Brief Review

The Adipocyte and Hemostatic Balance in Obesity
Studies of PAI-1

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Plasminogen activator inhibitor 1 is the primary physiological inhibitor of plasminogen activation in vivo, and elevations in plasma PAI-1 appear to compromise normal fibrin clearance mechanisms and promote thrombosis. PAI-1 is dramatically upregulated in obesity, a complex condition associated with increased risk for myocardial infarction, accelerated atherosclerosis, hypertension, glucose intolerance, insulin resistance, hyperinsulinemia, and NIDDM. In spite of the apparent link between elevated PAI-1 levels and thrombotic disease, little is known about the origin of this plasma inhibitor in obesity/NIDDM or about the signals that control its biosynthesis. Potential insights into the underlying molecular events have come from recent studies of genetically obese mice and of cultured adipocytes. These studies are reviewed here.*

They emphasize the key role played by the adipocyte, a cell whose numbers, size, and metabolic activity are grossly altered in obesity/NIDDM. They also suggest that multiple cytokines, hormones, and growth factors may be involved, and they raise the possibility that the abnormal expression of other hemostatic genes by adipocytes in obesity/NIDDM may also contribute to the cardiovascular complications of this disorder. In this regard, our preliminary studies indicate that TF gene expression is elevated in the adipose tissues of the obese mouse.

Properties of PAI-1

PAI-1 appears to be the primary physiological inhibitor of plasminogen activation in blood, since it is the only PAI found complexed with single-chain tissue-type PA in carefully collected human plasma, and the second-order rate constant for its interaction with tissue-type PA and urokinase-type PA (≈3.5×10^7 [mol/L]^-1·s^-1) is at least two orders of magnitude higher than that of other PAs.1–4 The normal concentration of PAI-1 protein in human plasma ranges from 6 to 80 ng/mL with a geometric mean at 24 ng/mL, whereas that of tissue-type PA is 5 to 10 ng/mL. Abnormalities in the concentration of PAI-1 are frequently associated with vascular disease. For example, the inhibitor is elevated in a variety of thrombotic conditions, including myocardial infarction and deep venous thrombosis. Elevated PAI-1 also correlates with thrombosis in animal models, and transgenic mice that overexpress PAI-1 have been reported to develop venous thrombosis.5 On the other hand, the absence of PAI-1 in humans leads to life-long bleeding problems presumably resulting from the development of a hyperfibrinolytic state, and disruption of the PAI-1 gene in mice is also associated with a mild hyperfibrinolytic state as manifested by increased resistance to endotoxin-induced thrombosis.5 Finally, neutralizing plasma PAI-1 activity with specific antibodies or by the use of PAI-1 inhibitors4,6 enhances spontaneous or tissue-type PA–mediated thrombolysis. These observations emphasize that imbalances in the PA/PAI-1 ratio are likely to promote either thrombosis or bleeding. As summarized in the next section, this balance is severely disturbed in obesity/NIDDM.

PAI-1 itself is a single-chain glycoprotein (13% carbohydrate) of ≈M, 50 000 and consists of 379 amino acids, although some amino-terminal heterogeneity has been reported. It lacks cysteines but contains multiple methionines, which may account for its susceptibility to irreversible inactivation by oxidizing agents. The reactive center of the inhibitor (Arg346/Met347) is contained within the exposed "strained loop" region at the carboxy terminus of the molecule and serves as a pseudosubstrate for the target serine protease. Inhibition of PAs by PAI-1 occurs in a rapid and stoichiometric manner, resulting in the formation of a covalent bond between the two molecules. The inhibitor is consumed in the process, giving rise to the term "suicide inhibitor." PAI-1 is ≈30% homologous with α1-antitrypsin and antithrombin III and is thus a member of the serine protease inhibitor (serpin) superfamily. The general structure of active PAI-1 is similar to that of other serpins, since it appears to be in a stressed conformation that is sensitive to thermal denaturation. The human PAI-1 gene is ≈12.2 kb long, is organized into 9 exons and 8 introns, and is located on the long arm of chromosome 7. It specifies two distinct transcripts of ≈2.3 and 3.2 kb long, which are colinear from their 5' ends and are formed by alternative polyadenylation.

The lack of cysteine residues (and hence disulfide bonds) in PAI-1 may in turn account for its biological instability in solution. In this regard, the inhibitor has been detected in both an active and an inactive state. It is synthesized in the active form but is unstable in solution and rapidly decays into the

*Because of strict limitations on references, a number of reviews are cited. The reader is referred to these reviews for more details and citations.
inactive or “latent” form. This transition is associated with a large conformational change in the molecule, and latent PAI-1 can be “activated” by denaturants and other molecules known to cause conformational changes in proteins. Interestingly, 75% to 80% of PAI-1 in platelets is present in the latent form in α-granules. The detection of PAI-1 mRNA and antigen in megakaryocytes (reviewed in Reference 7) suggests that PAI-1 may be deposited into the α-granules during the maturation of these cells. The presence of a large storage pool of latent PAI-1 in platelets raises the possibility that mechanism(s) may exist for the activation of this form of PAI-1 in vivo. In spite of the potential clinical importance of this possibility, experimental support for such a mechanism is lacking. In fact, there appears to be sufficient active PAI-1 released from platelets at sites of arterial thrombi to inhibit local fibrinolysis (reviewed in Reference 8). Platelet-derived PAI-1 may account for the known resistance of platelet-rich thrombi to thrombolytic therapy. PAI-1 is also present in the extracellular matrix of a variety of cultured cells, bound to the glycoprotein Vn. Vn stabilizes the inhibitor in its active conformation, thus increasing its biological half-life. In this regard, the majority of PAI-1 in blood is active and circulates in complex with Vn. Vn may also alter the specificity of PAI-1. When considered together, these observations suggest that Vn is a cofactor for PAI-1.

Although PAI-1 is present at low concentrations in plasma, its relatively short half-life in blood (<10 minutes) suggests a high biosynthetic rate. Moreover, its concentration rapidly increases in response to a variety of agents or changes in physiological state, indicating that the amount of PAI-1 in plasma is subject to dynamic regulation. In spite of this, the origin of plasma PAI-1 under normal and pathological conditions remains to be defined. The human liver has been reported to contain relatively high levels of PAI-1 mRNA, and PAI-1 has been detected in human spleen, kidney, lung, placenta, uterus, and myocardium. These observations should be interpreted with some caution, since most tissues were obtained under stressed conditions (eg, after trauma, from patients in poor health, or after major surgery), and PAI-1 is an acute-phase protein in humans.

The distribution of PAI-1 mRNA and protein has been extensively studied in rodent models under conditions that minimize the problems associated with the interpretation of human studies. In the rat, PAI-1 mRNA was detected mainly in the lung, with low levels in the heart. It was widely distributed in the mouse, with the highest concentration in the aorta, lung, heart, and adipose tissue. In contrast to human studies, little or no PAI-1 mRNA was detected in the murine liver, muscle, or spleen. The finding of detectable levels of PAI-1 in many human and rodent tissues raises the possibility that plasma PAI-1 may originate from a variety of tissues under normal conditions and suggests that common cells within these tissues (eg, vascular endothelial cells, smooth muscle cells, etc) are responsible for its production. In this regard, PAI-1 was originally called the “endothelial cell inhibitor” because it was produced at high concentrations by most species of cultured endothelial cells. However, in situ hybridization analysis failed to detect PAI-1 mRNA in normal murine endothelium in vivo, although it was consistently detected in vascular smooth muscle cells within most tissues. These results suggest that the high expression of PAI-1 by cultured endothelium may be an artifact of cell culture. PAI-1 gene expression in vitro and in vivo is induced by endotoxin, TNF-α, TGF-β, and a variety of other growth factors, cytokines, hormones, and proteinases. The diversity of this list implies that the regulatory region of the PAI-1 gene must be unusually complex, containing DNA sequences that are either directly or indirectly responsive to all of these molecules.

PAI-1, Obesity, and Cardiovascular Disease. It is now alarmingly clear that obesity (ie, excess energy storage) is rapidly increasing in Western societies (eg, some 30% of adults in the United States are now defined as clinically obese), and with it there is a steadily increasing risk for obesity-associated disorders including cardiovascular disease. Although billions of dollars are spent each year to treat the accelerated atherosclerosis, increased risk for fatal myocardial infarction, hypertension, insulin resistance, and NIDDM that frequently accompany obesity, the molecular changes in obesity that promote these conditions are far from resolved. The fact that human obesity is a polygenic disorder with complex environmental and behavioral characteristics has made obesity research one of the more difficult areas of investigation in the medical sciences. However, recent studies of genetically obese mice and rats have produced breakthroughs in several areas of obesity-related research, primarily because obesity in these animals appears to be monogenic (ie, to result from mutations in single genes). In this regard, five different genes, all mapped to different chromosomal locations, have been shown to cause distinct syndromes of spontaneous obesity with severe insulin resistance in mice. These include the obese (ob), diabetes (db), tubby (tub), lethal yellow (Ay) and fat (fat) mutations. Genes encoding intercellular adhesion molecule 1 (ie, ICAM-1) and the leukocyte integrin αmβ2 (MAC-1) have also been implicated in the regulation of adipose tissue mass. These animal studies, together with studies of cultured adipocytes, have provided fundamental new information about the factors and cells that may be responsible for elevated PAI-1 in obesity/NIDDM.

Adipose Tissue as a Source of PAI-1 in Obesity

The possibility that adipose tissue itself may directly contribute to the elevated expression of PAI-1 in obesity has recently gained considerable attention. Initial clues for such a hypothesis came from the observation that the adipose tissue of the mouse contained relatively high levels of PAI-1 mRNA. Moreover, clinical studies demonstrated that weight loss due to surgical treatment, diet, etc, significantly reduced plasma PAI-1 levels in obese humans. These
findings were noteworthy not only because adipose tissue was known to secrete a variety of proteins into blood but also because in obese animals, the size and number of adipocytes and thus, the amount of adipose tissue mass, typically increases several-fold. Thus, in obesity the PAI-1 biosynthetic capacity of adipose tissue may approach or even exceed that of other tissues.

We have been able to extend these initial observations considerably by studying genetically obese (ob/ob) mice. Plasma PAI-1 activity was approximately fivefold higher in these mice than in their lean counterparts, and this elevation increased further as a function of age. Importantly, PAI-1 gene expression was significantly elevated in the epididymal, subcutaneous, and brown adipose tissues of obese mice compared with the lean controls. Although there was a generalized increase in PAI-1 mRNA in other major organs as well, these effects were small compared with the increases in the fat. In situ hybridization studies of adipose tissues from obese mice demonstrated elevated PAI-1 mRNA in adipocytes, vascular smooth muscle cells, and occasional endothelial cells (Reference 10; Figs 1 and 2). The key role of adipocytes in PAI-1 biosynthesis is emphasized by the demonstration that mature, fully differentiated 3T3-L1 adipocytes in culture produce significant levels of PAI-1 mRNA and protein. Expression of PAI-1 mRNA has also been demonstrated in the visceral and subcutaneous fat of obese rats and in adipose tissues from human subjects. In both cases, omental tissue explants produced significantly more PAI-1 antigen than subcutaneous tissues from the same individual, and in humans, cardiovascular risk is most closely correlated with android obesity.

Molecular Mechanisms of Elevated PAI-1 in Obesity

A variety of observations implicate specific hormones and/or cytokines in the increased expression of PAI-1 by adipose tissue in obesity. These include TNF-α, insulin/proinsulin, and TGF-β. Triglycerides and free fatty acids may also stimulate PAI-1 gene expression in adipocytes.

**TNF-α**

Adipose tissue synthesizes TNF-α, and expression of this cytokine is chronically elevated in adipose tissue from obese mice and humans. The expression of TNF-α by adipose cells, particularly in the context of obesity, may interfere with certain aspects of insulin signaling, such as the tyrosine kinase activity of the insulin receptor, and thus contribute to insulin resistance. Interestingly, TNF-α is known to stimulate PAI-1 biosynthesis by a variety of cultured cells and by many murine tissues in vivo, and administration of TNF-α to lean mice significantly increased PAI-1 mRNA in the adipocytes, adventitial cells, and vascular smooth muscle cells in the adipose tissues (Reference 9; Figs 1 and 2, compare panels A and C). This pattern is similar to the pattern of PAI-1 mRNA observed in the adipose tissues of obese mice (Figs 1 and 2, compare panels B and C). Again, the central role of the adipocyte is emphasized by the finding that TNF-α also induced PAI-1 expression in mature 3T3-L1 adipocytes. Recent studies show that human adipose tissue explants also respond to exogenous TNF-α with increased PAI-1 mRNA and protein expression and that the addition of pentoxifylline (an inhibitor of TNF-α mRNA synthesis) decreased PAI-1 mRNA and protein. Taken together, these observations support the hypothesis that the chronic elevation in TNF-α that occurs locally in the
adipose tissues in human and rodent obesity may act via an autocrine manner to stimulate PAI-1 biosynthesis by the adipocyte and other cells in the adipose tissue. This cytokine may thus contribute to the elevated plasma PAI-1 levels observed in obesity/NIDDM.

Insulin/Proinsulin
Evidence in support of the view that insulin may play a role in the elevation of PAI-1 comes from observations that hyperinsulinemic NIDDM patients often display reduced fibrinolysis, possibly because of elevated plasma PAI-1.16 Moreover, conditions that increase endogenous plasma insulin levels (eg, a high-calorie, high-carbohydrate meal) were associated with increases in plasma PAI-1, whereas conditions that reduced endogenous insulin (eg, fasting or treatment with metformin or troglitazone, an insulin-sensitizing agent) were associated with decreases in plasma PAI-1.16,24 Direct administration of insulin into rabbits25 and mice10 and into the forearm vascular bed of human volunteers26 significantly increased the level of plasma PAI-1. Although these observations suggest that insulin directly contributes to the elevated PAI-1 in obesity/NIDDM, many other human studies failed to demonstrate elevated plasma PAI-1 in response to acutely or chronically administered insulin (reviewed in References 16 and 24). Thus, whereas some animal and clinical studies show a very strong correlation between insulin and plasma PAI-1, other studies are inconsistent and appear to depend on the method of insulin administration, the metabolic state of the individual, and whether the patient is lean or obese. Although it is difficult to reconcile these differences, it is possible that sufficient levels of plasma insulin were not achieved in some of the human studies. It is also possible that long-term insulin treatment may suppress endogenous insulin and proinsulin secretion.27,28 Another possibility is that proinsulin or proinsulin split products, rather than insulin per se, are involved, since NIDDM patients have an elevated basal ratio of plasma proinsulin to immunoreactive insulin and the concentration of PAI-1 in the plasma of these patients correlates more with the concentration of proinsulin and des28,29 proinsulin than with insulin.30 Recently, final observations raise the possibility that insulin resistance rather than hyperinsulinemia may be involved.31 Obviously, further studies are required to resolve these apparent inconsistencies in the human and animal insulin data.

The results of animal studies have been more consistent than the human studies and provide additional insights into underlying mechanisms. For example, in the rabbit, plasma PAI-1 antigen and liver mRNA were shown to increase slightly after administration of insulin or proinsulin.25 Similarly, intraperitoneal administration of insulin to lean mice increased PAI-1 antigen in the plasma and increased PAI-1 mRNA modestly (less than twofold) in a variety of tissues, including the liver.10 However, the major effect of insulin on PAI-1 gene expression in the mouse was in the adipose tissue (a fivefold to sevenfold increase), a tissue not examined in the rabbit. Thus, adipose tissue may be the primary insulin-responsive tissue in the mouse, at least in terms of PAI-1. Insulin induced PAI-1 mRNA primarily in adipocytes (Reference 10; Figs 1 and 2, compare panels A and D) but had no apparent effect on PAI-1 biosynthesis in large-vessel endothelial cells (Fig 1D). These observations are consistent with the lack of insulin response in cultured endothelial cells32 and indicate that the induction of PAI-1 by insulin in adipose tissue is relatively specific for the adipocyte. In this respect, PAI-1 mRNA and antigen also were induced by insulin in cultured 3T3-L1 adipocytes, with the level of induction being considerably higher than that reported for other cell types.16 Although insulin was shown to stimulate PAI-1 mRNA expression in cultured hepatocytes,29,33 it had only modest effects on PAI-1 gene expression by the murine liver.10 These results point to the importance of adipose tissue and more specifically to the adipocyte in insulin-mediated PAI-1 induction in obesity.

TGF-β
The multifunctional cytokine TGF-β stimulates PAI-1 biosynthesis by a large variety of cultured cells,3 and infusion of TGF-β into rabbits26 and mice15,19 dramatically increased plasma PAI-1 activity and induced PAI-1 mRNA in numerous tissues. In the mouse, the most TGF-β-responsive tissue in terms of PAI-1 appeared to be adipose tissue (References 15 and 19; Figs 1 and 2, compare panels A and E). TGF-β did not induce PAI-1 mRNA expression in large-vessel endothelial cells in adipose tissue (Fig 1E), in agreement with previous studies in the kidney.3 TGF-β also stimulated PAI-1 gene expression by cultured mouse19,21  and human12 adipocytes. Interestingly, the level of TGF-β mRNA was significantly higher in the adipose tissue of both ob/ob and db/db mice when compared with their lean counterparts,19 and this increase again was due to increased expression of TGF-β mRNA by mature adipocytes and stroma/vascular cells.

The increase in TGF-β gene expression in adipose tissue in obesity may have broad implications in the pathophysiology of obesity and its related complications. Besides stimulating PAI-1 biosynthesis, TGF-β is mitogenic for preadipocytes and inhibits the differentiation of preadipocytes into adipocytes in vitro (reviewed in Reference 19). Thus, the augmented expression of TGF-β in the adipose tissue of obese mice may increase adipocyte precursor cell proliferation and contribute to the excessive cellularity of the fat depots associated with the obese phenotype. Obesity and NIDDM are also associated with characteristic long-term complications, including microvascular kidney disease and atherosclerosis,16,33 and several investigations have demonstrated elevated TGF-β levels in the glomeruli in human and experimental diabetes (reviewed in References 19, 34, and 35). Taken together, these observations suggest that the increased expression of TGF-β in adipose tissue in obesity may contribute to the pathologies associated with these conditions.

Triglycerides and Free Fatty Acids
Increased levels of both triglycerides and free fatty acids are associated with obesity and insulin resistance,11-13 and these molecules were shown recently to stimulate PAI-1 expression by 3T3-L1 adipocytes.36 These results raise the possibility that triglycerides and free fatty acids may also contribute to the elevated PAI-1 in obesity.
TF is the major cellular initiator of the coagulation cascade and also serves as a cell surface receptor for activation of factor VII. A number of clinical studies have shown an increase in TF-mediated coagulation and in factor VII activity/antigen in obese and NIDDM patients. In preliminary studies, we demonstrated that TF mRNA expression also is elevated in the epididymal and subcutaneous fat pads from obese mice compared with their lean counterparts. TF expression in adipose tissue was localized to adipocytes, adventitial cells surrounding blood vessels, and other unidentified stromal vascular cells. Although administration of TNF-α or insulin to lean mice increased TF gene expression (threefold or twofold, respectively), TGF-β increased it sixfold to eightfold. These results suggest that TGF-β may play a major role in the elevated TF expression in adipose tissue of the obese and that TF itself may contribute to the increased cardiovascular risk associated with obesity and related NIDDM. Again, the adipocyte appears to play a key role in TF gene expression in obese mice.

**Summary and Perspectives: The Adipocyte as a Central Player in Obesity-Related Cardiovascular Disorders**

It is becoming increasingly apparent from studies in vivo and in vitro that adipocytes may synthesize and secrete a number of proteins (reviewed in References 13, 18, 19, and 21; Fig 3). Thus, the simple paradigm that the adipocyte is merely a fat storage cell is rapidly evolving into one that views this cell as a metabolically active secretory cell that can alter the composition of the blood. In this regard, adipocytes are known to express TF, leptin, TNF-α, PAI-1, and TGF-β, and the synthesis/secretion of these proteins is upregulated in adipocytes from obese animals and humans. Adipocytes also secrete lipoprotein lipase, and they are an important source of apolipoprotein E, extrahepatic cholesteryl ester transfer protein, and angiogenin. Interestingly, angiogenin II increases the expression of PAI-1 in vivo and in vitro and elevates PAI-1 antigen (reviewed in Reference 42). Adipocytes are also able to secrete three major proteins of the alternate complement pathway, including adipin (factor D), factor c3, and factor B. The capacity of adipocytes to synthesize significant amounts of sex steroids has also been shown. Taken together, these observations emphasize that adipocytes are able to secrete proteases, protease inhibitors, hormones, growth factors, and cytokines, and it seems likely that some of these proteins contribute to the thrombotic and cardiovascular risk associated with obesity (Fig 3). Because obesity is associated with an excess of adipose tissue, a condition that frequently includes an elevated number of fat cells, the increase in adipose tissue mass in obesity would tend to exacerbate the overproduction of these molecules, whether the effects are achieved locally or systemically.

In summary, the mechanisms that lead to the elevations in plasma PAI-1 in obesity/NIDDM are obviously complex and appear to involve adipose tissue itself, as well as multiple cytokines, hormones, and growth factors. The PAI-1 mRNA profiles induced in the adipose tissue of insulin-, TNF-α-, and TGF-β-treated mice, when superimposed, appear to largely recapitulate the characteristic profile seen in the adipose tissue of obese mice (Figs 1 and 2; compare panel B with panels C, D, and E). This fact, together with the observations that insulin, TNF-α, and TGF-β are elevated in obesity and induce PAI-1 in the plasma and adipose tissue of lean mice, certainly suggest the involvement of these mediators in the regulation of PAI-1 in obesity. They may therefore promote the increased risk for cardiovascular disease in obesity/NIDDM. These considerations emphasize the importance of the adipocyte and excess adipose tissue mass as a central player for the synthesis and secretion of proteins and other molecules that may contribute to the development of pathophysiological complications associated with obesity.

**Acknowledgments**

This work was supported by grants to D.J.L. from the National Institutes of Health, Bethesda, Md (HL47819), and Novartis Pharmaceuticals. The authors thank Dr P. Bell for his critical comments and suggestions and M. McRae for typing the manuscript.

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**KEY WORDS:** adipocyte [adipose tissue] insulin [TNF-α] TGF-β
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doi: 10.1161/01.ATV.18.1.1
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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