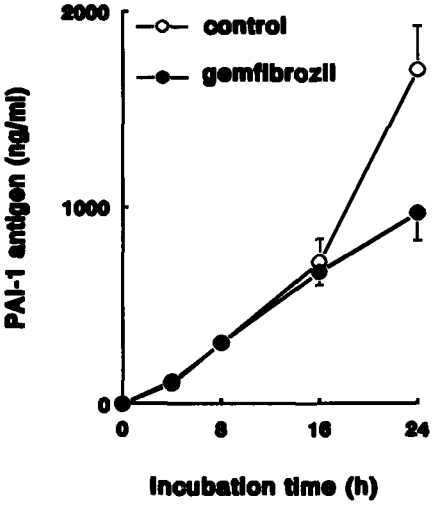


**Figure 2. Time course of PAI-1 antigen production by cultured simian hepatocytes incubated with or without gemfibrozil**
Cynomolgus monkey hepatocytes were incubated for various times up to 24 h in the presence (●) or absence (○) of 1 mmol/L gemfibrozil, and the conditioned media were analyzed for PAI-1 antigen as described in the Methods section. The experiment shown is representative for five independent experiments in duplicate. Values shown are means ± range.

**Effect of gemfibrozil on PAI-1 mRNA levels and transcription**

To determine whether the accelerated increase in PAI-1 antigen levels and inhibition thereof by fibrates was reflected at the mRNA level, we performed Northern analysis. As shown in Figure 3, there is no marked difference in PAI-1 mRNA levels between control and 1 mmol/L gemfibrozil-treated hepatocytes at 8 h. At 16 h, however, PAI-1 mRNA levels are strongly elevated in control, but not in gemfibrozil-treated hepatocytes, and this difference is maintained at 24 h. These mRNA data explain the different PAI-1 protein production rates in control and gemfibrozil-treated hepatocytes between 16 and 24 h (Fig. 2). The transient, about two-fold induction of PAI-1 mRNA by gemfibrozil at 4 h was consistently found in three independent experiments, and could be suppressed by an inhibitor of protein kinase C (see below). No such induction was observed with the other three fibrates (data not shown).

To determine whether the differences in PAI-1 mRNA levels in control and gemfibrozil-treated hepatocytes were the result of different PAI-1 transcription rates, we performed a nuclear run-on assay. Figure 4 shows that hepatocytes incubated with gemfibrozil have a 2-fold lower PAI-1 transcription rate than control cells.

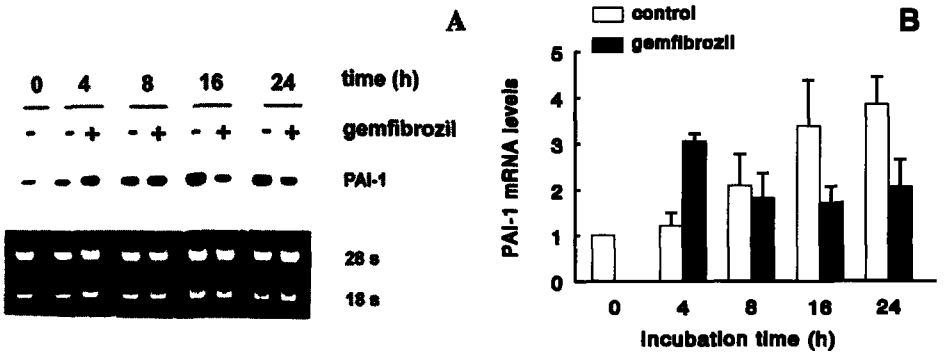


**Figure 3. Time course of the effect of gemfibrozil on PAI-1 mRNA levels in cultured simian hepatocytes**
Cynomolgus monkey hepatocytes were incubated with (+) or without (-) 1 mmol/L gemfibrozil for various times up to 24 h. Total RNA was isolated, and 5 μg of RNA was analyzed by Northern blotting for PAI-1 mRNA. Equal loading was checked by ethidium bromide staining of 18S and 28S ribosomal RNA. The experiment shown in panel A is representative for three independent experiments. The signals for PAI-1 mRNA were quantified by densitometry and adjusted for the corresponding GAPDH mRNA signals. The amount of PAI-1 mRNA present at the different time points is expressed relative to that found at t=0. The results shown in panel B are means ± S.D. of three independent experiments.

### Role of Protein Kinase C in PAI-1 expression

The transient increase in PAI-1 mRNA levels observed with gemfibrozil at 4 h (Fig. 3) is probably due to activation of PKC, since a similar rapid increase in PAI-1 mRNA levels was also seen with the specific PKC activator PMA, and both effects could be suppressed by the PKC inhibitor, Ro 31-8220 (Fig. 5). To assess whether modulation of PKC activity also plays a role in the suppression of the accelerated increase in PAI-1 synthesis between 16 and 24 h (see Fig. 2), we examined the effect of PMA and Ro 31-8220 on PAI-1 synthesis over a 24 h period (Table 1). Opposite to gemfibrozil, PMA increased PAI-1 levels, thus reflecting the strong induction of PAI-1 mRNA levels at 4 h (Fig. 3). The PKC inhibitor Ro 31-8220 suppressed the effect of PMA, but had no effect on PAI-1 production under control conditions. Together with the finding that clofibric acid, which did not transiently activate PKC after 4 h, was almost as effective as gemfibrozil in suppressing PAI-1 synthesis after a 24 h incubation period, these results indicate that fibrates suppress PAI-1 synthesis through a

mechanism independent of PKC.

**Table 1: Effect of PMA and Ro 31-8220 on PAI-1 synthesis in simian hepatocytes**

| compound | PAI-1 synthesis (% of conrol) |
|---|---|
| PMA | 169 ± 6 |
| Ro 31-8220 (10 µmol/L) | 107 ± 3 |
| PMA (100 nmol/L) + Ro 31-8220 (10 µmol/L) | 108 ± 3 |
| Gemfibrozil (1 mmol/L) | 25 ± 6 |

Cynomolgus monkey hepatocytes were incubated for 25 h with incubation medium containing gemfibrozil (1 mmol/L), the PKC inhibitor Ro 31-8220 (10 µmol/L) or solvent (control). 1 h after the start of the experiment, the PKC activator PMA (100 nmol/L) was added. At the end of the incubation, media were collected and analyzed for PAI-1 antigen as described in the Methods section. Values given are means ± range of two independent experiments performed in duplicate and expressed as a percentage of the control values (171 and 290 ng/ml, respectively).

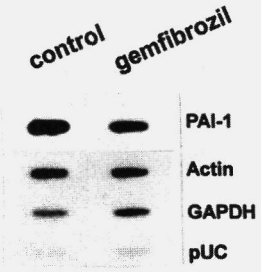


**Figure 4. Analysis of PAI-1 gene transcription rate after incubation of cultured simian hepatocytes with gemfibrozil.** Cynomolgus monkey hepatocytes were incubated for 48 h in the absence (control) or presence of 0.6 mmol/L gemfibrozil. Nuclei were isolated and used for run-on assays as described in the Methods section. Actin and GAPDH are controls for variation in mRNA labeling and pUC served as a control for nonspecific hybridization.

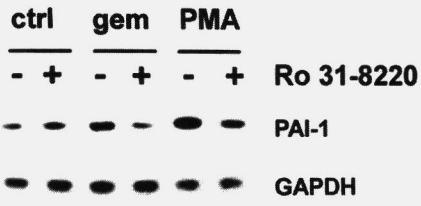


**Figure 5. Effect of gemfibrozil and PMA on PAI-1 mRNA levels in simian hepatocytes incubated in the presence or absence of Ro 31-8220** Cynomolgus monkey hepatocytes were incubated for 5 h with incubation medium with (+) or without (-) 10 µmol/L of the PKC inhibitor Ro 31-8220. 1 h after the start of the experiment, gemfibrozil (1 mmol/L), the PKC activator PMA (100 nmol/L) or solvent (ctrl) were added. At the end of the incubation, total RNA was isolated and 5 µg of RNA was analyzed by Northern blotting for PAI-1 mRNA and for GAPDH mRNA as acontrol for equal mRNA loading. The experiment shown is representative for three independent experiments.

**Effect of fibrates on the induction of PAI-1 by EGF and TGF-ß**

As shown in Figure 6, epidermal growth factor (EGF; 5 ng/ml) and transforming growth factor-ß (TGF-ß; 5 ng/ml) induce PAI-1 mRNA levels about 3-fold in primary hepatocytes after a 4 and 6 h incubation period, respectively. The EGF-mediated induction of PAI-1 mRNA levels was suppressed for over 50% by the tyrosine protein kinase inhibitor tyrphostin (30 µg/ml; data not shown), a compound known to interfere with EGF-receptor mediated signaling.[31] The induction of PAI-1 mRNA could not be inhibited, however, with 1 mmol/L gemfibrozil added to the cells 1 h prior to the addition of EGF or TGF-ß (Fig. 6). Similar negative results were obtained with 1 mmol/L clofibric acid, clofibrate and bezafibrate (data not shown). Because the effect of fibrates on PAI-1 synthesis became apparent after 16 h, we repeated the experiment after a 16 h preincubation with the fibrates. Again, no quenching effect on PAI-1 mRNA

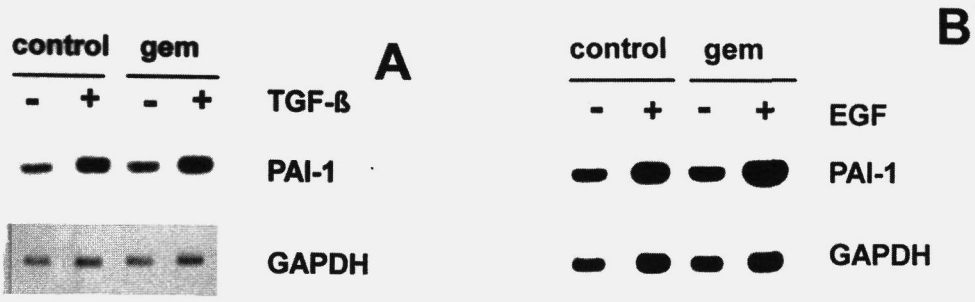


**Figure 6. Effect of gemfibrozil on PAI-1 mRNA levels in simian hepatocytes incubated in the presence or absence of TGF-ß or EGF.** Cynomolgus monkey hepatocytes were incubated for 7 h (panel A) or 5 h (panel B) with incubation medium containing solvent (control) or 1 mmol/L of gemfibrozil (gem). 1 h after the start of the experiment, solvent (-), 5 ng/ml TGF-ß (+, panel A) or 5 ng/ml EGF (+, panel B) was added. At the end of the incubation, total RNA was isolated, and 5 µg of RNA was analyzed by Northern blotting for PAI-1 mRNA and for GAPDH mRNA as a control for equal mRNA loading. The experiment shown is representative for three independent experiments.

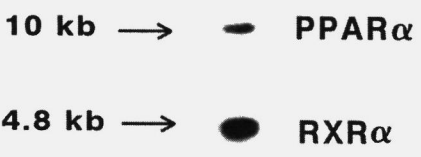


**Figure 7. Simian hepatocytes express PPARα and RXRα mRNA** Cynomolgus monkey hepatocytes were incubated for 24 h with incubation medium. At the end of the incubation, total RNA was isolated, and 5 µg of RNA was analyzed by Northern blotting for PPARα and RXRα mRNA expression. The experiment shown is representative for three independent experiments.

induction by EGF or TGF-ß was observed (data not shown). These data indicate that fibrates do not interfere with growth factor receptor mediated signaling.

**Studies on a role of PPAR in the inhibition of PAI-1 synthesis by gemfibrozil**

We next considered a possible role for the peroxisome proliferator-activated receptor (PPAR) in the inhibition of PAI-1 synthesis by gemfibrozil. As shown in Figure 7, cynomolgus monkey hepatocytes express the mRNAs of PPARα and retinoid-X-receptor α (RXRα), another steroid hormone receptor with which PPARα interacts to form heterodimers. Since the RXRα ligand, 9-*cis* retinoic acid, has been demonstrated to enhance PPAR action,[32] we tested the effect of gemfibrozil, 9-*cis* retinoic acid, and combinations thereof on PAI-1 synthesis. As shown in Figure 8, 10 µmol/L 9-*cis* retinoic acid was almost as effective as 1 mmol/L gemfibrozil in inhibiting PAI-1 synthesis: in three independent experiments, 9-*cis* retinoic acid and gemfibrozil decreased PAI-1 synthesis to 65±4% and 59±6% of control values (mean ± SD), respectively. The inhibitions of PAI-1 synthesis by 9-*cis* retinoic acid and gemfibrozil in these experiments were of the same magnitude as the corresponding accelerated increases in PAI-1 synthesis under control conditions (data not shown). These data suggest that 9-*cis* retinoic acid, like gemfibrozil, only prevents the accelerated increase in PAI-1 production, but does not affect the uninduced PAI-1 synthesis rate. Combinations of suboptimal concentrations of the two ligands cooperatively decreased PAI-1 synthesis (data not shown), but the maximal effect never exceeded the inhibiting effect seen with the optimal concentration of gemfibrozil as illustrated in Figure 8 for 1 µmol/L 9-*cis* retinoic acid and 1 mmol/L gemfibrozil. Taken together, these data suggest that 9-*cis* retinoic acid and gemfibrozil interfere with the same PAI-1 stimulatory pathway. They do not necessarily imply, however, that 9-*cis* retinoic acid and gemfibrozil act via the same regulatory pathway or mechanism.

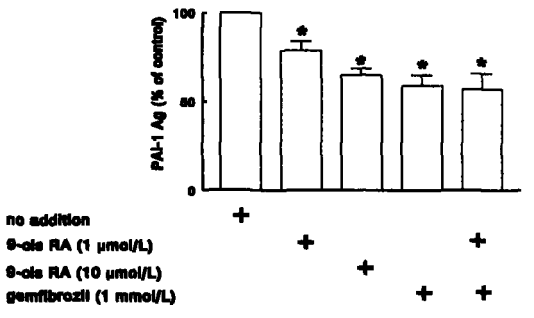


**Figure 8. Effect of gemfibrozil and 9-*cis* retinoic acid on PAI-1 synthesis in simian hepatocyte.** Cynomolgus monkey hepatocytes were incubated for 24 h with incubation medium containing solvent (no addition), 9-*cis* retinoic acid (1 µmol/L or 10 µmol/L) and/or gemfibrozil (1 mmol/L), and the conditioned media were analyzed for PAI-1 antigen as described in the Methods section. Results presented are means ± S.D. of 3 independent experiments performed in duplicate; the data are expressed as percentage values of controls. Values significantly different from control values are indicated with an asterisk ($p<0.05$).

## Discussion

In this study, we demonstrated a suppressive effect of fibrates on PAI-1 synthesis and PAI-1 mRNA levels in cultured cynomolgus monkey hepatocytes independent of changes in concentrations of triglycerides in the culture medium. We found that fibrates inhibit PAI-1 synthesis in a dose-dependent way and that different fibrates differ in their capacity to suppress PAI-1 production. Fibrates appeared to attenuate the accelerated increase in PAI-1 synthesis occuring under basal culture conditions. The changes in PAI-1 protein synthesis correlated closely with changes in PAI-1 mRNA and could also be demonstrated at the transcriptional level. Therefore, fibrates act selectively on a pathway that stimulates the activation of PAI-1 gene transcription. The regulatory mechanism by which this suppressive effect is brought about remained unknown, but could involve PPAR$\alpha$/RXR$\alpha$.

Our finding that the suppression of PAI-1 synthesis by fibrates is a direct effect, i.e. independent of lowering triglyceride levels, is in agreement with *in vivo* studies which show that simply reducing triglycerides is not sufficient to lower PAI-1 in patient populations with elevated triglyceride and PAI-1 levels.[1,33,34] Furthermore, our *in vitro* observation that fibrates inhibit an induction of PAI-1 synthesis rather than basal PAI-1 expression, is comparable to findings reported for the human hepatoma cell line Hep G2[8] and is in agreement with the *in vivo* situation where fibrates lower only elevated PAI-1 levels in patients.[1] Similarly, our *in vitro* finding that different fibrates which are equipotent in lowering triglyceride levels can differ in their efficacy to lower PAI-1 synthesis, parallels *in vivo* results. For example, we found in our cultured cynomolgus monkey hepatocytes that gemfibrozil and clofibric acid were potent PAI-1 suppressors while clofibrate and bezafibrate were not. Similarly, gemfibrozil but not bezafibrate was found to decrease enhanced PAI-1 levels in type IV hypertriglyceridemic patients.[7,35,36]

Our observation that PAI-1 synthesis in the simian hepatocytes is induced during basal culture conditions resembles similar findings in Hep G2 cells.[37] PAI-1 synthesis in Hep G2 was found to be induced by an autocrine factor, secreted during cell culture. The nature of this factor has not been identified until now, but had no similarity to any steroid, retinoid, growth factor or cytokine, factors known to induce PAI-1.[38] Similarly, the factor(s) responsible for the accelerated production of PAI-1 in cynomolgus monkey hepatocytes remained elusive.

The obscurity of the PAI-1 inducing factor hampered a rational approach to understand the mechanism by which fibrates suppress PAI-1 expression. We found that gemfibrozil rapidly and transiently increased PAI-1 mRNA expression in a manner comparable to the PKC activator PMA, albeit much weaker, and this could be inhibited with the PKC inhibitor, Ro 31-8220. Activation of PKC by fibrates has also been reported in rat hepatocytes.[9,10] However, specific activation or inhibiton of PKC did not prevent the accelerated increase in

PAI-1 during culture. Also, clofibric acid, which did not activate PKC, was as effective as gemfibrozil in suppressing PAI-1 production. These results make it unlikely that fibrates exert their PAI-1 suppressing action by interference with a PKC-dependent pathway. We could also exclude an effect of fibrates on the signal transduction pathways activated by growth factors like EGF and TGF-ß. We found that EGF and TGF-ß did induce PAI-1 mRNA levels in simian hepatocytes, but this could not be prevented by fibrates. Apparently, not every PAI-1 induction is inhibited by fibrates. This is also true *in vivo*. For example, gemfibrozil did not lower elevated PAI-1 levels in patients with a history of venous thrombosis[33] or in men with combined hyperlipoproteinemia.[34]

The finding that a number of fibrates are potent activators of the nuclear receptor PPAR suggested the possibility that this receptor mediates the beneficial action of fibrates on PAI-1. Indeed, PPARα and its heterodimeric partner, RXRα, are both expressed in cultured cynomolgus monkey hepatocytes, and the ligand for RXRα, 9-*cis* retinoic acid, also suppressed PAI-1 production. Two mechanisms have been described how PPAR/RXR can interfere with gene transcription and which might be relevant for PAI-1 expression. PPAR/RXR can antagonize transcriptional activation by competing with another transcription factor for binding to the same *cis*-acting element, as described by Keller et al.[39] Secondly, inhibition can be due to mechanisms involving DNA-independent negative interferences such as squelching. Sakai et al[16] recently showed that PPARα downregulates transcription of the glutathione transferase-P gene in rat hepatocytes through squelching of the transcription factor c-Jun. Interference with c-Jun activity has also been reported for the RXRα receptor.[40] In this context it is of interest that c-Jun is an important factor in PAI-1 gene transcription in the human hepatoma cell line Hep G2 (Arts and Kooistra, unpublished data). If PPAR activation is important for inhibition of PAI-1 expression by fibrates, then gemfibrozil and clofibric acid should be much stronger activators of PPAR activity in our system than bezafibrate or clofibrate. As a measure for PPAR activity we examined the expression of the acyl-CoA oxidase (ACO) gene. ACO is a peroxisomal target of PPARs, and its level has been found to be elevated manyfold in the livers of fibrate-treated rodents.[41,42] In contrast to rodents, however, ACO mRNA levels in cynomolgus monkey hepatocytes were only slightly increased upon treatment with fibrates (maximal increases of 150% were found after 24 h incubation with 1 mmol/L gemfibrozil). This is in agreement with previous reports showing that fibrates poorly induce peroxisome proliferation and peroxisomal ß-oxidation enzymes like ACO, in both human and cynomolgus monkey hepatocytes.[43,44] Ultimately, transfection experiments with dominant negative PPAR/RXR mutants, antisense technology or the use of PPAR/RXR knock out mice are needed to answer the question whether PPAR and RXR are indeed involved in mediating the fibrate-induced decrease in PAI-1 production.

**Acknowledgements**

This study was supported by a grant from the Netherlands Heart Foundation (90.267) and by a grant from the Netherlands Organisation for Scientific Research (NWO) (900-523-181). We gratefully acknowledge Elly de Wit, Diana Neele and Allard Kaptein for technical help with the simian hepatocyte isolations.

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*CHAPTER 4*

# Fibrate-modulated expression of fibrinogen, plasminogen activator inhibitor-1 and apolipoprotein A-I in cultured cynomolgus monkey hepatocytes

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*Thrombosis and Haemostasis 1998; 80:942-948*

## Abstract

Fibrates are used to lower plasma triglycerides and cholesterol levels in hyperlipidemic patients. In addition, fibrates have been found to alter the plasma concentrations of fibrinogen, plasminogen activator inhibitor-1 (PAI-1) and apolipoprotein A-I (apo A-I). We have investigated the *in vitro* effects of fibrates on fibrinogen, PAI-1 and apo A-I synthesis and the underlying regulatory mechanisms in primary monkey hepatocytes.

We show that fibrates time- and dose-dependently increase fibrinogen and apo A-I expression and decrease PAI-1 expression in cultured cynomolgus monkey hepatocytes, the effects demonstrating different potency for different fibrates. After three consecutive periods of 24 hours the most effective fibrate, ciprofibrate (at 1 mmol/L), increased fibrinogen and apo A-I synthesis to 356 % and 322 % of control levels, respectively. Maximum inhibition of PAI-1 synthesis was about 50 % of control levels and was reached by 1 mmol/L gemfibrozil or ciprofibrate after 48 hours. A ligand for the retinoid-X-receptor (RXR), 9- *cis* retinoic acid, and specific activators of the peroxisome proliferator-activated receptor-α (PPARα), Wy14,643 and ETYA, influenced fibrinogen, PAI-1 and apo A-I expression in a similar fashion, suggesting a role for the PPARα/RXRα heterodimer in the regulation of these genes. When comparing the effects of the various compounds on PPARα transactivation activity as determined in a PPARα-sensitive reporter gene system and the ability of the compounds to affect fibrinogen, PAI-1 and apo A-I antigen production, a good correlation ($r=0.80$; $p<0.01$) between PPARα transactivation and fibrinogen expression was found. Apo A-I expression correlated only weakly with PPARα transactivation activity ($r=0.47$; $p=0.24$), whereas such a correlation was absent for PAI-1 ($r=0.03$; $p=0.95$). These results strongly suggest an involvement of PPARα in the regulation of fibrinogen gene expression.


## Introduction

Fibrates are widely used hypolipidemic drugs, very effective in lowering plasma cholesterol and triglycerides.[1,2] In addition to these favourable lipoprotein profile-altering effects, fibrates have been shown to modulate the plasma levels of other cardiovascular risk factors, such as fibrinogen and plasminogen activator inhibitor-1 (PAI-1).[1,3] Although the exact mechanism of action of these drugs is not fully elucidated, there is increasing evidence that the peroxisome proliferator-activated receptor-α (PPARα) plays a centrol role in regulating lipid metabolism.[4,5] PPARα belongs to the nuclear hormone receptor superfamily. Activated PPARα heterodimerizes with another nuclear receptor, the retinoid-X-receptor (RXR), and alters the transcription of target genes after binding to specific response elements (PPREs). Several studies have demonstrated a direct involvement of PPARα in the fibrate-modulated gene expression of hepatic apo A-I and apo A-II, the major apolipoproteins in HDL,

lipoprotein lipase and apo C-III, both involved in triglyceride lowering, and several enzymes implicated in β-oxidation such as acyl-CoA oxidase (ACO).[4] The importance of PPARα in fibrate-induced modulation of these genes and in lowering cholesterol and triglycerides was recently underlined in experiments with PPARα-deficient mice.[6]

Given the reported modulating effects of fibrates on fibrinogen and PAI-1 *in vivo*, the question arises whether PPARα is also involved in regulating these haemostatic genes. Therefore, we studied the effects of fibrates on the expression of fibrinogen, PAI-1 and, for comparison, apo A-I in primary hepatocyte cultures of cynomolgus monkey (*Macaca fascicularis*). First we established that two fibrates, gemfibrozil and ciprofibrate, directly influence fibrinogen, PAI-1 and apo A-I mRNA levels and antigen production in this *in vitro* model. Then, we compared the efficacy of various fibrates, two specific PPARα-activators (Wy14,643 and 5,8,11,14-eicosatetraynoic acid [ETYA])[7] and a ligand for RXR (9-*cis* retinoic acid) to alter fibrinogen, PAI-1 and apo A-I synthesis with the capacity of the compounds to activate PPARα. Because ACO, which is usually used as a measure of PPARα activity in rodents, is relatively insensitive to PPARα-activating compounds in cynomolgus monkey hepatocytes,[8] we have used a stably transfected CHO cell line –containing the chloramphenicol acetyltransferase (CAT) reporter gene under control of the PPRE present in the rat ACO promoter– to assess the PPARα-activating capacity of the various compounds.

## Materials and Methods

### Materials

Clofibric acid and clofibrate were obtained from Sigma Chemical Co. Bezafibrate was provided by Boehringer Mannheim BV (Almere, The Netherlands). Gemfibrozil was a gift from Dr B. Bierman, Warner-Lambert (Hoofddorp, The Netherlands). Ciprofibrate was a gift from Dr M. Riteco, Sanofi Winthrop (Maassluis, The Netherlands). Fenofibric acid was a gift from Dr A. Edgar, Laboratoires Fournier (Daix, France). Wy14,643 (pirixinic acid) was obtained from Campro (Veenendaal, The Netherlands). 5,8,11,14-eicosatetraynoic acid (ETYA) was a gift from Dr G. Veldink, University of Utrecht (Utrecht, The Netherlands). 9- *cis* retinoic acid was a gift from Drs M. Klaus and C. Apfel, Hoffmann-LaRoche Ltd (Basel, Switzerland). Stock solutions of fibrates (1 mol/L), Wy14,643 (100 mmol/L), ETYA (100 mmol/L) and 9- *cis* retinoic acid (10 mmol/L) were prepared in dimethyl sulphoxide (DMSO) and kept at -20°C. Before use, fibrate stocks were diluted in incubation medium and placed on a rollerbank for 2 hours to ensure complete dissolution of the fibrates.

Deoxycytidine 5[α-$^{32}$P]triphosphate (3 Ci/mol) and the Megaprime kit were obtained from Amersham Nederland BV (Almere, The Netherlands). The Tintelize enzyme immunoassay kit for determination of PAI-1 antigen was from Biopool. CAT expression was determined with the Boehringer Mannheim CAT ELISA kit. Bradford protein reagent was from Biorad. Other materials used in the methods described below have been specified in detail in relating references or were purchased from standard commercial sources.

**Isolation and culture of cynomolgus monkey primary hepatocytes**

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys ( *Macaca fascicularis*, 1.5 to 3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at that institute. The isolation procedure was essentially as described for human hepatocytes [9,10] with a few modifications as described by Kaptein et al. [11]The cells were seeded in culture dishes at a density of $2\times10^5$ viable cells per square centimeter and were maintained in Williams' medium E supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 135 nmol/L insulin, 50 nmol/L dexamethasone, 2 mmol/L L-glutamine (Flow Laboratories), 100 IU/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL kanamycin, at 37°C in a 5% $CO_2$/95% air atmosphere. After 16 hours, the nonadherent cells were washed from the plates and the remaining cells refreshed with the same medium as described above. After 8 hours, the medium was changed to incubation medium in which the amount of insulin was lowered to 10 nmol/L. Experiments were started 24 hours after hepatocyte isolation. Conditioned media were obtained by incubating cells at 37°C for various times with incubation medium containing the appropriate test compound or stock solvent (DMSO; final concentration 0.1% [vol/vol]). The media were refreshed every 24 hours. Conditioned media were centrifuged for 4 minutes at 5000g in a Beckman Microfuge centrifuge to remove cells and cellular debris, and the samples were kept at -20°C until use. The cells were washed twice with ice-cold PBS and used for isolation of RNA.

**Transactivation activity of PPARα**

To evaluate the PPARα-activating capacity of compounds, we used an *in vitro* test system developed by Drs K. Hoffman and R. Tynes, Novartis (Basel, Switzerland). In short, Chinese hamster ovary K1 cells (CHO cells) were stably transfected with a pBLCAT5 vector containing a pentamer of the rat ACO PPRE in front of the chloramphenicol acetyltransferase (CAT) gene. After *neo*-selection, the clones were selected with or without addition of clofibric acid for the expression of CAT. Inducible clones with low background activity were chosen for cotransfection with pCMV Vector-1 expression vectors containing the inserts for the murine PPARα, the human RXRα and the human liver fatty acid-binding protein (FABP). Expression of the CAT reporter gene was shown to be dependent on the degree of PPAR α activation (R. Tynes, unpublished results).

The stably transfected CHO cells were grown at 37°C in a 5% $CO_2$/95% air atmosphere in 25 $cm^2$ flasks in DMEM supplemented with 1 mg/mL G418-sulphate, 50 mg/L L-proline, 12 mmol/L L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% (vol/vol) heat-inactivated fetal bovine serum. The media were refreshed every 2 or 3 days. Subcultures were obtained by trypsin/EDTA treatment at a ratio of 1:20. Experiments were performed at 80% confluency in 10 $cm^2$ dishes in duplicate. After 48 hours of incubation with the appropriate concentration of test compound or stock solvent, cells were washed twice with ice-cold PBS. Cells were collected in 300 µl 0.25 mol/L Tris/HCL (pH 7.8) using a rubber policeman and lysed by 4 cycles of freezing in liquid nitrogen and thawing at 37°C. Extracts were centrifuged for 10 minutes at 4°C in a Beckman Microfuge centrifuge to remove cellular debris. Aliquots of the supernatants were used for determination of protein concentration (Bradford assay) and CAT-ELISA.

**Northern Blot Analysis**

Total RNA was isolated from at least $2\times10^6$ simian hepatocytes according to Chomczynski and Sacchi. [12] RNA was fractionated by electrophoresis in a 1% (wt/vol) agarose gel under denaturing conditions using 1 mol/L formaldehyde, [13] and blotted to Hybond-N filter according to manufacturer's instructions. The filters were hybridized overnight at 63°C in hybridization mix, consisting of 7% (wt/vol) SDS, 0.5 mol/L

$Na_2HPO_4/NaH_2PO_4$ buffer (pH 7.2), and 1 mmol/L EDTA, containing 3 ng of [ $\alpha$-$^{32}$P]dCTP-labelled probe per mL. After hybridization with fibrinogen, PAI-1, apo A-I or 18S ribosomal probe, the filters were washed twice with 2× SSC (1× SSC being 0.15 mol/L NaCl, 0.015 mol/L $Na_3$citrate pH 7.0), 1% (wt/vol) SDS, and twice with 1 × SSC, 1% SDS for 20 minutes at 63°C. The filters were then exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C. The intensities of the bands present were determined on a Fujix Bas 1000 phosphoimager and expressed relative to the 18S ribosomal signal.

**cDNA probes**

cDNA probes used were a 2.2 kb *Pst* 1 fragment of the human apo A-I genomic DNA, provided by Dr S.E. Humphries[14]; a cDNA probe for the Bß chain of human fibrinogen, provided by Dr S. Lord (Chapel Hill, USA); a 2.5 kb *Eco*RI fragment of the human PAI-1 cDNA[15]; and a 3.8 md *Eco*RI fragment of the human 18S ribosomal DNA.[16]

**Assays**

Apo A-I antigen was measured in triplicate by an ELISA procedure using polyclonal antibodies to human apo A-I, both as catching and tagging antibodies.[17] Fibrinogen accumulation in the media was measured by an ELISA using monoclonal antibodies against the carboxy terminus of the A $\alpha$ chain as catching antibody and monoclonal antibodies against the amino terminus of the A $\alpha$ chain as tagging antibody.[18] PAI-1 antigen levels were determined using an adapted Tintelize assay from Biopool as described by Arts et al, [8] CAT expression was determined using a Boehringer Mannheim CAT ELISA kit.

**Statistical analysis**

The data are presented as means ± S.E.M. The significance of treatment with the various components was assessed by Student's paired *t*-test. Because fibrinogen, PAI-1, apo A-I and CAT values were slightly skewed, natural logarithm-transformed values were used in the correlation analysis. Correlation coefficients were determined using Pearson's correlation analysis.

## Results

### Effect of gemfibrozil and ciprofibrate on fibrinogen, PAI-1 and apo A-I synthesis in cultured simian hepatocytes

Cynomolgus monkey hepatocytes were incubated with 1 mmol/L of gemfibrozil or ciprofibrate for three consecutive periods of 24 hours, with the incubation medium refreshed after each period. As shown in Fig 1, both fibrates time-dependently increased fibrinogen and apo A-I synthesis, and decreased PAI-1 synthesis, with ciprofibrate showing the strongest effects. During the third 24-hour incubation period, gemfibrozil and ciprofibrate increased fibrinogen production to 251 ± 21 % ($p<0.01$) and 356 ± 79 % ($p<0.001$) and apo A-I production to 186 ± 16 % ($p<0.01$) and 322 ± 15 % ($p<0.001$) of control levels, respectively. With both fibrates PAI-1 levels decreased to about 50% of control levels during the second 24-hour incubation period; prolonged incubation did not further lower PAI-1 synthesis

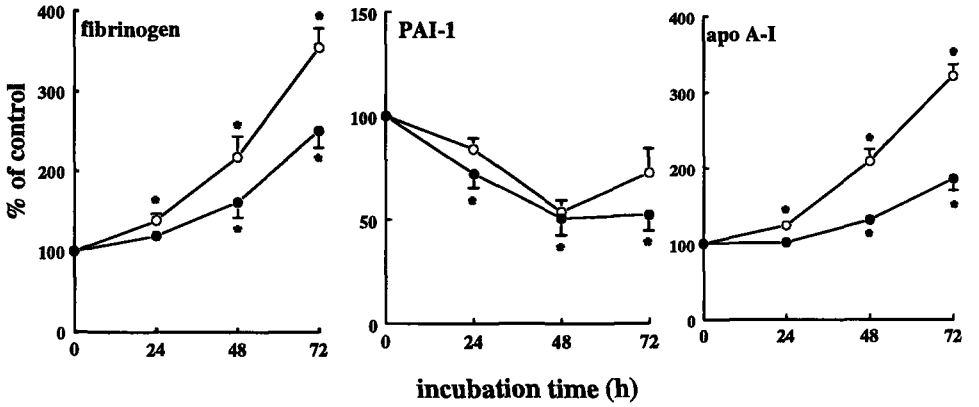


**Figure 1. Time course of the effects of fibrates on fibrinogen, PAI-1 and apo A-I antigen production in primary cultures of simian hepatocytes.** Cynomolgus monkey hepatocytes were incubated for three consecutive periods of 24 hours with 1 mmol/L gemfibrozil ( •), 1 mmol/L ciprofibrate ( ○), or vehicle (controls). Conditioned media were collected every 24 hours and analysed for fibrinogen, PAI-1 and apo A-I antigen as described in the Methods section. Results are means ± s.e.m. of six independent experiments performed in duplicate. The data are expressed as percentage values of controls. Control values ranged between 0.45 mg/mL and 4.54 mg/mL for fibrinogen, between 358 ng/mL and 1657 ng/mL for PAI-1 and between 253 ng/mL and 1634 ng/mL for apo A-I in the different experiments. * $p < 0.05$ versus control values

Fig 2 illustrates the results of experiments in which the cultured simian hepatocytes were incubated with three concentrations of ciprofibrate (0.1, 0.3 and 1.0 mmol/L) for two periods (PAI-1) or three periods of 24 hours (fibrinogen and apo A-I). As depicted in Fig 2A, ciprofibrate concentration-dependently increased fibrinogen and apo A-I synthesis and decreased PAI-1 synthesis. Similar, albeit less marked, results were obtained after incubation with gemfibrozil (data not shown). To analyse these effects further, total RNA was isolated from fibrate-treated and control cells at the end of the incubation period, and subjected to Northern analysis. Fig 2B shows a representative experiment, in which fibrinogen and apo A-I mRNA levels increase and PAI-1 mRNA levels decrease with increasing ciprofibrate concentration, in accordance with the antigen data shown in Fig 2A.

### Effect of 9-*cis* retinoic acid, Wy14,643 and ETYA on fibrinogen, PAI-1 and apo A-I synthesis

Previously, we have shown that PPARα and its heterodimerization partner, RXRα, are expressed in cynomolgus monkey hepatocytes (8). As a first approach to finding evidence for a role of the PPARα/RXRα heterodimer in the regulation of fibrinogen, PAI-1 and apo A-I expression, we evaluated the effect of two specific PPARα-activators, Wy14,643 and

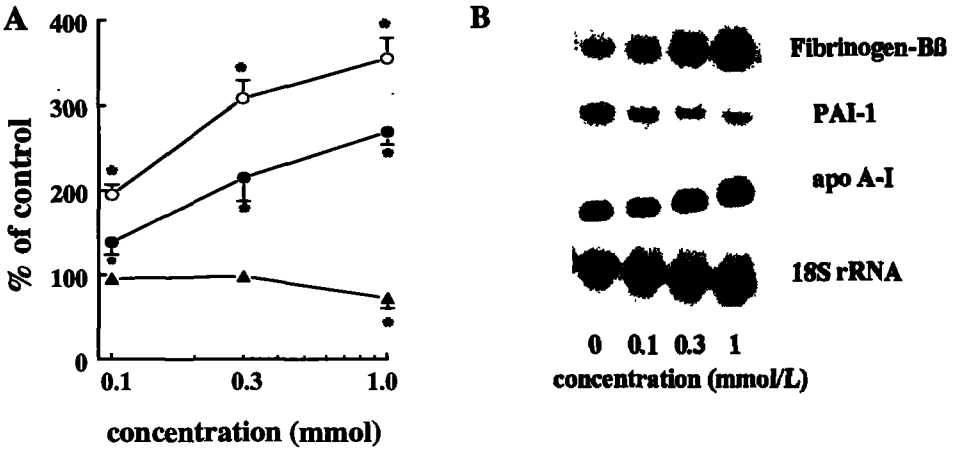


**Figure 2. Concentration dependency of the effect of ciprofibrate on fibrinogen, PAI-1 and apo A-I antigen production and mRNA levels in primary cultures of simian hepatocytes.** Cynomolgus monkey hepatocytes were incubated with 0.1, 0.3 or 1.0 mmol/L ciprofibrate or vehicle for two (PAI-1) or three (fibrinogen and apo A-I) consecutive periods of 24 hours, and conditioned media were analysed for fibrinogen, PAI-1 and apo A-I antigen as described in the Methods section. At the end of the final incubation period, total RNA was isolated and analysed by Northern blotting for fibrinogen, PAI-1 and apo A-I mRNA. A. Antigen data for PAI-1 (▲) after 48 hours and fibrinogen (○) and apo A-I (●) after 72 hours of incubation. Results are means ± s.e.m. of six independent experiments in duplicate. The data are expressed as percentage values of controls. Control values ranged between 0.45 mg/mL and 4.54 mg/mL for fibrinogen, between 358 ng/mL and 1657 ng/mL for PAI-1 and between 253 ng/mL and 1634 ng/mL for apo A-I in the different experiments. B. Signals for PAI-1 mRNA after 48 hours and fibrinogen and apo A-I mRNA after 72 hours of incubation. Equal loading was checked by hybridising with an 18S rRNA cDNA probe. *p<0.05 versus control values

ETYA, and of the RXR ligand, 9-*cis*-retinoic acid on this expression. Similarly as seen with fibrates, 9-*cis* retinoic acid time-and concentration-dependently increased fibrinogen and apo A-I synthesis and decreased PAI-1 synthesis. As shown in Fig 3 and Table 1 for a 72-hour incubation period, 1 µmol/L 9-*cis* retinoic acid stimulated fibrinogen and apo A-I antigen levels to 226 ± 9 % ($p<0.001$) and 204 ± 21 % ($p<0.05$) of control levels, respectively, and decreased PAI-1 antigen levels to 73 ± 5 % ($p<0.01$) of control levels. No additive or synergistic effect was seen when the hepatocytes were exposed to a combination of 9-*cis* retinoic acid and fibrates (data not shown). After 72 hours incubation in the presence of 100 µmol/L of the specific PPARα activators Wy14,643 or ETYA, fibrinogen levels were increased to 188 ± 10 % ($p<0.001$) and 150 ± 24 % ($p<0.05$) and apo A-I levels to 127 ± 18 % ($p<0.05$) and 134 ± 22 % ($p=0.05$) of control levels, respectively; ETYA lowered PAI-1 levels to 64 ± 11 % ($p<0.01$) of control levels after 72 hours, but Wy14,643 was without significant effect on PAI-1 synthesis ($p=0.58$) (Table 1).

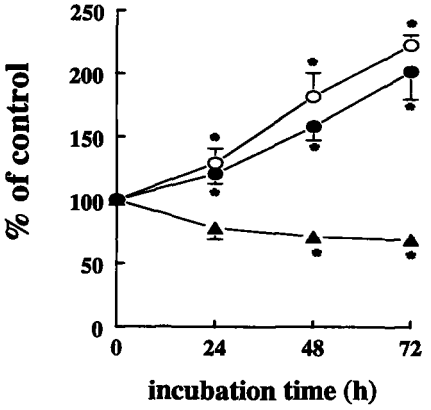


**Figure 3. Effect of 9-*cis* retinoic acid on fibrinogen, PAI-1 and apo A-I antigen production in primary cultures of simian hepatocytes.** Cynomolgus monkey hepatocytes were incubated with 1 μmol/L 9- *cis* retinoic acid or vehicle for three consecutive periods of 24 hours, and conditioned media were analysed for fibrinogen, PAI-1 and apo A-I antigen as described in the Methods section. Results for PAI-1 (▲) after 48 hours of incubation and for fibrinogen (○) and apo A-I (●) after 72 hours of incubation are means ± s.e.m. of six independent experiments in duplicate. The data are expressed as percentage values of controls. Control values ranged between 0.45 mg/mL and 4.54 mg/mL for fibrinogen, between 358 ng/mL and 1657 ng/mL for PAI-1, and between 253 ng/mL and 1634 ng/mL for apo A-I in the different experiments. * $p < 0.05$ versus control values.

## Relationship between induced PPARα transactivation activity and fibrinogen, PAI-1 and apo A-I synthesis

The above data show that fibrates time- and dose-dependently increase fibrinogen and apo A-I expression and decrease PAI-1 production in cultured simian hepatocytes. The finding that 9-*cis* retinoic acid, Wy14,643 and ETYA influence fibrinogen, PAI-1 and apo A-I in a

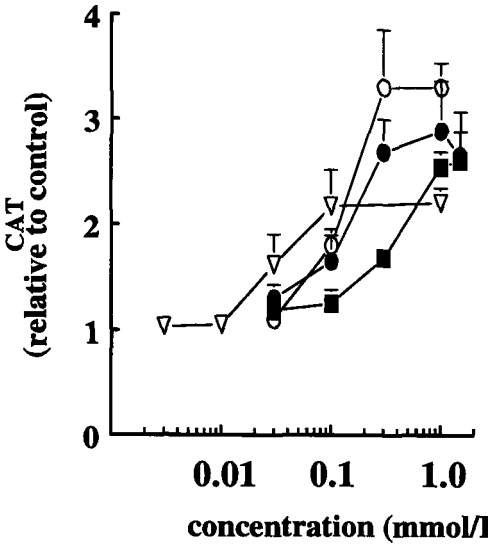


**Figure 4. Effect of various hypolipidemic compounds on transactivation activity of PPARα utilizing a CAT reporter gene system.** A CAT reporter gene cell line for measurement of PPARα transactivation activity was made by stably transfecting CHO cells with a pentamer of the ACO PPRE in front of the CAT gene and the expression vectors for PPARα, RXRα and FABP (see Methods section). The cells were incubated for 48 hours with different concentrations of ciprofibrate (○), bezafibrate (●), clofibric acid (■) or Wy14,643 (▽) in duplicate. Higher concentrations of test compounds than those shown were toxic to the cells. After incubation, cell extracts were prepared and assayed for CAT antigen and cell protein. CAT values are expressed as a percentage of control values (i.e. CAT in cells incubated with vehicle only) and normalized for cell protein. Mean values ± s.e.m. of at least five independent experiments performed in duplicate are shown.

similar fashion, suggests a possible involvement of the PPARα/RXRα heterodimer in the regulation of these three genes. To examine whether the changes in expression of fibrinogen, PAI-1 and apo A-I by the various hypolipidemic compounds and the retinoid are related to differences in induction of PPARα transactivation activity, we used recombinant CHO cells stably containing the reporter gene CAT –under the control of a pentameric PPRE– and expression vectors for PPARα, RXRα and FABP (see Methods section). Representative dose-response curves for the PPARα activators ciprofibrate, bezafibrate, clofibric acid and Wy14,643 are shown in Fig 4. PPARα elicited significant transcriptional activation of the CAT reporter in the absence of added compounds. This intrinsic transactivation ranged from 20 to 30 % of the maximum activation achieved in the presence of fibrates. The high intrinsic activity remained when the serum used in the medium was treated with activated charcoal in order to remove potential agonists, and is possibly due to endogenous ligands such as fatty acids. All compounds tested produced concentration-related increases in CAT activity (Fig 4) and showed marked differences in potency (Table 1).

**Table 1: Effect of various hypolipidemic compounds on transactivation activity of PPARα and the production of fibrinogen, PAI-1 and apo A-I antigen.**

| Compound | CAT expression (relative to control) | fibrinogen (% of control) | PAI-1 (% of control) | apo A-I (% of control) |
|---|---|---|---|---|
| Ciprofibrate | 3.5 ± 1.1 | 329 ± 22 | 77 ± 9 | 268 ± 22 |
| Bezafibrate | 2.6 ± 0.7 | 223 ± 8 | 78 ± 7 | 109 ± 9 |
| Fenofibric acid | 2.1 ± 0.2 | 212 ± 35 | 65 ± 3 | 150 ± 9 |
| Gemfibrozil | 1.9 ± 0.4 | 190 ± 8 | 64 ±16 | 200 ± 3 |
| Clofibrate | 1.6 ± 0.1 | 184 ± 2 | 88 ± 4 | 125 ± 6 |
| Clofibric acid | 1.6 ± 0.2 | 176 ± 11 | 74 ± 6 | 146 ± 14 |
| ETYA | 2.1 ± 0.8 | 188 ± 10 | 64 ± 11 | 134 ± 22 |
| Wy14,643 | 2.1 ± 0.6 | 150 ± 24 | 101 ± 5 | 127 ± 18 |
| 9-*cis* retinoic acid | 1.9 ± 0.2 | 226 ± 9 | 73 ± 5 | 204 ± 21 |

A CAT reporter gene cell line for measurement of PPAR α transactivation activity (as described in the legend to Fig. 4.) was incubated for 48 hours with 300 µmol/L of the different fibrates, 1 µmol/L 9- *cis* retinoic acid, 100 µmol/L Wy14,643 or 100 µmol /L ETYA. After incubation, cell extracts were prepared and assayed for CAT antigen and cell protein. CAT values are expressed as a percentage of control values and normalized for cell protein. Parallel, cynomolgus monkey hepatocytes were incubated with the compounds at the above concentrations or vehicle for three consecutive periods of 24 hours, and conditioned media were analysed for

fibrinogen, PAI-1 and apo A-I antigen as described in the Methods section. Results shown for PAI-1 are after 48 hours of incubation and those for fibrinogen and apo A-I after 72 hours of incubation, and expressed as percentage values of controls. All data shown are means ± s.e.m. of six independent experiments performed in duplicate.

Different maximal CAT activities were observed for different compounds, suggesting that in addition to different binding affinities, also differential transactivation efficiencies may occur due to differences in ligand-induced receptor conformational changes. We have compared the ability of numerous compounds to influence the synthesis of fibrinogen, PAI-1 and apo A-I with their ability to induce CAT expression (Table 1). Association analysis showed a strong and significant correlation between CAT expression and fibrinogen synthesis ($r=0.81$: $p<0.01$) (Fig 5). A weak, not-significant correlation was seen between CAT expression and apo A-I synthesis ($r=0.47$: $p=0.24$), whereas the ability of the compounds to induce CAT expression showed no relation to their effects on PAI-1 synthesis ($r=0.03$: $p=0.95$). Similarly as seen for the effect on fibrinogen, PAI-1 and apo A-I synthesis, a combination of fibrates and 9-*cis* retinoic acid showed no additive or synergistic effect on CAT expression.

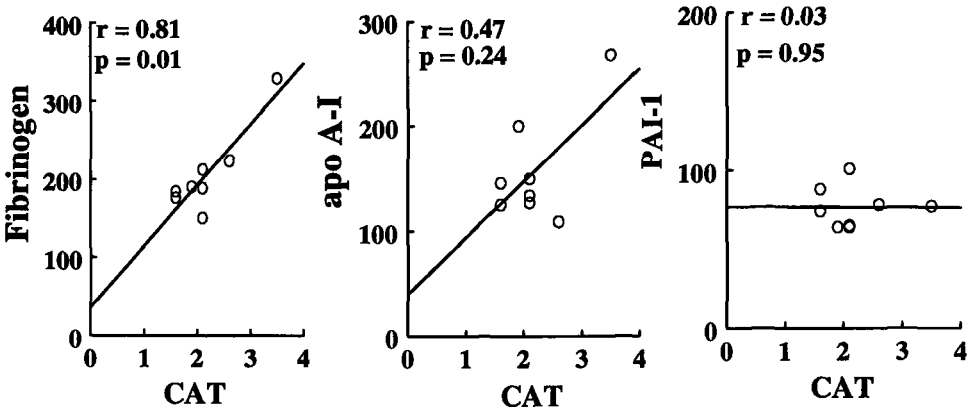


**Figure 5. Scatter plots showing the relationship between induction of fibrinogen, PAI-1 and apo A-I antigen levels and PPARα transactivation activity.** The results obtained with a series of eight PPAR α activators with respect to fibrinogen, PAI-1 and apo A-I antigen production on the one hand and CAT expression on the other as shown in Table 1 were subjected to Pearson correlation analysis. Fibrinogen, PAI-1, apo A-I and CAT distributions were slightly skewed, and therefore natural logarithm-transformed values were used in the analysis.

## Discussion

In this study, we demonstrate that hypolipidemic fibrates can directly influence the synthesis of fibrinogen, PAI-1 and apo A-I in cultured cynomolgus monkey hepatocytes. Our experiments show that fibrates time- and dose-dependently increase fibrinogen and apo A-I antigen and mRNA levels and decrease PAI-1 expression, with different fibrates showing different potencies towards these three genes. In view of recent findings indicating that the hypolipidemic action of fibrates involves activation of the nuclear receptor PPARα (bound to its heterodimeric partner RXR), we investigated a possible role for PPARα in the fibrate-induced changes in fibrinogen, PAI-1 and apo A-I expression. When comparing the effects of six different fibrates, the RXR-ligand 9-*cis* retinoic acid and the PPARα-activators Wy14,643 and ETYA on the synthesis of fibrinogen, apo A-I and PAI-1 with their PPARα-activating potency as determined in an *in vitro* gene reporter system, we found a strong correlation between the potency of a compound to activate PPARα and to induce fibrinogen. A weak relationship was seen between the relative potency values for PPARα and apo A-I, and no correlation could be demonstrated between the relative potency values for PPARα and PAI-1. Although conclusions drawn from an association analysis are only inferential, our data suggest that in addition to its role in the hypolipidemic effect of fibrates, PPARα is also important in regulating the haemostatic factor fibrinogen. Our results also indicate that fibrates can alter the expression of other factors independently of their potency to induce PPARα transactivation, as is the case for PAI-1 and (partly) for apo A-I.

Fibrinogen is a powerful and independent risk factor for cardiovascular disease.[19,20] Therefore, it is important to understand the mechanism(s) involved in the regulation of this protein in order to identify ways of decreasing elevated plasma levels. Our finding that PPARα-activating compounds upregulate fibrinogen production in hepatocytes is of particular relevance in that respect, because of the capacity of PPARα to be activated by dietary compounds (e.g. fatty acids) and pharmacological compounds (e.g. fibrates). In accordance with this, an increase in fibrinogen production was found in HepG2 cells after treatment with polyunsaturated fatty acids[21] which have been shown to be PPARα activators.[7,22] Also, the relationship between free fatty acid mobilization and fibrinogen synthesis, as reported by several investigators,[23-25] may be explained in that way. Considering the involvement of fibrates in both PPARα activation and fatty acid metabolism, the net effect of fibrates on plasma fibrinogen levels *in vivo* would be the resultant of these processes. This may explain the variable results observed for different fibrates with respect to changes in plasma fibrinogen levels.[26-28] It seems therefore that the activation state of PPARα is a crucial factor in controlling fibrinogen expression.

Activated PPARs heterodimerize with RXR and alter the transcription of target genes after binding to specific response elements, PPREs, consisting of direct repeats of AGGTCA-related sequences with a spacing of 1 base pair (DR-1).[5] A search for PPREs in the proximal

1.6 kb region of the fibrinogen B β-chain promoter revealed two putative PPREs, namely ATGTCGTGGGTCC at position -1143 bp and TGAGGAGTGCCCT at position -259 bp. Interestingly, we also found an increase in fibrinogen synthesis after treatment with the RXR ligand, 9-*cis* retinoic acid. This is consistent with the data of Nicodeme et al[29] who showed that fibrinogen production is stimulated by activation of RXR in rats *in vivo* and in cultured rat hepatocytes. Because RXR, apart from being a heterodimeric partner of PPAR α, can also bind as a homodimer to and activate transcription from DR-1 response elements, we cannot exclude the possibility that RXR/RXR homodimers rather than PPAR/RXR heterodimers are also involved in the effects of 9-*cis* retinoic acid on fibrinogen expression.

We observed a weak, positive correlation between PPAR α transactivation and the induction of apo A-I synthesis in simian hepatocytes. This is in contrast to a report by Peters et al[6] who showed, using PPAR α-deficient and wild-type mice, that PPAR α plays an active role in the down-regulation of apo A-I gene expression by fibrates. Apparently, species-specific differences in the regulation of apo A-I exist. This was elegantly demonstrated in a study by Berthou et al[30] who showed a stimulating effect of fibrates on human apo A-I expression in human apo A-I transgenic mice containing a human genomic DNA fragment driving apo A-I expression in liver, whereas endogenous mouse apo A-I expression decreased markedly. The 5'-flanking region of the cynomolgus monkey apo A-I gene is virtually identical to that of the human apo A-I gene.[31] Characterization of the human apo A-I promoter elements by promoter reporter studies in HepG2 cells revealed both a PPAR α-independent, down-regulatory element at position -41 to +91 bp and a PPAR α-dependent, upregulatory element at postition -214 to -192 bp. The overall regulation of the human apo A-I gene (and probably also the simian apo A-I gene) is therefore the resultant of a delicate interplay between these two regulatory elements which is dependent on the identity of the fibrate used. This might explain why no significant correlation was observed between the potency of the various compounds tested to activate PPAR α and the ability to increase apo A-I production.

In this and a previous study[8] we showed that fibrates suppress PAI-1 expression in cultured cynomolgus monkey hapatocytes, and similar findings have been reported for HepG2 cells.[32,33] The molecular mechanism by which fibrates suppress PAI-1 remains unknown however. In the present study we found no correlation between the suppression of PAI-1 synthesis and the ability of the various compounds to transactivate PPAR α, suggesting that fibrates can alter PAI-1 expression independently from PPAR α activation. Recently, Eriksson et al[34] identified a very-low-density lipoprotein response element (VLDLRE) in the promoter region of the human PAI-1 gene which showed some homology with a PPAR response element.[34] Inasmuch as this VLDLRE is able to bind PPAR and to mediate fibrate responses requires further research.

In conclusion, the present investigation has provided a molecular explanation, viz. activation of PPAR α, for the effects of fibrates on plasma fibrinogen levels. Considering that

a variety of fatty acids can activate PPARα,[7] plasma fibrinogen levels may reflect the endogenous lipid and lipoprotein metabolism in humans. The stimulating effect of fibrates on apo A-I expression in the simian hepatocytes may also partly be accounted for by activation of PPARα, but such a direct mechanism could not be demonstrated for the fibrate-induced lowering of PAI-1.

**Acknowledgements**

This study was supported by a grant from the Netherlands Organisation for Scientific Research (NWO: 900-523-181). We gratefully acknowledge Elly de Wit and Diana Neele for technical help with the simian hepatocyte isolations.

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*CHAPTER 5*

# Fibrates suppress fibrinogen gene expression in rodents via activation of the peroxisome proliferator-activated receptor-α

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## Abstract

Plasma fibrinogen levels have been identified as an important risk factor for cardiovascular diseases. Among the few compounds known to lower circulating fibrinogen levels in humans are certain fibrates. We have studied the regulation of fibrinogen gene expression by fibrates in rodents. Treatment of adult male rats with fenofibrate [0.5 % (wt/wt) in the diet] for 7 days decreased hepatic Aα-, Bβ- and γ-chain mRNA levels to 52 ± 7 %, 46 ± 8 % and 81 ± 19 % of control values, respectively. In parallel, plasma fibrinogen concentrations were decreased to 63 ± 7 % of controls. The suppression of fibrinogen expression was dose-dependent, and was already evident after 1 day at the highest dose of fenofibrate tested [0.5 % (wt/wt)]. Nuclear run-on experiments demonstrated that the decrease in fibrinogen expression after fenofibrate occurred at the transcriptional level, as exemplified for the gene for the A α-chain. Other fibrates tested showed similar effects on fibrinogen expression and transcription. The effect of fibrates is specific for peroxisome proliferator-activated receptor-α (PPARα), because a high-affinity ligand for PPARγ, the thiazolidinedione BRL 49653, lowered triglyceride levels but was unable to suppress fibrinogen expression. Direct evidence for the involvement of PPARα in the suppression of fibrinogen by fibrates was obtained using PPARα-null (-/-) mice. Compared to (+/+) mice, plasma fibrinogen levels in (-/-) mice were significantly higher (3.20 ± 0.48 vs 2.67 ± 0.42 g/L). Also, hepatic fibrinogen A α-chain mRNA levels were 25 ± 11 % higher in the (-/-) mice. Upon treatment with 0.2 % (wt/wt) fenofibrate, a significant decrease in plasma fibrinogen to 77 ± 10 % of control levels and in hepatic fibrinogen Aα-chain mRNA levels to 65 ± 12 % of control levels was seen in (+/+) mice, but not in (-/-) mice. These studies show that PPARα regulates basal levels of plasma fibrinogen and establish that fibrate-suppressed expression of fibrinogen in rodents is mediated through PPARα.


## Introduction

Many cross-sectional and case-control studies and numerous prospective cohort studies have identified elevated plasma fibrinogen levels as an independent risk factor for coronary heart disease, stroke and peripheral vascular disease. In addition, several cardiovascular and metabolic risk factors such as smoking, hypertension, hyperlipoproteinemia and diabetes are also associated with high plasma fibrinogen concentrations (for a review see,[1] and references therein). Interpretations of the relationship between fibrinogen and coronary heart disease are interesting and unresolved, but most likely reflect low grade inflammatory processes associated with atherogenesis.[2]

The recognition that fibrinogen is an important factor in the promotion of various disease states has led to the search for specific therapies intended to reduce plasma fibrinogen levels.

Although many different pharmacological approaches and strategies for therapeutic modulation of fibrinogen have been tested, the efficacy of the different treatments to lower plasma fibrinogen in humans is limited and the mode of action unidentified.[1,3] Among the few compounds that consistently lower circulating fibrinogen levels are some, but not all of the fibrates.[1,4] Fibrates are widely used hypolipidemic drugs, very effective in lowering elevated plasma triglyceride and cholesterol levels.[5] There is increasing evidence that at least part of the action of fibrates on lipid metabolism is exerted via the peroxisome proliferator-activated receptor-α (PPARα).[6] PPARα is a member of the nuclear receptor family of transcription factors, a diverse group of proteins that mediate ligand-dependent transcriptional activation and repression.[7] Several studies have demonstrated a direct involvement of PPARα in the fibrate-modulated gene expression of hepatic apo A-I and apo A-II, the major apolipoproteins in HDL, of lipoprotein lipase and apo C-III, both major determinants of plasma triglyceride levels, and of several enzymes implicated in fatty acid β-oxidation such as acyl-CoA oxidase (ACO).[6] The importance of PPARα in these fibrate-induced changes in gene expression and in lowering plasma triglyceride and cholesterol levels was confirmed in experiments using PPARα-deficient mice.[8-10] More recently, activation of PPARα by fibrates was also shown to inhibit the action of inflammatory cytokines by antagonizing the activities of the transcription factor, nuclear factor-κB (NF-κB).[11]

Given the reported suppressive effect of certain fibrates on plasma fibrinogen levels in humans, the hypothesis that PPARα is involved in regulating fibrinogen gene expression was tested. To that end, we first established the effect of fibrates on fibrinogen expression in rats. The fibrate-induced decrease of fibrinogen expression is regulated at the transcriptional level, as shown by nuclear run-on experiments, and is accompanied by a concomitant increase in ACO mRNA level and gene transcription, indicating PPARα activation. To establish the role of PPARα in fibrinogen gene expression, we studied the effect of fibrates in PPARα-null mice. Plasma fibrinogen concentrations were significantly higher in PPARα-null (–/–) mice than in wild-type (+/+) mice. Upon treatment with fibrate, a significant decrease in plasma fibrinogen levels and hepatic fibrinogen gene expression was observed in (+/+) mice but not in (–/–) mice. Our data provide strong evidence for an important role of PPARα in the suppression of fibrinogen gene expression and may explain fibrate-induced reductions of fibrinogen in humans.

## Material and Methods

**Reagents**

Fenofibric acid, gemfibrozil and ciprofibrate were kind gifts of Drs. A. Edgar (Laboratoires Fournier, Daix, France), B. Bierman (Warner-Lambert, Hoofddorp, The Netherlands) and M. Riteco (Sanofi Winthrop, Maassluis, The Netherlands), respectively. BRL 49653 was generously provided by Dr. Berthellon (Lipha Merck, Lyon, France). Bezafibrate was obtained from Boehringer Mannheim (Almere, The Netherlands).

**Animal studies**

Animal studies were carried out in compliance with European Community specifications regarding the use of laboratory animals. Details of experimental conditions have been described previously. [12] Briefly, male Wistar rats (three months old) were divided in groups of 4 animals each and treated for 7 days with fenofibrate mixed at the indicated concentrations (by mass) in standard rat chow. The food intake was recorded every 2 days throughout the treatment period. None of the treatments caused major changes in the amount of food consumed by the animals. Because each rat consumed approximately 20 g of chow per day, doses of 0.5 %, 0.05 % and 0.005 % (wt/wt) correspond to 320, 32, and 3 mg of fibrate/kg of body weight/day. In a subsequent experiment, rats were treated with 0.5 % (wt/wt) fenofibrate for different time periods up to 14 days, followed by a wash-out period varying from 1 to 14 days. In a second series of experiments, male Sprague-Dawley rats (3 months old) were divided in groups of 4 animals each and treated for three days with BRL 49653 (10 mg/kg of body weight/day), fenofibrate (200 mg/kg of body weight/day) or 10 % (wt/v) carboxymethylcellulose (vehicle for gavage) by gavage, twice a day. At the end of the treatment period, rats were fasted overnight, weighed and sacrificed by exsanguination under ether anaesthesia between 8 and 10 a.m. Blood was collected by aortic puncture and part of it was used for serum preparation. The other portion was incubated with 0.1 volume of trisodium citrate [3.8 % (wt/v)] to prevent coagulation, and platelet-free plasma was prepared for determination of fibrinogen. Livers were removed immediately, rinsed with 0.9 % (wt/v) NaCl, weighed, frozen in liquid nitrogen, and stored at -70°C until RNA preparation. In a third series of experiments, male Sv/129 homozygous wild-type (+/+) and PPARα-null (-/-) mice[8] (10 -12 weeks of age) were fed for 17 days with either a standard mouse chow or one containing 0.2 % (wt/wt) fenofibrate. At the end of the treatment period, the animals were fasted for 4 hours, weighed and sacrificed by exsanguination under ether anaesthesia. For determination of plasma fibrinogen levels, blood was collected from a small tail-cut using potassium-EDTA Microvette CB 300 tubes (Sarstedt, Nümbrecht, Germany). Livers were removed immediately, weighed, rinsed with 0.9 % (wt/v) NaCl, frozen in liquid nitrogen, and stored at -70 °C until RNA preparation.

**Rat hepatocyte isolation and culture**

Rat hepatocytes were isolated and cultured as described previously. [13] Briefly, hepatocytes were isolated by perfusion with 0.05 % (wt/v) collagenase and 0.005 % (wt/v) trypsin inhibitor. After a 4-hour attachment period in Williams E medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 135 nmol/L insulin, 50 nmol/L dexamethasone, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin, the nonadherent cells were washed from the plates and the remaining cells refed. After 16 hours, the medium was changed to incubation medium in which the amount of insulin was lowered to 10 nmol/L. Experiments were started 20 hours after isolation. Conditioned media were obtained by incubating cells at 37°C for 72 hours with incubation medium containing the appropriate test compound or stock solvent [DMSO; final concentration 0.1 %(v/v)]. The media were changed every 24 hours. Conditioned media were centrifuged for 4 minutes at 5000g to remove cells and cellular debris, and the samples were kept at -20°C until use. The cells were washed twice with ice-cold PBS and used for isolation of RNA.

**Serum triglycerides and plasma fibrinogen measurements**

Serum triglycerides were determined using a commercially available kit to measure total serum triglycerides (Boehringer Mannheim). Fibrinogen concentrations in plasma and conditioned media were measured by an ELISA procedure, using polyclonal antibodies to rat fibrinogen both as catching and tagging antibodies. [14]

**RNA analysis**

RNA was isolated from liver and cultured cells by the acid guanidinium thiocyanate/phenol/chloroform method.[15] Northern and dot-blot analysis of total cellular RNA were performed as described. [12] All probes were labeled with a Megaprime kit, yielding an average activity of 0.5 μCi/ng DNA. Filters were hybridized with 3 ng of [α-$^{32}$P]dCTP-labelled probe per mL as described. [16] Mouse fibrinogen cDNA probes used were provided by F. Razaee from our institute, and were a 1.2 kb fragment of the mouse fibrinogen A α-chain cDNA; a 1.0 kb fragment of the mouse fibrinogen B β-chain cDNA; a 0.6 kb fragment of the mouse fibrinogen γ-chain cDNA. Other cDNA probes used were a 2.0 kb *Sac* I fragment of the rat ACO cDNA, provided by Dr. T. Osumi, [17] and a 3.8 mDa *Eco*RI fragment of the human 18S ribosomal DNA. [18] The intensities of the signals were determined using a Fujix Bas 1000 phosphoimager and expressed relative to the signal of the 18S ribosomal RNA band.

**Isolation of nuclei and transcriptional rate assay**

Nuclei were prepared from livers of untreated rats and rats treated for 14 days with 0.5 % (wt/wt) of different fibrates in rat chow, exactly as described by Gorski et al. [19] Transcription run-on assays were performed as described by Nevins.[20] Equivalent amounts of labeled nuclear RNA were hybridized for 36 hours at 42°C to 5 μg of purified cDNA probes immobilized on Hybond C Extra filters (Amersham, Arlington Heights III). The following cDNAs were spotted: a mouse fibrinogen A α-chain cDNA probe, a rat ACO cDNA probe and a chicken ß-actin cDNA probe. As a control, 5 μg of vector DNA was applied to the filter. After hybridization, filters were washed at room temperature for 10 minutes in 0.5 x SSC (1 x SSC being 0.15 mol/L NaCl, 0.015 mol/L $Na_3$citrate) and 0.1 % (wt/v) SDS, and twice for 30 minutes at 65°C, and subsequently exposed to X-ray (X-OMAT-AR, Kodak) film. The intensities of the signals present were determined by scanning densitometry (Bio-Rad GS670 Densitometer) and expressed relative to the signal of the β-actin mRNA band.

**Statistical analysis**

The data are presented as means ± SD. The significance of treatment was assessed by an unpaired Student's- *t* test, with exception of the dose-response and time-course experiments in which analysis of variance was used to evaluate the results. For comparison of the wild type and PPAR α-deficient mice also an unpaired Student's- *t* test was used. Differences were considered significant at $p<0.05$.

## Results

**Fibrates decrease hepatic fibrinogen gene expression and plasma fibrinogen concentrations** Adult male rats were treated for 7 days with different concentrations [0.005, 0.05 or 0.5 % (wt/wt)] of fenofibrate mixed in standard rat chow, and analyzed for hepatic fibrinogen gene expression and plasma fibrinogen levels (Fig. 1). Fibrinogen is secreted as a fully assembled dimer, with each half composed of three non-identical polypeptide chains, Aα, Bβ and γ. Fenofibrate treatment decreased hepatic fibrinogen Aα-, Bβ- and γ-chain mRNA as well as plasma fibrinogen levels in a dose-dependent fashion. At the highest dose (0.5 %) of fenofibrate tested, fibrinogen mRNA levels were reduced to 52 ± 7 %, 46 ± 8 % and 81 ± 19 % of control values for the Aα-, Bβ- and γ-chain, respectively (Figs 1A and B). This weaker effect of fibrates on the γ-chain was consistently found in all experiments

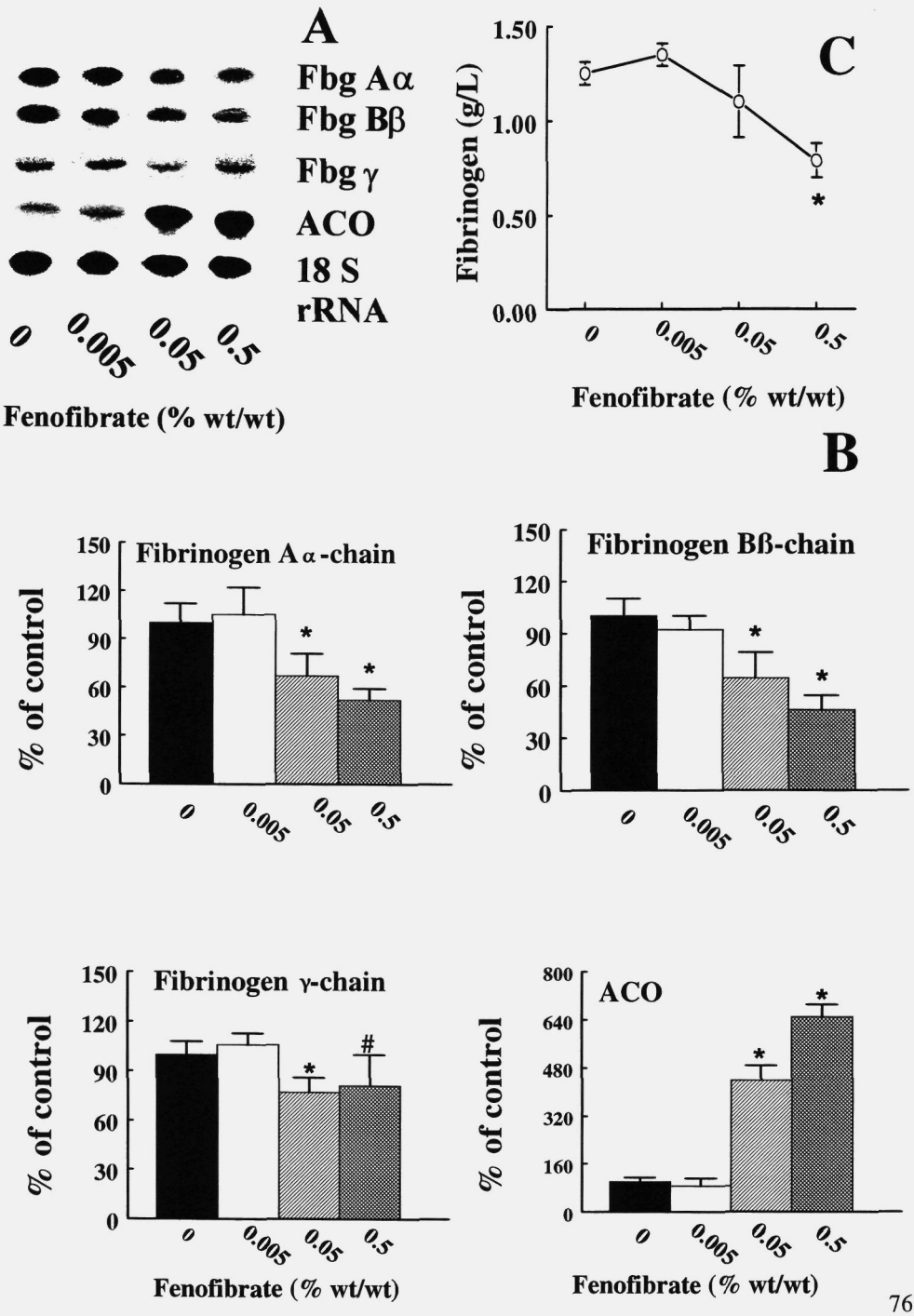
A
Fbg Aα
Fbg Bβ
Fbg γ
ACO
18 S rRNA
0
0.005
0.05
0.5
Fenofibrate (% wt/wt)
C
Fibrinogen (g/L)
1.50
1.00
0.50
0.00
Fenofibrate (% wt/wt)
B
Fibrinogen Aα-chain
% of control
Fibrinogen Bß-chain
Fibrinogen γ-chain
ACO
Fenofibrate (% wt/wt)

**Figure 1. Dose-dependent effect of fenofibrate on hepatic fibrinogen mRNAs and plasma fibrinogen levels.** Adult male rats were treated with 0.005, 0.05 or 0.5 % of fenofibrate [(wt/wt) in rat chow] for 7 days and compared with animals on rat chow only. Total RNA was extracted from livers and analyzed by Northern blotting for fibrinogen Aα-, Bβ-, and γ-chain mRNA, and ACO mRNA. Equal loading was checked by hybridizing with an 18S rRNA cDNA probe. Plasma fibrinogen levels were measured as described in the Methods section. Data shown are from a representative experiment with four animals per experimental group. (A) Representative Northern blot analysis of fibrinogen (Fbg) A α-, Bβ-, and γ-chain mRNA, ACO mRNA and 18S rRNA. (B) Signals for the three fibrinogen chain mRNAs and ACO mRNA were quantified by densitometry and adjusted for the corresponding rRNA signals. Data are expressed relative to that found in untreated animals. Results are means ± SD of four animals. (C) Plasma fibrinogen data are means ± SD of four animals. Statistically significant differences ($p<0.05$) are indicated by an asterisk; # $p = 0.13$.

control values (Fig. 1C). At the lowest dose (0.005 %) of fenofibrate tested, fibrinogen mRNAs in the liver and plasma fibrinogen levels were not significantly affected. Hepatic mRNA levels of ACO, the rate-limiting enzyme in peroxisomal β-oxidation, the induction of which by fibrates is strictly PPARα mediated,[8] showed a dose-dependent response to fenofibrate-treatment comparable to that of fibrinogen, reaching an approximately sixfold increase at a fenofibrate dose of 0.5 %.

When rats were treated with 0.5 % (wt/wt) fenofibrate for different periods of time, the mRNA levels of fibrinogen Aα-chain, the presumed rate-limiting chain in the assembly of the mature fibrinogen molecule in rats,[21] were found to be decreased to 55 ± 3 % of control levels after just 1 day of fenofibrate treatment (Fig. 2). Fibrinogen A α-chain mRNA concentrations decreased only slightly further upon prolonged treatment, reaching 44 ± 9 % and 41 ± 3 % of control values after 7 and 14 days of treatment, respectively.

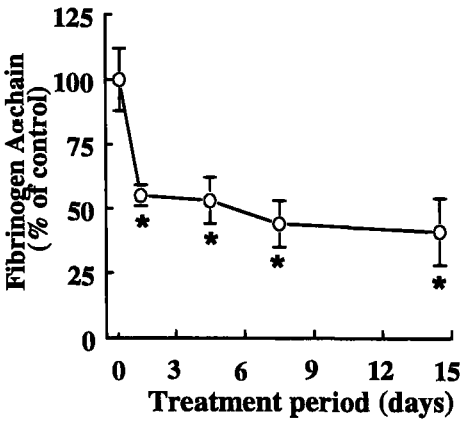


**Figure 2. Time-dependent effect of fenofibrate on fibrinogen A α-chain mRNA levels.** Adult male rats were treated with 0.5 % (wt/wt) fenofibrate for different time periods. Total RNA was extracted from livers and analyzed for fibrinogen A α-chain mRNA levels by dot blot analysis as described in the "Methods" section. Equal loading was checked by hybridizing with an 18S rRNA cDNA probe. Values are means ± SD of three animals and presented as percentage of control values. Statistically significant differences ($p<0.05$) are indicated by an asterisk.

To examine whether the observed down-regulation of plasma fibrinogen and hepatic fibrinogen gene expression is a general characteristic of fibrates rather than a specific effect of fenofibrate, we also tested the effect of other fibrates. In rats exposed for 14 days to 0.5 % (wt/wt) of gemfibrozil or bezafibrate, or 0.05 % (wt/wt) of ciprofibrate, fibrinogen A α-chain mRNA levels were reduced to 74 ± 12 %, 53 ± 3 % and 59 ± 2 % of control values, respectively.

**Down-regulation of fibrinogen expression is due to a direct effect of fibrates on hepatocytes**

Fibrates are known to cause extensive peroxisome proliferation and hepatomegaly in rodents.[6] In the present study we found that treatment of rats with 0.05 % (wt/wt) and 0.5 % (wt/wt) of fenofibrate for 14 days increased liver weights 1.4- and 2.0-fold, respectively. To exclude the possibility that the suppressive effects of fenofibrate on fibrinogen expression are due to changes in liver structure and/or function, we performed wash-out experiments: male rats were treated for 14 days with 0.5 % (wt/wt) of fenofibrate, after which the fibrate was withdrawn from the food. At the start of the wash-out period, hepatic fibrinogen A α-chain mRNA levels were 51 ± 9 % of control levels (Fig. 3). Fibrinogen A α-chain mRNA levels increased to 76 ± 13 % of control values within 1 day after withdrawal of fenofibrate, and reached control levels after 4 days, staying constant thereafter. These findings indicate that fibrates decrease fibrinogen expression reversibly and independent of changes in liver structure and/or function.

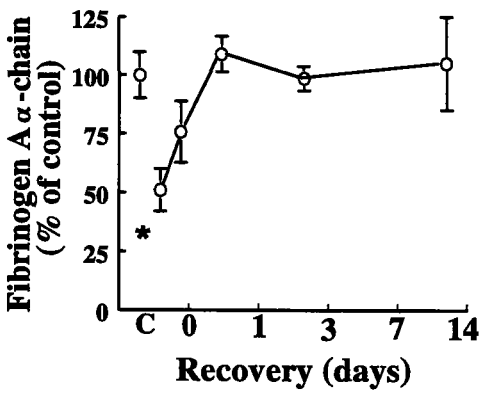


**Figure 3. Effect of cessation of fenofibrate treatment on fibrinogen A α-chain mRNA levels.** Adult male rats were treated with 0.5 % (wt/wt) of fenofibrate for 14 days, after which fenofibrate was withdrawn from the food. Total RNA was extracted from livers and analyzed for fibrinogen Aα-chain mRNA at different time point after cessation of fenofibrate treatment. Equal loading was checked by hybridizing with an 18S rRNA cDNA probe. Values are means ± SD of three animals per group and presented as percentage of control values obtained from untreated animals (C). Statistically significant differences ($p<0.05$) are indicated by an asterisk.

To find further evidence for a direct effect of fibrates on hepatic fibrinogen expression, we investigated whether these effects are also observed in primary cultures of rat hepatocytes. Treatment of rat hepatocytes for 72 hours with ciprofibrate or the active form of fenofibrate, fenofibric acid, reduced fibrinogen production dose-dependently to 62 ± 13 % and 59 ± 2 % of control values, respectively at the highest concentration of the fibrate tested (1 mmol/L)

(Fig. 4). The reduction of fibrinogen antigen levels was reflected in a reduction of fibrinogen Aα-chain mRNA levels (data not shown), indicating that fibrates suppress fibrinogen gene expression in a direct manner.

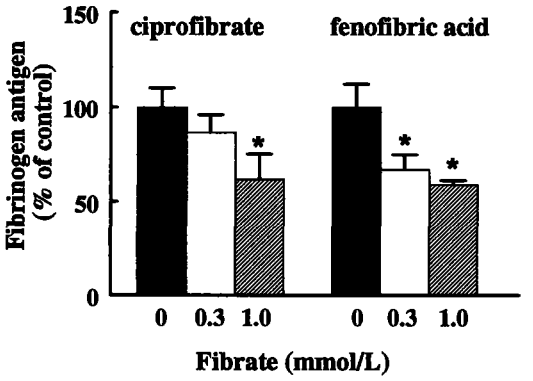


**Figure 4. Effect of ciprofibrate and fenofibric acid on fibrinogen production in primary cultures of rat hepatocytes.** Isolated rat hepatocytes were incubated with 0.3 or 1 mmol/L ciprofibrate or fenofibric acid or vehicle for three consecutive periods of 24 hours. The conditioned media were analyzed for fibrinogen antigen as described in the Methods section. Results are means ± SD of three independent experiments performed in duplicate. The data are expressed as percentage values of controls. Statistically significant differences ($p<0.05$) are indicated by an asterisk.

**Fibrates suppress fibrinogen gene transcription**

To assess the effects of various fibrates on fibrinogen gene transcription rate, nuclear run-on transcription assays were performed on nuclei prepared from livers of untreated (control) rats or rats treated for 14 days with 0.5 % (wt/wt) of fenofibrate or ciprofibrate. Both fibrates decreased fibrinogen Aα-chain transcription rate (to 24 % and 45 % of control levels for fenofibrate and ciprofibrate, respectively) and increased ACO transcription rate (to 373 % and 589 % of control levels for fenofibrate and ciprofibrate, respectively ) (Figs 5A and B), reflecting activation of PPARα.

**Fibrinogen gene expression is suppressed by PPARα but not by PPARγ activators**

In addition to their PPARα activating capacity, fibrates are also known to activate, albeit much more weakly, PPARγ.[22] To verify that the suppressive effect of fibrates on fibrinogen is mediated via activation of PPARα rather than PPARγ, we compared the effects of fenofibrate with the effects of the antidiabetic drug thiazolidinedione, BRL 49653, previously shown to be a high affinity ligand for PPARγ.[23] Rats treated for 3 days with 400 mg/kg/day fenofibrate or 10 mg/kg/day BRL 49653 by gavage showed significantly reduced plasma triglyceride levels (to 69 ± 12 % and 68 ± 8 % of control levels for fenofibrate and BRL 49653, respectively). However, whereas treatment with fenofibrate reduced fibrinogen Aα-chain mRNA levels to 55 ± 3 % of control levels, BRL 49653 did not affect fibrinogen expression (115 ± 11 % of control levels) (Fig. 6), indicating that the suppressive effect of fibrates on fibrinogen levels requires PPARα activation.

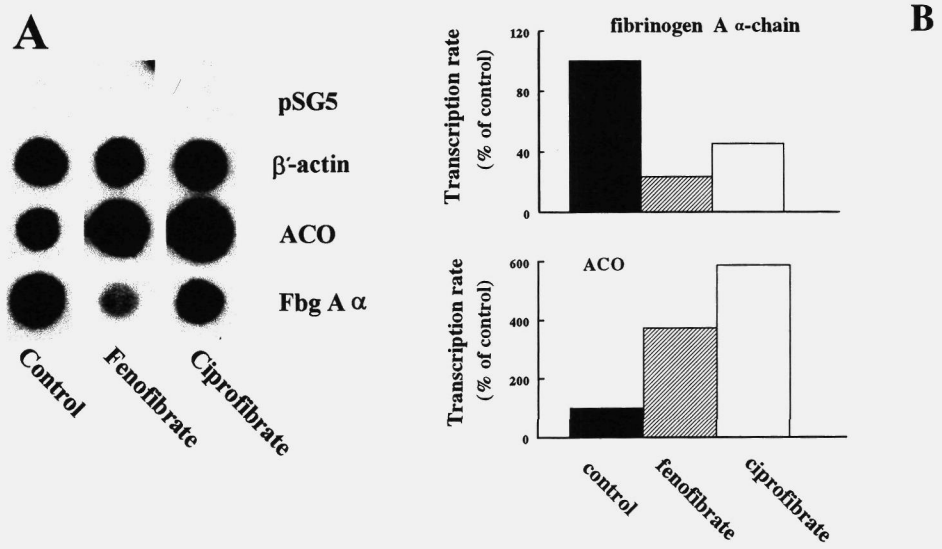


**Figure 5. Effect of fibrates on fibrinogen and ACO gene transcription.** Nuclear run-on assays were performed on nuclei obtained from livers of control rats and rats treated with 0.5 % (wt/wt) fenofibrate or 0.5 % (wt/wt) ciprofibrate for 14 days as described in the Methods section. The data shown are of a representative experiment. (A) Autoradiogram showing vector (pSG5), β-actin, ACO and fibrinogen Aα-chain (Fbg Aα) signals. (B) Signals were quantified by densitometric scanning and adjusted for the corresponding β-actin signal. Data are expressed as percentage values relative to that in control nuclei.

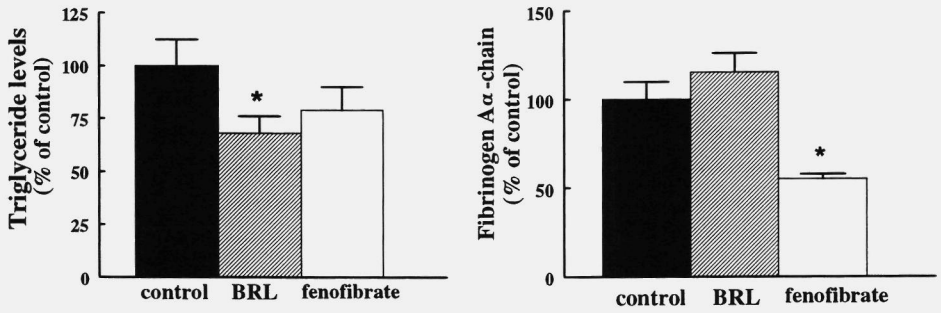


**Figure 6. Effect of BRL 49653 and fenofibrate on fibrinogen A α-chain mRNA levels in rats.** Adult male rats were treated with 10 mg/kg of body weight/day BRL 49653 or 400 mg/kg of body weight/day fenofibrate by gavage twice a day for 3 days. Plasma triglyceride levels were determined as described in the Methods section. Total RNA was extracted from livers and analyzed for fibrinogen A α-chain mRNA by Northern blotting. Equal loading was checked by hybridizing with an 18S rRNA cDNA probe. Values are means ± SD of two independent experiments (with four animals per group) and presented as percentage of control values. Statistically significant differences ($p<0.05$) are indicated by an asterisk.

**PPARα-null mice are refractory to the suppressive effects of fibrates on fibrinogen expression**

To establish a direct role of PPARα in the regulation of fibrinogen gene expression, we studied fibrinogen expression and its response to fenofibrate in PPARα-null (-/-) mice. Compared with wild type (+/+) mice, plasma fibrinogen levels were significantly higher in (-/-) mice, being 2.67 ± 0.42 g/L and 3.20 ± 0.48 g/L, respectively (Table 1).

**Table 1: Effects of fenofibrate on plasma fibrinogen levels in PPARα-null (-/-) and wild type (+/+) mice**

| | Fibrinogen (g/L) | |
|---|---|---|
| Treatment | wild type (+/+) | PPARα-null (-/-) |
| Control (n=7) | 2.67 ± 0.42 | 3.20 ± 0.48¶ |
| Fenofibrate (n=7) | 2.06 ± 0.26 | 2.87 ± 0.23¶ |
| Difference | -0.61 ± 0.31 (p= 0.007) | -0.33 ± 0.36 (p = 0.13) |

Wild type (+/+) and PPARα-null (-/-) mice (n=7) were treated with 0.2 % (wt/wt) fenofibrate mixed in chow for 17 days. Values are means ± SD. ¶ Statistically significant ($p<0.05$) difference between wild type versus PPARα-null mice.

Hepatic fibrinogen Aα-chain mRNA levels were 25 ± 11 % higher in the (-/-) mice (Fig. 7). Upon treatment with 0.2 % (wt/wt) fenofibrate for 17 days, liver weights were increased to 277 % of controls in (+/+) mice, while no change in liver weights of (-/-) mice was observed (data not shown). Fenofibrate significantly decreased plasma fibrinogen levels in (+/+) mice (-0.61 ± 0.31 g/L; p=0.007) but not in (-/-) mice (-0.33 ± 0.36 g/L; p=0.13) (Table 1). Consistent with the antigen data, fibrinogen Aα-chain mRNA levels were significantly decreased in (+/+) mice (-35 ± 12 %; p=0.04) but not in the fibrate-treated (-/-) mice (+1 ± 13 %; p=0.9) (Fig. 7). These results indicate that PPARα is involved in the suppression of basal levels of plasma fibrinogen, and that fibrate-suppressed expression of fibrinogen in wild-type mice is dependent on PPARα activation.

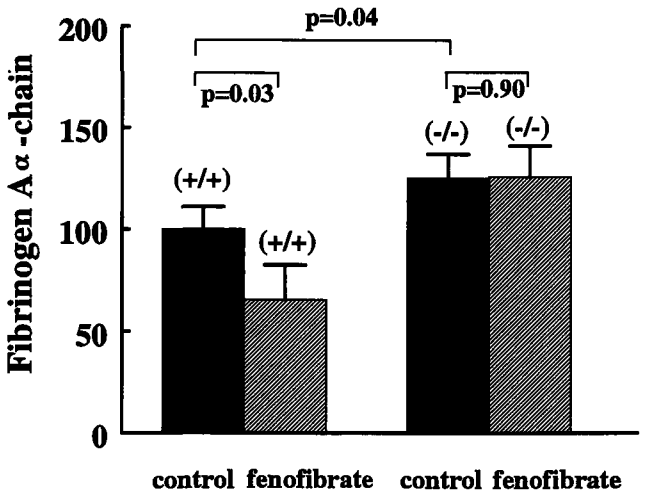


**Figure. 7. Effect of fenofibrate on fibrinogen Aα-chain mRNA levels in wild-type versus PPARα-null mice.** Wild type (+/+) and PPARα-null (-/-) mice were treated with 0.2% (wt/wt) fenofibrate mixed in chow for 17 days. Total RNA was extracted from livers and analyzed for fibrinogen Aα-chain by Northern blotting. Equal loading was checked by hybridizing with an 18S rRNA cDNA probe. Values are means ± SD of seven animals per group and presented as percentage values of control, untreated wild type mice.

## Discussion

Fibrates reportedly lower plasma fibrinogen in humans, but the regulatory mechanism of this effect remains to be clarified. Here, we show that activation of the nuclear hormone receptor PPARα mediates the suppression of fibrinogen gene transcription by fibrates in rodents. A direct involvement of PPARα in fibrinogen gene expression was provided using PPARα-null (-/-) mice. Basal levels of plasma fibrinogen were significantly higher in the (-/-) mice than in (+/+) mice, and fibrates suppressed fibrinogen gene expression and plasma levels in (+/+) mice only. These observations clearly establish PPARα as a key regulatory factor in fibrinogen gene expression in rodents and may explain the suppressive effect of fibrates on plasma fibrinogen levels in humans.

The fibrinogen molecule is arranged as a dimer, with each monomer composed of three non-identical polypeptide chains: Aα, Bβ and γ.[24] The three fibrinogen chains are encoded by three separate, closely linked genes situated on the same chromosome and located in the sequence γ, Aα and Bβ, with the last one in opposite transcriptional orientation to the first one.[25] It has been reported that in rat hepatocytes, the amount of Aα-chain limits the rate of assembly of the fibrinogen molecule.[21] whereas in human hepatoma cells the amount of Bβ-chain appears to limit assembly.[26,27] We found that, at least in rats, the inhibition of gene expression by fibrates was evidently not confined to the Aα-chain, but equally affected the Bβ-chain and, albeit to a lesser extent, the γ-chain. Recently, Binsack et al. reported that also in the human hepatoma cell line HepG2 bezafibrate suppressed Aα-, Bβ- and γ-chain mRNA levels.[28] These findings corroborate the concept of the coordinated expression of the three

fibrinogen chains in both rats and humans.[27]

Our results show that PPARα has an important role in mediating the effects of fibrates on fibrinogen expression. Whereas several genes involved in lipid metabolism -apo A-I, lipoprotein lipase and acyl-CoA synthetase- are positively regulated by PPARα,[29-31] the genes encoding the three fibrinogen chains are negatively regulated by PPARα, like apo CIII.[6] Although the coordinate suppression of gene transcription of the three fibrinogen chains by fibrates would suggest a shared regulatory mechanism, the exact molecular mechanism by which PPARα acts is not understood. Further experiments, including functional analysis of the regulatory regions of the genes encoding for the fibrinogen chains, will be necessary to elucidate the precise mechanism of transcriptional repression of fibrinogen gene expression by PPARα.

Fibrates are also implicated in suppressing elevated fibrinogen levels under inflammatory conditions. Many reports link the inflammatory mediator IL-6 to elevated fibrinogen expression,[32,33] and indeed IL-6 responsive elements have been identified in the promoters of the different rat and human fibrinogen genes.[34-36] Recent evidence indicates that activated PPARα can interfere negatively with cytokine-induced signalling pathways.[11,37] It is thus conceivable that PPARα also plays an important role in down-regulating cytokine-increased fibrinogen gene expression.

We found significantly higher basal plasma fibrinogen levels in PPARα-null (-/-) mice than in wild-type (+/+) mice, suggesting that PPARα is involved in modulating basal fibrinogen expression. Several endogenous ligands have been identified such as long chain fatty acids (palmitic acid, linoleic acid, arachidonic acid) and eicosanoids (leukotriene B4, 8(S)-hydroxyeicosatetraenoic acid)[22,38] which could account for PPARα activation under basal conditions. Therefore, changes in endogenous fatty acid profiles as a result of changes in environmental and life-style factors may explain reported intra-individual variation in fibrinogen levels of about 10-15 %.[39-41] Similarly, the recent identification of structural and functional polymorphisms in human PPARα[42] may be relevant for understanding regulation of plasma fibrinogen levels. It would be interesting to delineate the role of abnormal PPAR α activity in patients with disturbed fibrinogen and lipid levels by genetic linkage studies.

It is important to recognize that fibrates down-regulate fibrinogen expression via the same transcription factor as that identified for reducing circulating triglyceride and cholesterol levels, i.e. activated PPARα. Other lipid lowering drugs such as HMG CoA reductase inhibitors (statins) and PPARγ activators (thiazolidinediones) show no significant consistent effects on fibrinogen levels. For example, lovastatin therapy has resulted in minor fibrinogen reductions in hypercholesterolemic patients[41] or actually increased fibrinogen[43], while reductions were seen in a single reported study with pravastatin therapy in familial hypercholesterolemia patients.[44] In the present study, we found no effect of the thiazolidinedione, BRL 49653, on plasma fibrinogen levels in rats, while triglyceride levels

were lowered to a similar extent as with fenofibrate. These results further emphasize that the lowering effect of fibrates on fibrinogen are not the result of lowered triglyceride levels. Because both elevated plasma fibrinogen levels and elevated plasma lipids have been identified as key risk factors for cardiovascular diseases,[1] the identification of a common, specific molecular target, PPARα, that is suitable for application of modern drug discovery provides a new lead for therapy. Such a novel compound specifically activating PPARα may prove superior to existing fibrates, in the action of which other, as yet unidentified molecular mechanisms are also involved.[4,45]

## Acknowledgement

We gratefully acknowledge Sabine Post for technical help with the rat hepatocyte isolation and Karin Toet for technical assistance. We also thank Farhad Rezaee for providing the mouse fibrinogen Aα-, Bβ- and γ-chain cDNA probes.

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*CHAPTER 6*

# Relationship between visceral fat and plasminogen activator inhibitor-1 in overweight men and women before and after weight loss

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*Thrombosis and Haemostasis, in press*

## Abstract

This study was aimed at evaluating the relationship between visceral fat accumulation and plasma plasminogen activator inhibitor-1 (PAI-1) levels in healthy, obese men and women undergoing weight loss therapy. The subjects, 25 men and 25 premenopausal women, aged between 26 and 49 years, with an initial body mass index between 28 and 38 $kg/m^2$, received a controlled diet for 13 weeks providing a 4.2 MJ/day energy deficit. Magnetic resonance imaging was used to measure visceral and subcutaneous abdominal fat. Our results show that before weight loss visceral fat was significantly correlated with PAI-1 in men ($r = 0.45$; $p < 0.05$), but not in women ($r = -0.15$; ns). The association between visceral fat and PAI-1 in men remained significant after adjustment for age and total fat mass, and multiple linear regression analysis showed a significant independent contribution of visceral fat to plasma PAI-1 levels. Both visceral fat areas and PAI-1 levels decreased significantly with weight loss in both men and women. Changes in visceral fat area were related to changes in PAI-1 in women ($r = -0.43$; $p = 0.05$) but not in men ($r = -0.01$; ns); however, this association in women disappeared after adjustment for total fat mass. We conclude that there is a relationship between visceral fat and PAI-1 in obese men but not in obese women, and that PAI-1 levels decrease substantially (52%) by weight loss, but this change is not related to changes in visceral fat mass *per se*.


## Introduction

Obesity, defined as an accumulation of excess body fat, is a common condition in affluent societies, and represents a major health problem. Obesity is an independent risk factor for atherosclerosis and cardiovascular disease, and a major contributor to morbidity and mortality[1]. Several studies have indicated that obesity is associated with an impaired fibrinolytic activity, mainly as a result of an increased plasma level of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of tissue-type plasminogen activator (t-PA)[2-4]. The pathophysiological importance of elevated PAI-1 and a low fibrinolytic activity is illustrated by work showing elevated plasma levels of PAI-1 in young survivors of myocardial infarction[5], in subjects with deep vein thrombosis[6], and in patients with non-insulin-dependent diabetes mellitus (NIDDM)[7]. Increased PAI-1 also correlates with thrombosis in animal models, and transgenic mice that overexpress PAI-1 develop venous thrombosis[8]. Studies directed at understanding the role of fat tissue in elevating plasma PAI-1 levels in obese subjects may contribute to our insight in the underlying mechanisms of the pathophysiology of obesity.

It has been frequently shown that the location of the body fat deposits rather than their mass is a key factor in the development of obesity-linked disorders[9,10]. Accumulation of intra-abdominal visceral fat located in the mesenterium and omentum is a better predictor of coronary heart disease than is the body mass index (BMI, weight in kilograms divided by the square of the height in metres) in both men[9] and women[10]. A direct link between excess abdominal adipose tissue and attenuated fibrinolysis was recently suggested by several groups showing that plasma levels of PAI-1 are closely related to the visceral fat area but not to the subcutaneous fat area in obese and nonobese children and adults[11-14]. The question arises how corpulence influences plasma PAI-1 levels.

There is increasing evidence that adipocytes, in particular from visceral fat tissue, may directly contribute to the elevated expression of PAI-1 in obesity[15]. PAI-1 gene expression was significantly elevated in the adipose tissue of obese mice compared with their lean counterparts[16]. Expression of PAI-1 mRNA has also been demonstrated in the visceral and subcutaneous fat of obese rats[11] and in adipose tissues from human subjects[17]. In both cases, visceral tissues expressed significantly more PAI-1 than subcutaneous tissues from the same subject. In rats subjected to lesion in the ventromedial hypothalamus, an animal model of obesity, PAI-1 mRNA expression increased in visceral fat but not in subcutaneous fat or liver as obesity developed[11]. Recently, Alessi et al.[17] demonstrated that PAI-1 antigen production by explants of adipose tissue from visceral areas was higher than that from subcutaneous areas. However, a variety of observations also implicate factors other than visceral fat mass per se in determining plasma PAI-1 levels. For example, in humans a large variation in PAI-1 levels exists between individuals with the same degree of adiposity[3]. Also, abdominal obesity is accompanied by a variety of metabolic disorders such as hypertension, hyperlipidemia, insulin resistance and NIDDM (reviewed in[18]), disorders which themselves are associated with increased PAI-1 levels[7,19]. In addition, specific hormones and/or cytokines known to upregulate PAI-1 gene expression are elevated locally or systemically in obesity[1,20]. Furthermore, sex steroids influence circulating PAI-1 levels[21], while plasma levels of sex steroids are affected by adipose distribution[22].

In the present study we have evaluated the contribution of visceral fat to plasma PAI-1 levels in overweight men and women. In most, usually cross-sectional studies in which plasma PAI-1 levels and central adiposity have been found to be associated, adjustments were made for insulin and triglyceride levels but no adjustments have been made for potential confounders, such as total body fat and gonadal steroids, each of which may affect both the accumulation of visceral fat and PAI-1. In several studies, weight loss in overweight subjects as a result of surgical treatment[23] or diet[24] has led to reduced PAI-1 levels in these patients. In only a few studies the relations between changes in body fat distribution and changes in fibrinolysis parameters were determined. Folsom et al.[25] found a decline in PAI-1 to be correlated with declines in most anthropometric variables, and with correlations being

stronger for men than for women. We reasoned that proportional changes in visceral fat should correlate with proportional changes in PAI-1 levels, if visceral fat directly contributes to plasma PAI-1 levels. We also evaluated whether relations between (changes in) central adiposity and fibrinolysis parameters were attributable to other potential confounders, and which (metabolic) factors other than fat mass are associated with increased plasma PAI-1 concentrations.

## Materials and Methods

**Study population**

Subjects were participants in a weight loss study carried out by the University of Wageningen [26,27]. Participants were recruited through local newspaper advertisements. Fifty obese subjects (25 women and 25 men) were selected on the basis of their body mass index (BMI, weight in kilograms divided by the square of the height in metres; between 28 and 38 kg/m$^2$), premenopausal state, smoking behaviour (< 5 cigarettes per day), and drinking behaviour (< 2 alcoholic consumptions per day). All subjects were apparently healthy on clinical examination and medical history. Subjects with glycosuria and proteinuria were excluded. Throughout the study, none of the volunteers used any prescription medication known to affect the variables measured in this study, and none of the women used oral contraceptives. None of the subjects had been on a slimming diet for several months before the study. The study was approved by the Medical Ethical Committee of the Department of Human Nutrition, and each participant gave written informed consent before participation.

**Experimental design and diet**

Subjects were given a weight-maintenance diet before the weight-loss intervention. A standard Western-type food was supplied and individually tailored to meet each person's energy requirement, which was estimated from resting metabolic rate and physical activity pattern [28]. Body weights were recorded twice a week by the subjects, and energy intakes were adjusted to maintain individual weights. After a weight-stable period of 3 to 10 weeks, baseline measurements were performed to determine body composition and fat distribution, and blood samples were drawn. The subjects then received a 4.2 MJ/day energy-deficit diet during a period of 13 weeks. Individual energy deficits were based on estimated daily energy intake at the end of the weight-stable period preceding the period of energy restriction. At the end of the weight-loss period, all measurements were repeated and blood samples were collected.

The nutrient compositon of the diets remained the same throughout the experimental period and was calculated with the use of the Dutch computerized food-composition table (UCV). The diet consisted of 25% of energy (en%) as protein, 33 en% as total fat (11 en% as saturated fatty acids, 11 en% as monosaturated fatty acids, 11 en% as polyunsaturated fatty acids) and 42 en% as carbohydrates. The weight-stable diets consisted of conventional foods, whereas the energy-deficit diet was a combination of slimming products and conventional foods. The participants were encouraged not to change their lifestyle throughout the study. They were asked to record any sign of illness, deviations from the diet, medication used, and changes in smoking and activity patterns in a diary. Compliance to the diet was checked by weight control and meetings with trained dietitians every 2 weeks.

**Blood sampling and analyses**

Blood sampling was performed in the morning hours after an overnight fast of 11-13 hours, with an interval of 2 days. The mean concentration of the 2 samples was used for statistical analysis. Serum was prepared by low-speed centrifugation within 1 hour after venepuncture and stored at -80 °C until analysis. For the fibrinolysis assays, blood samples were collected in ice-cold CTAD tubes (1/10 volume of 0.11 mmol/L citric acid, 15 mmol/L theophylline, 3.7 mmol/L adenosine, 0.198 mmol/L dipyridamol (Becton Dickinson, Cedex, France) essentially as described in the protocol of the Leiden Fibrinolysis Working Party [29]. The blood samples were immediately centrifuged (2000 x g, 15 min at 4 °C), and plasma was snap frozen and stored at -80 °C until use. Before and after intervention samples were measured simultaneously.

Total serum cholesterol was determined by an enzymatic colorimetric method [30]. HDL cholesterol was measured by the same procedure following precipitation by dextran sulphate -Mg [2+31]. The LDL cholesterol concentration was calculated using the Friedewald equation [32]. Triglycerides and insulin were determined with commercially available kits (Boehringer Mannheim, Mannheim, Germany). PAI-1 activity was measured by a t-PA-based specific chromogenic assay ("Coatest", Kabi Diagnostica, Mölndal, Sweden). PAI-1 antigen was determined using commercially available enzyme immunoassay (EIA) kits ("Innotest" PAI-1 from Innogenetics, Zwijndrecht, Belgium and "Imulyse" PAI-1 from Biopool AB, Umeå, Sweden). Statistical analysis of the results obtained with both assays showed very similar relationships between PAI-1 and the various parameters. For the sake of being concise, only the data obtained with the Imulyse assay are shown. The EIA kit for determination of t-PA antigen was obtained from Biopool AB (Umeå, Sweden).

Levels of sex-hormone-binding-globulin (SHBG) were determined using the immunoradiometric assay of Farmos Diagnostica (Oulunsalo, Finland). Total testosterone (T) was measured by radio-immuno-assay (RIA) after extraction with diethylether as described previously [33]. Estrone (E1) and total 17β-estradiol (E2) were extracted with diethylether, purified and separated by chromatography on Sephadex LH-20 columns using toluene: methanol (92:8, v/v) as eluent, and quantified by RIA [33]. The percentages of free T and free E2 were calculated indirectly by the use of the equations described by Nanjee and Wheeler [34] and Moore et al.[35], respectively.

**Body composition and fat distribution**

All anthropometric measurements were made with the subjects wearing only swimming gear or underwear. Body weight was determined to the nearest 0.05 kg on a digital scale and body height was measured to the nearest 0.001 m using a wall-mounted stadiometer. BMI was calculated by dividing weight in kilograms by height in metres squared. The circumference of the waist was measured midway between the lower rib margin and the iliac crest at the end of a gentle expiration. The hip circumference was measured at the level of the widest circumference over the great trochanters. Both circumferences were measured to the nearest 0.001 m with the participant standing erectly. The waist-to-hip ratio (WHR) was calculated as a measure of fat distribution. Percentage body fat was calculated from total body density, as determined by underwater weighing, by applying the equation described by Siri [36].

Magnetic resonance imaging (MRI) scans were made on a whole-body scanner (GYROSCAN S15, Philips Medical Systems, Best, The Netherlands) using a 1.5-T magnetic field (64 Mhz) and a slice thickness of 10 mm. Transverse MRI-scans were taken midway between the lower rib margin and the iliac crest while subjects were lying supine. Imaging analysis to determine the visceral and subcutaneous fat areas was carried out as previously described[37].

**Statistical methods**

Deviations from normality of the distributions of the variables were checked within each sex. In case of a skewed distribution, natural logarithm-transformed values were used in statistical analysis. Nonparametric tests were used with respect to analyses involving PAI-1, which was skewed after logarithm-transformation. Differences in baseline characteristics as well as differences in responses due to weight loss between the sexes were tested by the Student's unpaired *t*-test. The effect of weight loss on variables within each sex was tested with the Student's paired *t*-test. Correlations between PAI-1/t-PA antigen levels and other variables were determined by Spearman's rank correlation analysis. Multiple linear regression analysis was performed to evaluate the relative contribution of visceral fat area to the variability in PAI-1 and t-PA antigen levels . Two-sided p-values $\leq 0.05$ were considered to be statistically significant. Results are expressed as means ± standard deviation (SD).

## Results

### Pre-diet baseline values

Sex-specific baseline characteristics of the obese subjects participating in the study are shown in Tables 1 and 2. The differences in variables between women and men were all statistically significant, except for age, BMI, total abdominal fat area, PAI-1 and insulin. Although women and men did not differ in total abdominal fat areas, women had substantially more subcutaneous abdominal adipose tissue, whereas men had larger visceral fat areas.

Simple correlation coefficients of PAI-1 and t-PA with body composition and body fat distribution variables, triglycerides, free testosterone and insulin are summarized in Table 3. Because PAI-1 activity and PAI-1 antigen data were very comparable, only PAI-1 antigen data are shown. Baseline PAI-1 levels in women were strongly negatively correlated with age, but such a correlation was absent in men. In women, PAI-1 levels were positively correlated with free testosterone levels, but not with visceral fat areas (see also Fig.1). In men, however, PAI-1 concentrations correlated with visceral fat areas (see also Fig.1), but not with free testosterone concentrations.

For t-PA plasma levels in women, positive correlations were found with fat mass, subcutaneous fat and total fat area, whereas in men, t-PA correlated positively with waist-to-hip ratio.

### Effects of weight loss

Within each sex group, all variables were significantly lower after a 13-week diet period, except for estrogens in women and testosterone in men, which did not significantly change, and HDL-cholesterol and sex hormone binding globulin (SHBG), which increased in both sexes (Tables 1 and 2). In men, elevated levels of estrone were found after weight loss. The diet-induced decreases in body weight and fat mass did not significantly differ between women and men. Women and men lost similar amounts of subcutaneous abdominal fat, but men lost significantly more visceral fat than women, and the decrease in the waist/hip ratio was also significantly larger in men.

**Table 1: Effect of Weight loss on Characteristics of Women (n=25)**

| Characteristics | Before | After | Change |
|---|---|---|---|
| **General characteristics** | | | |
| Age (years) | 38.4 ± 5.5 (27 - 46) | – | – |
| Weight (kg) | 85.9 ± 8.8 (72.3 - 100.5) | 74.5 ± 8.9 (60.0 - 92.7) | 11.4 ± 3.9 (4.0 - 19.3) *** |
| BMI ($kg/m^2$) | 31.3 ± 4.5 (27.7 - 35.6) | 27.0 ± 2.1 (22.5 - 30.6) | 4.3 ± 1.4 (1.5 - 7.1) *** |
| Total body fat (%) | 43.3 ± 4.5 (35.1 - 51.6) | 36.5 ± 5.7 (23.7 - 45.2) | 6.7 ± 2.9 (1.1 - 12.7) *** |
| Fat mass (kg) | 37.2 ± 6.0 (26.6 - 47.1) | 27.4 ± 6.2 (15.6 - 39.8) | 9.8 ± 3.6 (2.4 - 16.4) *** |
| WHR | 0.89 ± 0.07 (0.76 - 1.02) | 0.83 ± 0.05 (0.76 - 0.97) | 0.03 ± 0.03 (-0.02 - 0.09) |
| Glucose (mmol/l) | 5.2 ± 0.3 (4.7 - 5.8) | 5.0 ± 0.4 (4.4 - 5.6) | 0.3 ± 0.3 (-0.3 - 1.0) *** |
| Insulin (μU/ml) | 10.8 ± 6.7 (1.3 - 27.8) | 6.8 ± 4.6 (1.8 - 19.1) | 3.9 ± 5.9 (-10.4 - 14.9) *** |
| Triglycerides (mmol/l) | 1.3 ± 0.5 (0.7 - 2.4) | 1.0 ± 0.3 (0.4 - 2.0) | 0.3 ± 0.3 (-0.4 - 1.3) *** |
| HDL cholesterol (mmol/l) | 1.1 ± 03 (0.7 -1.6) | 1.2 ± 0.3 (0.6 - 1.7) | -0.03 ± 0.13 (-0.22 - 0.22) |
| **Abdominal fat areas ($cm^2$)** | | | |
| Visceral | 98 ± 31 (52 - 163) | 66 ± 26 (28 - 138) | 32 ± 23 (2 - 84) *** |
| Subcutaneous | 402 ± 104 (222 - 615) | 281 ± 88 (128 - 453) | 122 ± 61 (1 - 233) *** |
| Total | 501 ± 111 (325 - 698) | 351 ± 101 (172 - 576) | 150 ± 63 (25 - 258) *** |
| **Fibrinolytic factors** | | | |
| PAI-1 activity (IU/ml) | 17 ± 11 (0.5 - 34) | 7.6 ± 6.8 (0.5 - 24.8) | 9.8 ± 8.5 ( -4.2 - 26.7) *** |
| PAI-1 antigen (ng/ml) | 23 ± 14 (5-58) | 11 ± 7.8 (1.9 - 30.4) | 12 ± 11 (-7.1 - 38.9) *** |
| t-PA antigen (ng/ml) | 9.1 ± 2.9 (3.4 - 15.7) | 6.1 ± 2.0 (2.9 - 11.1) | 3.0 ± 1.9 (-0.9 - 7.0) *** |
| **Hormones** | | | |
| SHBG (mmol/l) | 28 ± 12 (11 - 57) | 38 ± 17 (15 - 88) | -10 ±-14 (-57 - 7) * |
| $E_1$ (pmol/l) | 241 ± 77 (50 - 425) | 235 ± 97 (155 - 655) | 5.7 ± 86 (-305 - 120) |
| Free $E_2$ (pmol/l) | 5.4 ± 2.9 (1.0 - 12.9) | 3.8 ± 3.3 (1.0 - 16.5) | 1.6 ± 2.9 (-5.2 - 7.1) |
| Free T (nmol/l) | 0.03 ± 0.01 (0.01 -0.05) | 0.03 ± 0.01 (0.01 - 0.04) | 0.01 ± 0.01 (0.002 - 0.02) |

Values are means ± SD (range)

BMI = body mass index, WHR = waist-hip ratio, PAI-1 = plasminogen activator inhibitor type-1, t-PA = tissue type plasminogen activator, SHBG = sex hormone binding globulin, $E_1$ = estrone, $E_2$ = 17β-estradiol, T = testosterone.

* $p<0.05$; ** $p<0.001$; *** $p<0.0001$ effect of weight loss

**Table 2: Effect of Weight loss on Characteristics of Men (n=25)**

| Characteristics | Before | After | Change |
|---|---|---|---|
| **General characteristics** | | | |
| Age (years) | 40.2 ± 6.2 (28 - 49) | – | – |
| Weight (kg) | 96.6 ± 8.4 (82.2 - 116) | 84.5 ± 8.9 (72.5 - 108.3) | 12.1 ± 3.1 (6.8 - 17.6) |
| BMI ($kg/m^2$) | 30.5 ± 2.2 (26.2 - 34.9) | 26.7 ± 2.6 (22.2 - 32.5) | 3.9 ± 1.0 (2.1 - 6.3) *** |
| Total body fat (%) | 31.9 ± 4.4 (23.2 - 39.3) | 25.4 ± 5.5 (14.0 - 36.5) | 6.4 ± 2.6 (1.9 - 11.4) |
| Fat mass (kg) | 30.8 ± 5.2 (20.7 - 41.2) | 21.7 ± 6.1 (10.5 - 35.5) | 9.1 ± 2.6 (4.4 - 14.9) |
| WHR | 0.97 ± 0.05 (0.84 - 1.08) | 0.91 ± 0.05 (0.79 - 0.99) | 0.06 ± 0.03 (-0.04 - 0.14) |
| Glucose (mmol/l) | 5.4 ± 0.4 (4.6 - 6.0) | 5.0 ± 1.0 (0.5 - 6.6) | 0.4 ± 0.8 (-0.6 - 4.6) * |
| Insulin (μU/ml) | 11.6 ± 6.7 (2.9 - 32.8 ) | 7.6 ± 3.6 (2.4 - 20.2) | 4.1 ± 5.8 (-3.3 - 24.3) |
| Triglycerides (mmol/l) | 1.9 ± 0.7 (1.0 - 3.8) | 1.2 ± 0.5 (0.6 - 2.5) | 0.7 ± 0.5 (-0.2 - 1.8) |
| HDL cholesterol (mmol/l) | 0.9 ± 0.2 (0.7 - 1.3) | 1.0 ± 0.2 (0.8 - 1.3) | -0.08 ± 0.09 (-0.28 - 0.07) |
| **Abdominal fat areas ($cm^2$)** | | | |
| Visceral | 155 ± 43 (66 - 264) | 95 ± 36 (25 - 177) | 61 ± 22 (20 - 105) *** |
| Subcutaneous | 312 ± 66 (202 - 456) | 202 ± 64 (105 - 342) | 109 ± 43 (42 - 234) *** |
| Total | 467 ± 78 (335 - 621) | 297 ± 90 (165 - 470) | 170 ± 48 (101 - 335) *** |
| **Fibrinolytic factors** | | | |
| PAI-1 activity (IU/ml) | 22 ± 10 (2 - 37) | 10 ± 10 (0.5 - 37) | 13 ± 10 (-6 - 28) *** |
| PAI-1 antigen (ng/ml) | 27 ± 15 (5 - 63) | 13 ± 13 (1 - 60) | 14 ± 11 (-7 - 41) *** |
| t-PA antigen (ng/ml) | 11.2 ± 4.0 (4.5 - 19.6) | 7.5 ± 3.6 (2.2 - 16.9) | 3.7 ± 2.7 (-1.3 - 9.4) *** |
| **Hormones** | | | |
| SHBG (mmol/l) | 17.1 ± 7.2 (6 - 33) | 22 ± 10 (7 - 52) | -4.4 ± 5.3 (-24.0 -7.0) ** |
| $E_1$ (pmol/l) | 140 ± 48 (50 - 225) | 163 ± 35 (115 - 235) | -23 ± 35 (-100 - 45) * |
| Free $E_2$ (pmol/l) | 2.3 ± 0.5 (1.5 - 3.6) | 1.7 ± 0.6 (0.9 - 2.8) | 0.5 ± 0.6 (-0.7 - 1.6) ** |
| Free T (nmol/l) | 0.42 ± 0.07 (0.27 - 0.57) | 0.40 ± 0.11 (0.12 - 0.58) | -0.03 ± 3.25 (-0.14 - 0.29) |

Values are means ± SD (range)
BMI = body mass index, WHR = waist-hip ratio, PAI-1 = plasminogen activator inhibitor type-1, t-PA = tissue type plasminogen activator, SHBG = sex hormone binding globulin, $E_1$ = estrone, $E_2$ = 17β-estradiol, T = testosterone.
* $p<0.05$; ** $p<0.001$; *** $p<0.0001$ effect of weight loss

**Table 3: Simple Spearman Correlation Coefficients of PAI-1 and t-PA with Subject Characteristics before and after Weight Loss in Women and Men**

| | Women | | | | Men | | | |
|---|---|---|---|---|---|---|---|---|
| | PAI-1 antigen | | t-PA antigen | | PAI-1 antigen | | t-PA antigen | |
| Characteristics | Before | After | Before | After | Before | After | Before | After |
| Age | - 0.55 ** | - 0.46 ** | - 0.01 | 0.06 | - 0.03 | - 0.10 | 0.24 | 0.11 |
| Fat mass | 0.38 | 0.41 * | 0.52 ** | 0.27 | 0.33 | 0.41 * | 0.17 | 0.50 ** |
| Visceral fat area | - 0.15 | 0.29 | 0.08 | 0.37 | 0.45 * | 0.55 ** | 0.28 | 0.49 ** |
| Subcutanous fat area | 0.06 | 0.17 | 0.64 *** | 0.35 | 0.06 | 0.36 | 0.18 | 0.93 * |
| Total fat area | 0.03 | 0.28 | 0.65 *** | 0.49 * | 0.34 | 0.22 | 0.35 | 0.49 ** |
| WHR | 0.01 | 0.25 | 0.06 | 0.26 | 0.10 | 0.11 | 0.38 * | 0.21 |
| Insulin | 0.15 | 0.41 * | 0.03 | 0.48 * | 0.32 | 0.22 | 0.07 | 0.16 |
| Triglycerides | 0.27 | 0.22 | 0.19 | 0.26 | 0.30 | 0.61 *** | 0.20 | 0.60 *** |
| Free T | 0.59 ** | 0.36 | 0.37 | 0.25 | 0.18 | 0.22 | - 0.10 | - 0.01 |

WHR=waist-hip ratio, PAI-1 = plasminogen activator inhibitor type-1, t-PA = tissue-type plasminogen activator, T = testosterone.
* $p<0.05$; ** $p<0.001$; *** $p<0.0001$

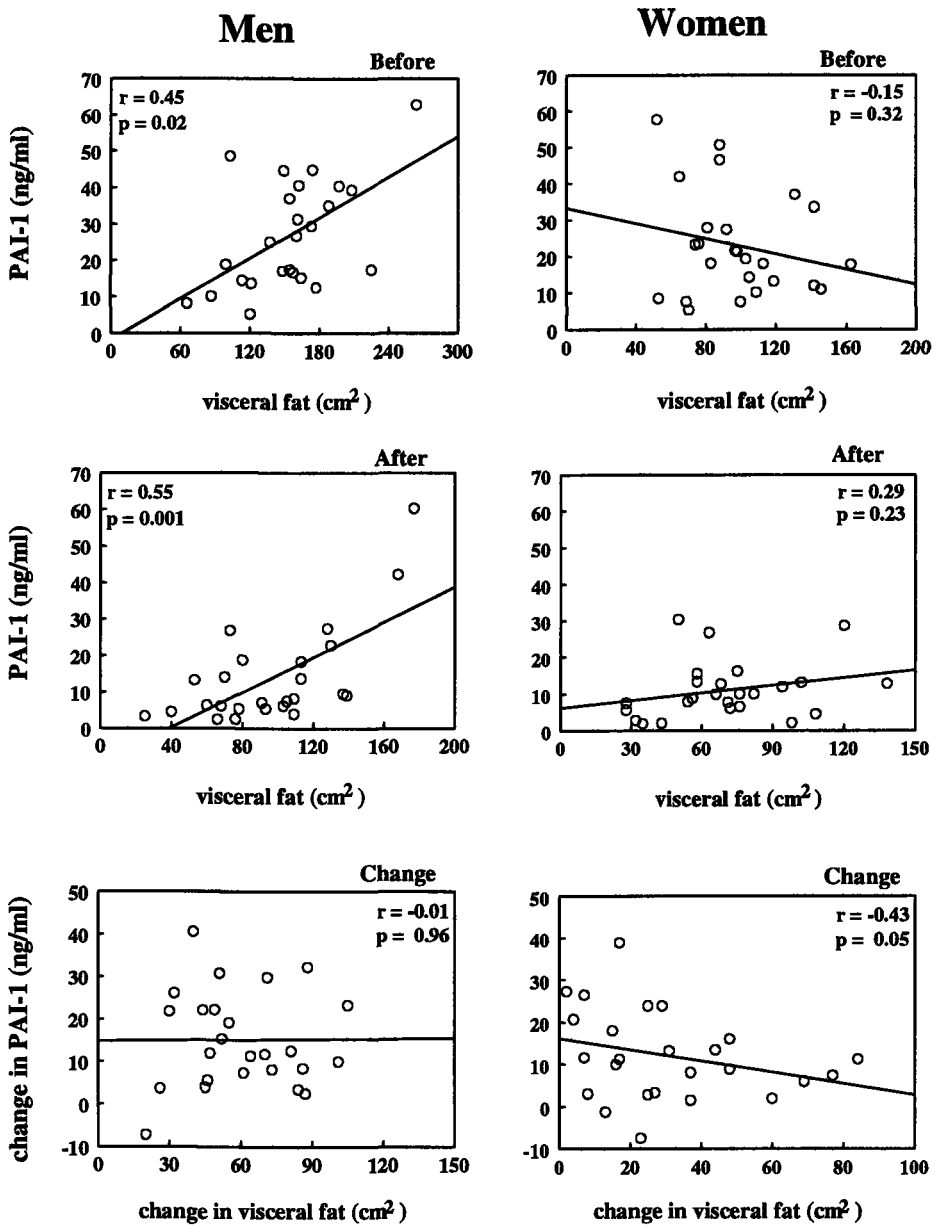


**Figure 1. Scatter plots showing the relationship between PAI-1 antigen level and visceral fat area in obese men (n = 25) and obese women (n = 25), before and after weight loss, and between changes inPAI-1 and visceral fat in response to weight loss.**

Established associations between PAI-1 and variables at baseline generally remained intact after weight reduction, albeit sometimes weaker (Table 3). However, some factors not correlated with PAI-1 at baseline, did so after the diet period. Notably, after weight loss PAI-1 positively correlated with fat mass and insulin in women and with fat mass and triglycerides in men. When comparing baseline and follow-up correlations for t-PA, much less consistency than for PAI-1 was observed. In women, positive correlations between t-PA and fat mass and subcutaneous fat area had disappeared after the diet period, whereas a correlation between t-PA and insulin became significant. In men, the correlation between t-PA and waist-to-hip ratio became insignificant after weight loss, while statistically significant correlations between t-PA and fat mass, triglycerides, and visceral, subcutaneous and total fat areas became apparent.

The associations between PAI-1 and visceral fat area before and after weight loss in men were not markedly affected if adjustments were made for age and total fat mass ($r = 0.55$; $p < 0.05$ before and after weight loss).

Sex-specific correlations between changes in PAI-1 and t-PA on the one hand and subject characteristics on the other are shown in Table 4 and Fig.1. Remarkably, none of the parameters that significantly correlated with PAI-1 in women or men before or after weight loss did so when analyzed for relationships with diet-induced changes in PAI-1 levels. Surprisingly, the diet-induced decrease in PAI-1 levels in women showed a negative correlation with changes in visceral fat area (Table 4 and Fig.1). However, this association was reduced to a non-significant level after adjustment for total fat mass. The strong relationship between PAI-1 and visceral fat area before and after weight reduction in men was totally absent for the diet-induced changes in these two parameters (Fig.1). For t-PA, diet-induced changes correlated negatively with changes in insulin in men. All other relationships as observed for t-PA before and after weight reduction were found to be irrelevant with respect to diet-induced changes in t-PA.

**Multiple linear regression analysis of PAI-1 and t-PA**

Multiple linear regression analysis showed that in women, the individual contribution of visceral fat area to the variability of plasma PAI-1 was low (0.7% and 7% before and after weight loss, respectively), whereas in men this parameter accounted for 28% and 44% of the variation in PAI-1 before and after weight loss, respectively. Changes in visceral fat area could explain 16% of the diet-induced changes in PAI-1 in women, but in men, such a contribution was found to be negligible.

The individual contributions of visceral fat area to the variability in plasma t-PA also were modest (between 6% and 15% in women and between 21% and 24% in men), and of the diet-induced changes in t-PA, no contribution at all of changes in visceral fat area could be found, beit in women or men.

**Table 4: Simple Spearman Correlation Coefficients of Changes in PAI-1 and t-PA with Changes in Subject Characteristics.**

| | PAI-1 antigen | | t-PA antigen | |
|---|---|---|---|---|
| Characteristics | Women | Men | Women | Men |
| Fat mass | 0.04 | - 0.25 | 0.33 | 0.08 |
| Visceral fat area | - 0.43 * | - 0.01 | 0.08 | 0.23 |
| Subcutanous fat area | 0.03 | - 0.08 | - 0.02 | 0.05 |
| Total fat area | - 0.09 | - 0.12 | - 0.01 | 0.09 |
| WHR | 0.06 | - 0.11 | 0.09 | 0.04 |
| Insulin | - 0.15 | - 0.32 | 0.02 | - 0.32 * |
| Triglycerides | 0.09 | 0.32 | - 0.03 | 0.27 |
| Free T | - 0.02 | 0.27 | - 0.15 | 0.23 |

WHR = waist-to-hip ratio, PAI-1 = plasminogen activator inhibitor-1, t-PA = tissue-type plasminogen activator, T = testosterone.* $p \leq 0.05$

## Discussion

Elevated plasma PAI-1 is a frequent finding in obesity, but the molecular basis of this connection is not fully understood. Our study was undertaken to investigate a possible relationship between visceral adipose tissue and plasma PAI-1 concentrations in overweight subjects undergoing weight loss therapy. We found that there is a significant correlation between visceral fat area and PAI-1 levels in obese men, but not in obese women. However, our findings provide no evidence for a significant contribution of visceral fat mass *per se* to plasma PAI-1 levels. Rather, our results suggest that the factors that lead to increased plasma PAI-1 in obesity are complex and gender specific, and may involve interactions between multiple variables.

We have found that in men a positive relationship exists between visceral fat area –as measured by precise MRI– and PAI-1 concentrations in plasma, also after adjustments had

been made for total fat mass and age. In multivariate analysis, the individual contribution of visceral fat accounted for 28% and 44% of the variation in plasma PAI-1 levels in men before and after weight loss, respectively. An association between plasma PAI-1 levels and visceral fat accumulation in men has also been described in previous studies, involving obese1[1,14] as well as non-obese male subjects[13,38,39].

In contrast to the studies in men, and also deviant from three recent cross-sectional studies conducted in obese and non-obese women[11,12,38], we observed no significant link between visceral fat and plasma PAI-1 levels in our experimental group of women. Although an explanation for these discrepancies is not immediately obvious, several points may be of relevance. In the study by Shimomura et al.[11] too, the visceral fat area appeared to be much more significantly related to plasma PAI-1 levels in men than in women. Giltay et al.[38] indeed showed that at baseline, the PAI-1 level in young, nonobese women was correlated significantly with the visceral fat area ($r=0.59$; $p=0.03$), but an even stronger correlation was found with total body fat ($r=0.70$; $p=0.006$). The question remains whether the association between PAI-1 and visceral fat area in this study is still significant if adjustment had been made for the potential confounder, total body fat. Interestingly, after cross-sex hormone administration to these transsexual women (and men), the plasma PAI-1 levels were no longer correlated with visceral fat. An important aspect of the study by Janand-Delenne et al.[12] is the wide range of body mass index (21-49 $kg/m^2$) and visceral adipose tissue (7-336 $cm^2$) of the women evaluated (cf., 23-31 $kg/m^2$ and 52-163 $cm^2$, respectively in our experimental group).

Diet intervention resulted in a significant reduction of plasma PAI-1 levels in both females and males, but no correlation between diet-induced changes in visceral fat area and PAI-1 in men was found. The significant inverse relationship between changes in visceral fat and PAI-1 in women disappeared when adjusted for total fat mass. These findings in women and men are not consistent with the notion that visceral fat accumulation is a major, independent determinant of plasma PAI-1, as reported by several groups[12-14,38,39]. However, much of this evidence is based on crude anthropometric measures such as BMI and waist/hip circumference ratios, and their implications for visceral fat accumulation may not be unequivocal[40]. Also, in many studies that have reported a relationship between fat distribution and PAI-1, no adjustment has been made for potential confounders such as total body fat and age. Therefore, based on the present study and the currently available evidence from literature a direct causal relationship between visceral fat mass *per se* and plasma PAI-1 levels is not proven and even questionable. The available data do not exclude the possibility, however, that the adipocyte, in an interaction between and in response to, systemic and/or local factors is a major site of synthesis of plasma PAI-1. In fact, the possibility that adipose tissue itself may directly contribute to plasma PAI-1 levels has recently gained considerable support. Studies of obese and non-obese rodents and humans demonstrate that adipose tissue is a

major site of PAI-1 mRNA expression, and pieces of adipose tissue in culture release significant amounts of PAI-1 protein, with the expression and secretion of PAI-1 being elevated in obesity[11,15,17,41].

In both animals and humans, omental fat tissue has been found to express more PAI-1 than subcutaneous tissue from the same subject[11,17,41]. The question arises which factors are of importance in increasing adipocyte PAI-1 synthesis. In obesity, both the size and number of adipocytes increase several-fold. Eriksson et al.[41] recently demonstrated that in human adipose tissue PAI-1 secretion is related to the lipid content and cell volume of the fat cells. In addition, specific hormones and/or cytokines known to influence size and number of fat cells and to upregulate PAI-1 gene expression are elevated systemically or locally in obesity[1]. For example, adipocytes are known to express TNFα and TGFβ, and the synthesis of these proteins is upregulated in adipocytes from obese subjects, as demonstrated for TNFα and TGFβ in rodents[15] and for TNFα in humans[42]. Both TNFα and TGFβ stimulate PAI-1 gene expression and induce PAI-1 in plasma and adipose tissue of lean mice[43,44]. Morange et al.[45] recently reported that PAI-1 production by human adipose tissue explants was significantly correlated with that of TNFα. Circulating TNFα is extremely low or undetectable in humans, even in obese patients who showed overexpression of TNFα in adipose tissue ([42]; T. Kooistra et al., unpublished data), suggesting direct action of TNFα in fat cells via an autocrine/paracrine loop.

It has been reported that insulin resistance is a major determinant of plasma PAI-1 levels, and that besides visceral fat accumulation, other elements of this syndrome are associated with elevated plasma PAI-1[46-48]. We found that a weight loss of about 12 kg resulted in a strong decrease in PAI-1 levels and in favourable changes in insulin resistance parameters in both women and men, but correlations between changes in PAI-1 and changes in triglyceride and fasting insulin levels were not significant in either sex. Similar results were reported by Folsom et al.[25] who also found that the reduction in PAI-1 levels was more related to the degree of weight loss than to changes in insulin or triglycerides, indicating that the exact nature of the connection between plasma PAI-1 levels and the cluster of variables defining the insulin resistance syndrome is not established yet.

Plasma PAI-1 and t-PA antigen were strongly correlated in our study, but the reason for the association between PAI-1 and t-PA is not precisely known[19]. It could be that PAI-1 and t-PA synthesis are regulated by the same factors. Like PAI-1, plasma t-PA is related to the variables belonging to the insulin resistance syndrome as shown in this and other studies[19]. Also, t-PA and PAI-1 levels have been found to be influenced by steroid hormones in a comparable manner[21,38,49,50]. Another explanation could be that t-PA antigen levels are mainly a reflection of the formation of t-PA:PAI-1 complexes, which have a delayed clearance compared to free t-PA[51]. t-PA antigen then accumulates in the presence of high plasma PAI-1 concentrations, as seen in obesity and other disease states[6,7]. This interaction between PAI-1

and t-PA further indicates that correlations between visceral fat accumulation and plasma PAI-1 levels should be interpreted with caution, and emphasizes that the mechanisms responsible for the high plasma PAI-1 in obesity are far from being elucidated.

## Acknowledgements

The intervention study was carried out at the Department of Human Nutrition of the Agricultural University in Wageningen, The Netherlands, and partly funded by grants of the Netherlands Heart Foundation (grant 87.067), the Agricultural University and Wander AG / Sandoz Nutrition Ltd, Bern, Switzerland. The authors thank K. van der Kooy, PhD and P. Deurenberg, PhD for their contribution to the organization of the study and A. Droop, RD. and S. Meyboom, RD for expert dietary advice. Medical screening of subjects was done by A.F.X. Giesen, MD and H.G. Martijn, MD. MRI scans were done with the help of C.J.G. Bakker, PhD of the University Hospital in Utrecht, The Netherlands, fibrinolytic parameters were measured by I. Huisveld, PhD of the Department of Physiology of the University of Utrecht, The Netherlands. The authors are also indebted to the participants for their cooperation and the members of the laboratory of the Department of Human Nutrition for help in conducting the study.

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*CHAPTER 7*

# Summay, discussion and future perspectives

## Summary

In **Chapter 1**, fibrinogen (Fbg) and plasminogen activator inhibitor-1 (PAI-1), key components of the coagulation and the plasminogen/plasmin systems, respectively, are introduced. Fbg is a plasma glycoprotein, secreted as a dimer of two Aα-, Bβ- and γ-polypeptides. During coagulation Fbg molecules polymerise into an insoluble fibrin network. Fibrin clots are removed by the plasminogen/plasmin system in which PAI-1 plays an important regulatory role by inhibiting the activity of tissue-type plasminogen activator (t-PA), a pivotal enzyme in the onset of the fibrinolytic process by converting plasminogen into plasmin. Both Fbg and PAI-1 are synthesized in liver hepatocytes. Being key factors of two systems involved in many (patho-) physiological processes, it is of relevance to understand how Fbg and PAI-1 gene expression are regulated. Because in this thesis emphasis has been on delineating the regulatory mechanisms by which a specific class of hypolipidaemic drugs, fibrates, modulate Fbg and PAI-1 expression, possible modes of action of fibrates are summarized. Particular attention is paid to the nuclear hormone receptor, peroxisome proliferator-activated receptor-α (PPARα), as a mediator of fibrate action in liver hepatocytes.

In **Chapter 2**, we sought to learn more about the variability in reported results of fibrates on plasma Fbg and PAI-1 levels. Therefore, the effects of two fibrates, gemfibrozil and ciprofibrate, on plasma levels of Fbg and PAI-1 were examined in primary hyperlipidaemic patients after six and twelve weeks of treatment, using different assay systems for Fbg and PAI-1. We found that although both fibrates effectively lowered triglyceride and cholesterol levels, no effect on the elevated PAI-1 levels was observed by fibrate treatment. With regard to Fbg, different effects were seen using different fibrates, assays or treatment periods. Fbg antigen levels were increased with gemfibrozil after six and twelve weeks of treatment, whereas there was no effect of ciprofibrate. Using a Clauss functional assay with either a mechanical end point or a turbidity-based end point, no significant change in Fbg levels was seen after six weeks of gemfibrozil treatment. However, after twelve weeks, gemfibrozil enhanced functional Fbg levels as assessed by the Clauss mechanical assay, but decreased Fbg levels when a Clauss assay based on turbidity was used. Remarkably, the functionality of Fbg (assessed by the ratio of functional Fbg to Fbg antigen) was found to be decreased by both fibrates. These results indicate that the diversity and variability in effects of fibrates on haemostatic variables may be due at least partly to intrinsic differences between the various fibrates, but in addition to different outcomes of the various assays used and differences in treatment periods.

The effects of fibrates on PAI-1 synthesis and the molecular mechanism(s) involved were further studied in primary cultures of cynomolgus monkey hepatocytes **(Chapter 3).** In the monkey hepatocytes fibrates attenuated the observed accelerated increase in PAI-1 synthesis occurring under basal culture conditions. Different fibrates were found to lower PAI-1

synthesis with different efficacy. A concomitant lowering effect on PAI-1 mRNA levels was observed, and nuclear run-on assays showed that fibrates suppress PAI-1 at the transcriptional level. This action of fibrates was shown not to involve protein kinase C (PKC) activation, as specific activation or inhibition of PKC did not interfere with the accelerated increase in PAI-1 synthesis during culture. Also, clofibric acid, which did not activate PKC, was as effective as gemfibrozil in suppressing PAI-1 production. We could also exclude an effect of fibrates on the signal transduction pathways activated by growth factors like epidermal growth factor (EGF) and transforming growth factor-β (TGF-β). We found that EGF and TGF-β did induce PAI-1 mRNA levels in simian hepatocytes, but this action could not be prevented by fibrates. As fibrates are known to activate PPARα, a possible role of this nuclear receptor was investigated too. We found that PPARα and its heterodimerization partner RXRα are both expressed in cultured cynomolgus monkey hepatocytes, indicating that basal conditions necessary for an involvement of PPARα/RXRα heterodimer are present. Interestingly, the specific ligand for RXRα, 9-*cis* retinoic acid, also suppressed PAI-1 production, pointing to a possible involvement of the PPARα/RXRα heterodimer.

A possible role of PPARα/RXRα in mediating the effects of fibrates on PAI-1 expression was further evaluated in a second study using primary monkey hepatocytes, which investigation is described in **Chapter 4.** In addition, the effects of fibrates on Fbg expression and a role of PPARα herein were determined. The approach chosen was to compare the effects of various fibrates and PPARα-activating compounds on Fbg and PAI-1 synthesis with their PPARα-activating capacity in order to establish a possible relationship. As a measure of PPARα activation the induction of the acyl-CoA oxidase (ACO), whose expression is known to be dependent on PPARα activation, was chosen. Because ACO mRNA levels were only slightly induced by fibrate treatment of our monkey hepatocyte cultures, we determined PPARα-activation in a more sensitive PPARα-dependent gene reporter system consisting of stably-transfected Chinese hamster ovary cells, containing a reporter gene under control of several PPAR responsive elements. When comparing the effects of six different fibrates, 9-*cis* retinoic acid and the specific PPARα-activators Wy14,653 and 5,8,11,14-eicosatetraynoic acid on the synthesis of Fbg and PAI-1 with their PPARα-activating capacity as determined in the *in vitro* reporter gene system, the potency of the compounds to activate PPARα and to induce Fbg were found to be strongly correlated, whereas no correlation between the PPARα activating potency of the compounds and their effects on PAI-1 synthesis was observed. Apo A-I, which was included as a control, correlated moderately with PPARα transactivating activity. These results suggest that fibrates can alter PAI-1 expression independently of PPARα activation. Interestingly, they strongly point to a role of PPARα in the fibrate-mediated effects on Fbg.

Because the conclusions drawn from the association analysis are only inferential, we further investigated the involvement of PPARα in the effects of fibrates on Fbg regulation in

**Chapter 5**. In adult male rats, fibrates dose-dependently decreased hepatic Fbg Aα-, Bβ and γ-chain mRNA levels. In parallel, plasma Fbg concentrations were decreased. The fibrate-induced decrease of Fbg expression in rats is regulated at the transcriptional level, as shown by nuclear run-on analysis, and is accompanied by a concomitant increase in ACO mRNA level and gene transcription, indicating PPARα activation. Using PPARα-deficient (-/-) mice a direct role of PPARα in the regulation of Fbg expression by fibrate was established; upon treatment with fibrate, a significant decrease in plasma Fbg levels and hepatic Fbg gene expression is observed in wild-type (+/+) mice, but not in (-/-) mice. Compared with (+/+) mice, basal plasma Fbg levels in (-/-) mice were significantly higher. These studies demonstrate that PPARα regulates basal levels of plasma Fbg and establish that fibrate-suppressed expression of Fbg in rodents is mediated through PPARα.

Recent studies suggests that next to liver, adipose tissue, especially the visceral fat area, may be an important source of plasma PAI-1. In **Chapter 6** the contribution of the amount of visceral fat area to plasma PAI-1 levels in obese men and women was investigated. We found a relationship between visceral fat area and plasma PAI-1 levels in moderately obese men. In contrast, in women no such relation was observed. Weight loss therapy for 13 weeks resulted in a significant decrease in plasma PAI-1 levels in both men and women. These changes in PAI-1 were, however, not related to changes in visceral fat area *per se*, indicating that variables other than just the amount of fat tissue are important for determining PAI-1 concentrations in the circulation.

In **Chapter 7**, the results of the studies are summarized and discussed, and suggestions for further research are made.

## Discussion and future perspectives

In this thesis, a role of PPARα in mediating basal as well as fibrate-suppressed expression of Fbg was established. In contrast, our studies are not indicative for an involvement of PPARα in modulation of PAI-1 expression by fibrates in hepatocytes, indicating that at least in this cell type, fibrates must affect PAI-1 synthesis independently from PPARα activation.

Two lines of evidence point to a key role of PPARα in regulating basal and fibrate-suppressed Fbg gene expression. First, in cultures of cynomolgus monkey hepatocytes a strong association exists between the effects of various compounds, including fibrates, on Fbg expression and their PPARα transactivation capacity. Second, in PPARα-deficient mice basal Fbg levels are increased as compared to wild-type mice; furthermore, no effect of fibrates on Fbg levels in PPARα- deficient mice was found, whereas in wild-type mice fibrates decreased Fbg levels. The differing effects of different fibrates on modulating plasma Fbg levels in humans could therefore be related to a differing potency to activate PPARα. This contention does not explain however, why, using one type of Fbg assay, an increasing effect of gemfibrozil on plasma Fbg levels was found, whereas ciprofibrate lowered plasma Fbg levels (Chapter 2). A complicating factor in the interpretation of such findings is that fibrates also mobilize free fatty acids, which are known activators of PPARα[1-3]. Several investigators actually have suggested that fibrates affect Fbg expression via modulation of free fatty acid metabolism[4,5]. Considering the effect of fibrates on fatty acid metabolism and the involvement of both fibrates and fatty acids on PPARα activation, the net effect of fibrates on plasma Fbg levels *in vivo* would be the resultant of these processes. Thus, besides differences in PPARα activating potency, changes in fatty acid metabolism may contribute to the variability in results observed for different fibrates with respect to changes in plasma Fbg levels.

Our studies show that intrinsic differences between fibrates, differences in patient groups and the usage of different assay systems can contribute to variability in effects of fibrates on plasma Fbg levels. The finding that different assays systems can yield different Fbg values has also been observed by others[6-10]. In addition to different assay principles by which Fbg levels can be determined, the use of different types of instruments and reagents have been found to influence the outcome of Fbg measurements[8-13]. To what extent these differences in outcome as a result of different methodology is related to the variability in reported effects of fibrates has not been looked into before. Our results strongly suggest that this is an important factor to consider in the explanation of the diversity in effects reported on fibrates.

We observed large differences in the effects of fibrates on Fbg levels using a functional Fbg assay versus an assay measuring Fbg antigen. It has been suggested that the variation between immunological and functional assays is related to the different molecular forms of Fbg present in plasma[14]. As the different forms exhibit different clotting behaviour[15,16], changes in the functionality of Fbg could result from an altered distribution of these forms.

However, this explanation is unlikely to be true for the observed differences in functional versus antigen Fbg levels after fibrate treatment in our study, since comparison of the molecular weight distribution of Fbg before and after fibrate treatment of the hyperlipidaemic patients did not reveal any difference in distribution pattern. An interesting alternative explanation would be that fibrates (possibly via PPARα) induce a factor that affects the functionality of the Fbg molecules without influencing the outcome of the Fbg antigen measurements.

Our findings also raise questions about the use of the different Fbg assays in the risk assessment of cardiovascular disease. It is not clear, which molecular form of Fbg is the most "dangerous" one and is related to the highest risk for cardiovascular disease. The most functional (i.e. the best clottable), high molecular weight form of Fbg has been found to make clots better lysable than clots made of a lesser clottable, low molecular weight form of Fbg[17,18]. It should be noted that in our study functionality of Fbg (assessed by the ratio of functional Fbg to Fbg antigen) was found to be decreased by both fibrates. Others have also suggested that the ratio of functional to antigen might be a of more relevance in the prediction and treatment of cardiovascular disease than measurement of either functional or Fbg antigen levels[14,19]. Comparative studies directed at studying the risk of the different forms are clearly necessary to answer the question which form is a better indicator and predictor of cardiovascular risk, and will eventually establish which Fbg assay is the best to use in risk assessment.

The exact mechanism by which PPARα downregulates Fbg expression is not known yet, but could occur in several ways, for each of which there is a precedent. Firstly, fibrates could suppress the occurrence of essential transcription factors necessary for Fbg transcription. For example, Hertz et al[20] showed that fibrates repress transcription of the apolipoprotein (apo) CIII gene by down-regulating the expression of the strong positive transcription factor, hepatocyte nuclear factor-4 (HNF-4). Secondly, fibrates could induce factors that suppress Fbg transcription directly. Such a mechanism has been observed in the suppression of apo A-I expression by fibrates in rodents, where the negative regulation of apo A-I expression by fibrates was shown to involve a PPARα-dependent induction of the nuclear factor Rev-erbα, which after binding to its responsive element suppresses transcription of the apo A-I gene[21]. Thirdly, PPARα may compete with other factors positively influencing Fbg transcription, as is the case in the apo CIII promoter were HNF-4 is displaced from the promoter by non-productive PPAR/RXR heterodimers[20]. Fourthly, as Fbg is an acute phase protein induced by IL-6, fibrates may interfere with the IL-6 induction of Fbg, by decreasing IL-6 levels or by interfering with IL-6 signalling. The latter hypothesis is supported by recent studies showing that activated PPARα can interfere negatively with IL-6-induced signalling pathways[22,23].

The stimulating effects of fibrates on Fbg expression observed in the monkey hepatocytes (Chapter 4) is in contrast with the Fbg lowering effects of most fibrates observed *in vivo* in

humans (Table 3 of the general introduction) and with our *in vivo* studies in rodents (Chapter 5). The reason for this discrepancy is unknown, but is unlikely to be related to *in vitro* versus *in vivo* conditions: we found a decrease of Fbg levels after fibrate treatment in primary rat hepatocytes (Chapter 5), which were cultured under similar conditions as the primary monkey hepatocytes. In addition, a fibrate-mediated suppression of Fbg levels was also found in cultures of the human hepatoma cell line HepG2 by Binsack et al.[24] Therefore, the stimulatory effect of fibrates on Fbg levels in the monkey hepatocytes versus the suppressive effect found in human and rodent hepatocytes is more likely to represent a species-specific effect. A species-specific difference in the effects of fibrates was also observed for apo A-I expression in humans versus rats. Studies of Vu-Dac et al[21] showed that this difference was related to differences in promoter context of a positive and a negative regulatory element. In humans, apo A-I is induced by activation of the PPAR$\alpha$/RXR$\alpha$ heterodimer bound to a PPRE, whereas a negative element is nonfunctional. In rats, apo A-I is downregulated by a PPAR$\alpha$-mediated increase of the negative regulatory protein, Rev-erb$\alpha$. The PPRE in rats was shown to be nonfunctional due to differences in the nucleotide sequence of this responsive element. The opposite effect of fibrates on Fbg levels in human/rodent versus monkey hepatocytes could also be due to differences in promoter context of the elements involved in fibrate-modulation of Fbg expression in these species. Another explanation may involve additional factors necessary for PPAR$\alpha$-mediated transcription. A number of co-activators or repressors that interact with PPAR/RXR heterodimers have been identified[25,26]. Differences in the presence of such factors may explain why Fbg expression is downregulated in human and rodent hepatocytes and upregulated in monkey hepatocytes.

The studies performed in this thesis do not provide an answer to the question by which mechanism(s) fibrates modulate PAI-1 expression in hepatocytes. Besides the observation that PAI-1 expression is altered independently from PPAR$\alpha$ activation, our results indicate that fibrates do not affect PAI-1 expression by interference with protein kinase C, growth factor signalling pathways or by lowering triglyceride levels. In the cynomolgus monkey hepatocytes fibrates appeared to attenuate the accelerated increase in PAI-1 synthesis occurring under basal culture conditions. This is in agreement with the *in vivo* situation in which fibrates only lower elevated PAI-1 levels in patients[27]. Comparison of the different clinical studies show that fibrates influence PAI-1 levels in type IV and type V (according to the Fredrickson classification) and in patients with previous myocardial infarction,[28-31] but not in type IIa or type IIb patients (Chapter 2 and [32,33]). This observation might be related to differences in the underlying causes of elevation of plasma PAI-1 levels in these patient groups. The mechanism(s) responsible for elevation of plasma PAI-1 in some patient groups is not clear. It has been suggested that fatty acids derived from VLDL-triglycerides are the mediators of elevation of plasma PAI-1 levels[34]. *In vivo* studies consistently have

demonstrated a strong positive correlation between the plasma VLDL-triglyceride and PAI-1 activity levels[35-37]. *In vitro* studies have shown that VLDL induces PAI-1 secretion in cultured endothelial cells and HepG2 cells[38-41]. Fibrates are known to affect fatty acid metabolism and as a result might decrease PAI-1 synthesis only in patients with high levels of PAI-1-inducing fatty acids. On the other hand, PAI-1 is known to behave as an acute phase reactant and to be induced by TNFα and IL-1. Therefore, elevation of PAI-1 could reflect an inflammatory response. Fibrates may, like for Fbg, lower PAI-1 by suppressing these inflammatory responses. In the case, in which both Fbg and PAI-1 are increased as a result of inflammatory reactions, activation of PPARα by fibrates may downregulate plasma Fbg as well as PAI-1 levels. It should be realized, however, that PPARα is mainly expressed in liver and to some extent in smooth muscle cells, whereas PAI-1 may originate from many cell types *in vivo*. Inasmuch, activation of PPARα by fibrates has a significant impact on overall PAI-1 synthesis *in vivo* under inflammatory conditions is not clear.

Recently, a role fat tissue, especially of the visceral fat area, in determining plasma PAI-1 levels was suggested[42-45]. We observed that there is no evidence for a significant contribution of visceral fat mass *per se* to plasma PAI-1 levels in obese men and women. However, *in vitro* studies demonstrated PAI-1 production in explants of adipose tissue[45,44]. Eriksson et al[44] suggested that it is the quality rather than the quantity that is important for the level of adipocyte synthesis of PAI-1. Their studies show that PAI-1 secretion is related to the lipid content and volume of the fat cells. Our finding that factors other than the amount of visceral fat area are important in determining circulating PAI-1 levels support this view. It is very well possible that the adipocyte, in an interaction between and in response to, systemic (e.g. insulin, triglycerides, cytokines) and/or local factors (e.g. TNFα) is a major site of plasma PAI-1. How these factors all link together is far from being elucidated but provides interesting and exciting research for the future eventually establishing the relationship between plasma PAI-1 and fat tissue.

## Future perspectives

In this thesis we have identified PPARα as a key regulatory component in the expression of basal Fbg levels. It is conceivable that PPARα also plays an important role in down-regulating increased Fbg gene expression under inflammatory conditions. In line with this are reports showing that other acute phase proteins are also regulated by fibrates and PPARα. In a report of Staels et al[22] treatment of hyperlipidaemic or healthy subjects with fenofibrate decreased the plasma concentration of IL-6, as well as the acute phase proteins, Fbg and C-reactive protein (CRP). Using PPARα-deficient mice, PPARα was shown to be involved in the expression of the acute phase protein α2 urinary globulin[46,47]. To obtain insight into the (patho-)physiological processes in which PPARα is involved and which could be modulated

by activation of this receptor, it is of great interest to study whether PPAR α can interfere with acute phase induced Fbg expression. In addition, it is important to resolve the exact mechanism by which PPARα suppresses Fbg expression during basal condition. Therefore, transfection experiments directed at the functional analysis of the regulatory regions of the genes encoding for the Fbg chains, will be necessary to elucidate this precise mechanism of transcriptional repression of Fbg gene expression by PPARα.

Resolving the mechanism(s) by which fibrates affect plasma PAI-1 levels, may help explaining why only in some patient groups PAI-1 levels are affected. It is therefore important the study the mechanism of elevation of circulating PAI-1 levels. It would be worthwhile to investigate whether PAI-1 in patients is elevated as a result of ongoing inflammatory processes and whether PAI-1 can be modulated by fibrates through PPARα activation during inflammation. The other PPAR isotype, PPARγ has also been reported to interfere with cytokine signalling.[23] As fat tissue is a major determinant of plasma PAI-1 and the major tissue expression PPARγ, it is interesting to investigate a role of this receptor in basal as well as cytokine mediated adipose PAI-1 expression.

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## Samenvatting voor de geïnteresseerde leek

De vorming van bloedstolsels (stolling) en het oplossen daarvan (fibrinolyse) spelen een belangrijke rol bij tal van processen in het lichaam, zoals bijvoorbeeld wondheling. Bij een teveel aan stolling of een verminderde fibrinolyse bestaat er een kans op trombose, een belangrijke oorzaak van hart- en vaatziekten, terwijl een slechte stolling of een versterkte fibrinolyse kan leiden tot bloedingen. Het zal daarom duidelijk zijn dat het belangrijk is dat de vorming van bloedstolsels en het oplossen ervan goed geregeld moet zijn in het lichaam.

Een stolsel ontstaat na een opeenvolging van reacties. In de laatste reactie wordt fibrinogeen, een eiwit dat in oplosbare vorm circuleert in de bloedbaan, omgezet in het onoplosbare fibrine. Het fibrine-netwerk vormt de basis van een stolsel. Als het stolsel zijn dienst heeft gedaan, moet het worden afgebroken. Dit gebeurt door het enzym plasmine, dat het fibrine netwerk in stukjes knipt. Plasmine ontstaat door activering van plasminogeen door plasminogeen-activatoren. Dit proces wordt geremd door plasminogeen-activator remmers waarvan plasminogeen-activator inhibitor-1 (PAI-1) de belangrijkste is. Een te veel aan PAI-1 betekent een minder snelle fibrinolyse, en daarom een belemmering voor het oplossen van stolsels.

Dit proefschrift richt zich op twee belangrijke componenten uit de stolling en de fibrinolyse, namelijk fibrinogeen en PAI-1. Verschillende patiënten-studies hebben laten zien dat zowel fibrinogeen als PAI-1 concentraties in het bloed beïnvloed kunnen worden door bepaalde medicijnen, de zogenaamde fibraten. Fibraten zijn geneesmiddelen die verhoogde concentraties aan vetten en cholesterol in het bloed effectief kunnen verlagen. Sinds een aantal jaren is bekend dat ze ook de concentraties van stollings- en fibrinolyse-factoren in het bloed (waaronder fibrinogeen en PAI-1) kunnen beïnvloeden, maar niet bekend is hoe fibraten dat doen. Ook is het opvallend dat er veel variatie in de beschreven effecten van fibraten op fibrinogeen en PAI-1 bestaat. Een overzicht van de in de literatuur beschreven effecten van fibraten op fibrinogeen en PAI-1 is gegeven in **hoofdstuk 1.** Daarnaast bevat dit hoofdstuk een samenvatting van wat bekend is over de mechanismen via welke de aanmaak van fibrinogeen en PAI-1 is geregeld en hoe fibraten deze regelmechanismen mogelijk kunnen beïnvloeden.

In **hoofdstuk 2** van dit proefschrift is getracht meer inzicht te krijgen in de oorzaken van de variabele effecten van fibraten op fibrinogeen en PAI-1. Onze resultaten laten zien dat in elk geval een deel van de variatie in fibrinogeen veranderingen o.i.v. fibraten verklaard kan worden door verschillende uitkomsten van verschillende meetmethoden, de behandelingsduur van fibraat-toediening, maar ook doordat verschillende fibraten een verschillende werking kunnen hebben. Zo werd bij het gebruik van één meetmethode om fibrinogeen te meten in onze patiënten met verhoogd cholesterol de fibrinogeen concentratie verlaagd door het ene

fibraat (ciprofibraat), terwijl een ander fibraat (gemfibrozil) de fibrinogeen concentraties in het bloed verhoogde. In dezelfde patiënten werd bij het gebruik van één methode een verlaging van de fibrinogeen concentratie in het bloed door ciprofibraat gevonden, terwijl bij het gebruik van een tweede methode om fibrinogeen te meten er geen effect van ciprofibraat op de fibrinogeen concentratie geconstateerd werd. Dat verschillende behandelperioden ook kunnen bijdragen aan de variatie, bleek uit het feit dat na 6 weken behandeling er nog geen effect van gemfibrozil op de fibrinogeen concentratie werd gezien, terwijl na 12 weken de fibrinogeen concentratie duidelijk verhoogd was. In deze studie bleek uit metingen van de PAI-1 concentraties in het bloed, dat noch gemfibrozil noch ciprofibraat een effect op PAI-1 hadden, ook niet als verschillende meetmethoden werden gebruikt. Dit geeft aan dat door anderen gevonden effecten van fibraten (inclusief gemfibrozil en ciprofibraat) waarschijnlijk niet het gevolg zijn van een direkt effect van fibraten op de aanmaak van PAI-1. Mogelijk wordt de PAI-1 aanmaak op een indirekte manier door fibraten beïnvloed.

Verder onderzoek was er vooral op gericht uit te zoeken hoe, d.w.z. via welk mechanisme, fibraten de aanmaak van PAI-1 en fibrinogeen beïnvloeden. In eerste instantie is dit werk uitgevoerd m.b.v. gekweekte levercellen, die zowel PAI-1 als fibrinogeen produceren. Later zijn de belangrijkste bevindingen nader onderzocht in proefdieren. Bij het ophelderen van de betrokken regelmechanismen is uitgegaan van bestaande kennis m.b.t. processen die van invloed kunnen zijn op het meer of minder produceren van PAI-1 en fibrinogeen in levercellen alsook van in de literatuur gesuggereerde werkingsmechanismen van fibraten.

In **hoofdstuk 3** is het onderzoek beschreven naar het effect van fibraten op een aantal bekende regelmechanismen voor de aanmaak van PAI-1. Hierbij is gebruik gemaakt van levercellen van een aap (*Macaca fascicularis*) die in zogenaamde celcultures in bakjes gekweekt kunnen worden. Onze studies laten zien dat fibraten de aanmaak van PAI-1 in deze cellen verlagen. Opvallend hierbij was dat alleen de versnelde aanmaak van PAI-1 die tijdens het kweken optrad, beïnvloed werd. De constante (basale) hoeveelheid PAI-1 die door deze cellen gemaakt wordt, werd niet beïnvloed in aanwezigheid van fibraat. Dit komt overeenkomt met de bevindingen in patienten, waar fibraten alleen verhoogde PAI-1 concentraties in het bloed beïnvloeden. Van drie onderzochte regelmechanismen betrokken bij de aanmaak van PAI-1 bleek er geen betrokken te zijn bij de verlaging van PAI-1 door fibraten in de ape-levercellen.

Hierna is gekozen voor een andere benadering om meer te weten te komen over het mechanisme via welk fibraten PAI-1 beïnvloeden. Recent is er namelijk een verklaring gevonden hoe fibraten de productie van verschillende eiwitten kunnen beïnvloeden. Hierbij beïnvloeden fibraten direct een factor die van belang is bij het vertalen van genetische informatie naar een eiwit. De informatie voor het maken van een eiwit ligt opgeslagen in een stukje erfelijk materiaal (het DNA) dat zich in de kern van een cel bevindt. Voor het omzetten

van een DNA-code in een eiwit wordt de code eerst omgezet in een tussen-code: het mRNA. Het omzetten van de DNA-informatie naar de mRNA-informatie heet transcriptie. De mRNA-code wordt vervolgens vertaald in een eiwit. Bekend is nu dat fibraten een factor activeren die de transcriptie kan beïnvloeden. Deze transcriptiefactor wordt de peroxisoom proliferator-geactiveerde receptor (PPAR) genoemd. PPAR behoort tot de familie van de hormoon-receptoren. Dit zijn eiwitten in de kern van een cel en die actief worden na binding van een hormoon of, zoals in het geval van PPAR, een fibraat. PPARs kunnen aan specifieke delen van het DNA binden en zo via transcriptie de aanmaak van eiwitten beïnvloeden. Er bestaan verschillende PPAR subtypen, waarvan het α-type voornamelijk in de lever voorkomt en door fibraten geactiveerd wordt.

In **hoofdstuk 4** is een mogelijke rol van PPARα in de verlaging van PAI-1 door fibraten in levercellen van de aap bestudeerd. Gelijktijdig is naar het effect van fibraten op Fbg en de rol van PPARα daarin gekeken. Na blootstelling aan fibraten bleek de fibrinogeen-productie in de ape-levercellen verhoogd te worden. De volgende stap was om te zien of de effecten van fibraten op fibrinogeen en PAI-1 in de ape-levercellen in verband stonden met de mate waarin PPARα werd geactiveerd. Omdat het niet mogelijk is PPARα-activiteit direct te meten in levercellen van de mens of aap, is gebruik gemaakt van een losstaand model-systeem waarin het wel mogelijk is om PPARα-activiteit te meten. Wanneer de effecten van verschillende verbindingen, waaronder fibraten, op de fibrinogeen en PAI-1 productie in ape-cellen werden vergeleken met hun PPARα-activerende capaciteit, werd er een sterk verband gevonden tussen de mate waarin deze verbindingen fibrinogeen-aanmaak stimuleren en de mate waarin zij PPARα activeren. Voor PAI-1 bleek er nauwelijks zo'n verband te bestaan, hetgeen suggereert dat fibraten in ape-levercellen PAI-1-synthese onafhankelijk van PPARα-activatie beïnvloeden. Daarnaast duiden de resultaten wel op een betrokkenheid van PPARα in de effecten van fibraten op fibrinogeen-productie.

Om te bepalen of PPARα inderdaad een rol speelt, in de beïnvloeding van fibrinogeen productie door fibraten, zijn de de effecten van fibraten op fibrinogeen-productie verder onderzocht in proefdieren (**hoofdstuk 5**). In eerste instante werd het effect van fibraten op de fibrinogeen-productie in mannelijke ratten bepaald. De experimenten lieten zien dat in ratten de fibrinogeen concentraties in het bloed verlaagd werden na fibraat-behandeling. Deze effecten waren ook op mRNA en transcriptie-niveau te zien, wat aangeeft dat fibraten plasma fibrinogeen concentraties verlagen door de transcriptie te remmen. De effecten werden alleen gevonden met PPARα activerende stoffen. De verlaging van de fibrinogeen-productie ging gepaard met een gelijktijdige verhoging van een eiwit waarvan al eerder was aangetoond dat het specifiek door PPARα beïnvloed wordt. Om direct te kunnen bewijzen dat PPARα betrokken is bij de regulatie van fibrinogeen is gebruik gemaakt van PPARα-deficiënte muizen. Dit zijn muizen die geen PPARα bezitten waardoor ze PPARα afhankelijke reacties niet vertonen. Onze studies laten zien dat fibraten in normale muizen (die wel PPARα

bezitten) de fibrinogeen-productie verlagen, terwijl in de PPARα-deficiënte muizen geen invloed van fibraten op fibrinogeen te zien was. Deze resultaten tonen duidelijk aan dat PPARα betrokken is in de effecten van fibraten op fibrinogeen. Daarnaast bleek ook dat de aanwezige hoeveelheid fibrinogeen in het bloed van de niet-met-fibraat behandelde PPARα-deficiënte muizen hoger ligt dan die van onbehandelde normale muizen, wat aangeeft dat PPARα tevens betrokken is bij de basale aanmaak van fibrinogeen.

Recente studies geven aan dat naast de lever ook het vetweefsel en met name het viscerale vet (het vet dat in de buikholte ligt) PAI-1 produceert. In **hoofdstuk 6** is de mogelijke bijdrage van het viscerale vet aan PAI-1 concentraties in het bloed van gematigd obese (zwaarlijvige) mannen en vrouwen onderzocht. In obese mannen werd een verband gevonden tussen de hoeveelheid visceraal vet en de PAI-1 concentraties in het bloed. Naarmate mannen meer visceraal vet hadden, nam de PAI-1 concentraties in het bloed toe. Er werd echter geen verband gevonden tussen de hoeveelheid visceraal vet en PAI-1 in obese vrouwen. Gewichtsverlies door een dieet ging gepaard met een sterke daling in PAI-1 bij zowel mannen als vrouwen. Deze verandering in PAI-1 was echter niet gerelateerd aan de veranderingen in de hoeveelheid visceraal vet. Dit suggereert dat er geen directe relatie is tussen de PAI-1 concentratie in het bloed en de hoeveelheid visceraal vet. Omdat studies van anderen hebben aangetoond dat het viscerale vet wel degelijk betrokken is bij de productie van PAI-1in obese mensen, betekenen onze resultaten dat het niet de kwantiteit van het vet is, maar de kwaliteit die een rol speelt. Waarschijnlijk zijn er andere factoren in het vet van belang voor het bepalen van de hoeveelheid PAI-1 in het bloed van obese mensen.

In **hoofdstuk 7** worden de resultaten uit de verschillende studies bediscussieerd en toekomstige punten voor vervolg-onderzoek aangegeven.

## Abbrevations

| | |
|---|---|
| ACO | acyl-CoA oxidase |
| AP | activator protein |
| C/EBP | CAAT/binding protein |
| DMEM | Dulbecco's modified Eagle's medium |
| EGF | epidermal growth factor |
| EIA | enzyme immune assay |
| FCS | fetal calf serum |
| Fbg | fibrinogen |
| HDL | high-density lipoprotein |
| HNF | hepatic nuclear factor |
| IL | interleukin |
| kb | kilo basepair(s) |
| LDL | low-density lipoprotein |
| MMP | matrix metalloproteinases |
| MRI | magnetic resonance imaging |
| PA | plasminogen activator |
| PAI | plasminogen activator inhibitor |
| PBS | phosphate buffered saline |
| PKC | protein kinase C |
| PMA | phorbol myristate acetate |
| PPAR | peroxisome proliferator activated-receptor |
| PPRE | peroxisome proliferator-response element |
| RID | radial immunodiffussion |
| RXR | retinoid X receptor |
| TGF | transforming growth factor |
| TNF | tumor necrosis factor |
| STAT | signal transducer and activator protein |
| TRE | PMA response element |
| t-PA | tissue-type plasminogen activator |
| u-PA | urokinase-type plasminogen activator |
| USF | upstream stimulatory factor |
| VLDL | very-low density lipoprotein |
| VLDLRE | VLDL response element |
| WE | Williams E medium |

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**Curriculum Vitae**

Maaike Kockx werd op 3 maart 1969 geboren in Hengelo, Overijssel. In 1988 behaalde zij haar VWO diploma op het Lyceum de Grundel te Hengelo. Dat zelfde jaar begon zij aan de studie biologie aan de Universiteit van Utrecht. Al snel raakte zij geinterresseerd in het medisch moleculaire onderzoek. Haar eerste stage verrichte zij gedurende negen maanden aan de vakgroep Moleculaire Celbiologie onder leiding van Prof. Dr. H.O. Voorma waar zij de expressie van heat shock proteines bestudeerde. Haar tweede stage doorliep zij aan de vakgroep Molecular Biology of the University of Massachusetts Medical Center in Worchester, Massachusetts, USA onder leiding van Prof. G. Stein, waar zij gedurende negen maanden de invloed van proliferatie remming in primaire en kanker botcellen vergeleek. Op 25 april 1994 behaalde zij haar doctorandus titel. Van mei 1994 tot november 1998 was zij als OIO werkzaam op een door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek gesubsidieerd project aan het Gaubius Laboratorium TNO-PG te Leiden. Gedurende deze periode werd onder begeleiding van Dr. T. Kooistra, Dr. H.M.G. Princen en Prof. P. Brakman het onderzoek verricht dat staat beschreven in dit proefschrift.

## Dankwoord

Vijf belangrijke jaren zijn voorbij. Een periode waar ik met veel plezier op terug kijk. Niet alleen omdat het uiteindelijk heeft geleid tot de totstandkoming van dit boekje, maar vooral ook om de vele leuke dingen er direct of indirect mee gepaard gingen. Graag grijp ik nu de kans om in mijn eigen woorden de mensen die er kortere of langere tijd zijn bij betrokken zijn geweest, te bedanken.

Natuurlijk allereerst jullie pap en mam aan wie ik dit boekje heb opgedragen. Ik weet dat er momenten waren waarin jullie dachten dat ik nog eerder m'n nek zou breken dan promoveren, maar het merendeel van de tijd hebben jullie in toch in mij geloofd. Wat er ook gebeurde, het was voor jullie altijd even belangrijk als voor mij. Mede dankzij jullie steun (pap, zonder jouw auto had het nog drie maanden langer geduurd!), hulp en medeleven is dit boekje er gekomen.

Linda: geweldig hoe je al die reisjes steeds maar weer voor me wist te regelen.

Teake: m'n hersenen kraken nog van alle "denklessen", waarin je probeerde mij enig inzicht bij te brengen. Dank voor al je geduld. Fijn dat je deur altijd openstond en ik met al m'n vragen "even" bij je langs kon. Zonder jouw hulp bij het interpreteren van de data en het bepalen van de volgende stap van het onderzoek was dit boekje er nooit gekomen. Ik ben er inmiddels van overtuigd dat geen spellingscheck uit de computer op kan tegen jouw nauwgezetheid in het gebruik van de Nederlandse of Engelse taal.

Hans: je was dan misschien wat minder direct bij het onderzoek betrokken, de bijdrage was er niet veel minder om. Het was erg prettig af en toe een wat andere kijk op de zaak te horen. Bedankt voor al je adviezen en correcties van de manuscripten (en Sabine bedankt voor het ontcijferen ervan!).

De leden van de Teake-clan: Janine, Mirian en Karin.
Kwijlend blijf ik terugdenken aan al onze toetjes-avonden!
Karin: jouw "tis allemaal maar psychisch" bracht me vaak met beide beentjes weer op de grond. Ik moet je alleen helaas mededelen dat van jouw overtuiging ik in vier à vijf jaar wat meer geduld zou aanleren, niets is geworden. Mirian en Janine: van alle AIO's stonden jullie het meest dichtbij. Gelukkig niet alleen op het lab, maar ook daarbuiten. Met jullie als paranimfen gaat het vast een stuk beter.

Diana, Martine, Mariëlle, Annemie, Sabine, Ellen en Geerten: alleen een andere AIO weet wat je soms moet doorstaan. Maar gedeelde frustratie blijkt gelukkig toch maar een halve!

Alle andere Gaubianen voor het medeleven tijdens mijn ietwat gehandicapte periodes. Marian bedankt voor al je hulp bij mijn dierexperimenten. Erna en Annemie bedankt voor de liften en de goede opvang toen ik plotseling dankloos was.

Johan Auwerx en Bart Staels bedankt voor de prettige samenwerking hier in Nederland en Lille, Frankrijk. Zo'n Frans lab is toch wel weer heel wat anders. My thanks to everybody in the lab (especially Inez, Philippe, Delphine, Anne-Marie, Bruno and Kristina), for being so helpful and trying to understand what I was saying. Laurent (thank God you speak english so well!), many thanks for taking care of me, when I was in Lille.

Jaap, jij ook bedankt voor de prettige samenwerking. Mijn statistiek is weer helemaal bijgespijkerd.

En natuurlijk alle vrienden, vriendinnen, bekenden en vage kennissen: Jo, Babette, Willie, Karin, Nelleke, Adriaan, Hetty, Lique, Karen, Partrick, Sjoerd, Jeroen, Erwin, Aino, Rene, David, Patrick, Roland (Ronald?), Peter, Bart, Willem en Paul (lang leve de e-mail!). Voor alle leuke uurtjes buiten het lab. En Marja, jij moet even apart genoemd worden. Weinig mensen zullen elkaar zoveel vertellen: best friends forever!

En dan als laatste Hans: je viel in de laatste periode binnen en was meteen met hart en ziel betrokken (niet alleen met mij, maar ook met m'n promotie en alles eromheen). Ik geloof niet dat we samen nog eens een Nederlandse samenvatting moeten maken, maar voor de rest zie ik onze toekomst erg zonnig in. Geweldig dat je "zomaar" meegaat naar een werelddeel dat ik zonodig moet verkennen!

Leiden, 26 juli 1999

Maaike