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Adipose tissue secretion of plasminogen activator inhibitor-1 in non-obese and obese individuals

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Summary High plasma plasminogen activator inhibitor-1 (PAI-1) activity is a frequent finding in obesity, and both PAI-1 and obesity are risk factors for cardiovascular disease. To study the mechanisms underlying increased PAI-1 levels in obese individuals, gene expression and secretion of PAI-1 were measured in human abdominal subcutaneous adipose tissue. A total of 32 obese, otherwise healthy subjects and 10 never-obese healthy subjects with a body mass index (BMI) of 42.6 ± 1.2 and 24.3 ± 1.9 kg/m^2 (mean ± SEM), respectively, were investigated. Plasma PAI-1 activity, adipose tissue PAI-1 secretion and adipocyte PAI-1 mRNA levels were increased sevenfold ($p < 0.0001$), sixfold ($p < 0.0001$) and twofold ($p < 0.05$), respectively, in the obese group. There were clear associations between adipose tissue secretion of PAI-1 and PAI-1 mRNA levels on the one hand and fat cell volume on the other ($r = 0.68$, $p < 0.0001$ and $r = 0.51$, $p < 0.01$, respectively, in the obese group). PAI-1 mRNA levels were also related to the amount of PAI-1 secreted among obese individuals ($r = 0.31$, $p = 0.09$). It is concluded that adipose tissue secretes significant amounts of PAI-1, that PAI-1 secretion from adipose tissue is increased in obesity, and that PAI-1 secretion is related to the lipid content and cell volume of fat cells. Plasma PAI-1 activity is elevated in obesity, at least in part due to increased gene expression in adipocytes, which, in turn, enhances PAI-1 secretion from adipose tissue. [Diabetologia (1998) 41: 65--71]

Keywords PAI-1, adipocyte, obesity, mRNA.

Obesity is an independent risk factor for the development of cardiovascular disease [1, 2] and is one component of the insulin resistance syndrome [3]. Other features of this common metabolic disorder are hypertriglyceridaemia, hyperinsulinaemia, hypertension and glucose intolerance. Recently, elevated plasma activity of plasminogen activator inhibitor-1 (PAI-1), the fast-acting inhibitor of tissue-type plasminogen activator and the main regulator of the endogenous fibrinolytic system, has also been included as a component of the insulin resistance syndrome [4]. High plasma PAI-1 activity is a common finding in obese individuals [5--8]. As elevated plasma PAI-1 activity is associated with manifest coronary heart disease (CHD) [9] and with increased risk of major cardiovascular events in patients with a history of cardiovascular disorders [10--12], impaired fibrinolytic function secondary to PAI-1 elevation is likely to increase the risk of cardiovascular disease in obese subjects.

Although human obesity is often associated with elevated levels of plasma PAI-1, limited information is available about the tissue and cellular origin of the excess circulating PAI-1 of the obese individual. However, adipose tissue has been suggested to be an important contributor to the plasma PAI-1 levels in a murine animal model, particularly under obese conditions with increased expression of tumour necrosis factor-α (TNFα) [13]. Furthermore, PAI-1 mRNA

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Abbreviations: PAI-1, Plasminogen activator inhibitor-1; CHD, coronary heart disease; TNF-α, tumour necrosis factor-α; TNA, total nucleic acid.

was found to increase in visceral fat during the development of obesity in a rat model [14]. Recently, Alessi et al. [15] demonstrated that human adipocytes produce PAI-1 under culture conditions and that omental fat produces more PAI-1 than does subcutaneous fat.

To investigate the mechanisms underlying the increased plasma levels of PAI-1 in human obesity, we measured the in vitro secretion rate and mRNA levels of PAI-1 in subcutaneous adipose tissue of obese and non-obese individuals.

Materials and methods

Subjects, blood sampling and biochemical methods. A total of 32 obese individuals (body mass index (BMI) 42.6 ± 1.2 kg/m^2, mean ± SEM) planned to undergo weight-reducing surgery were included (23 females and 9 males) along with a random control group (n = 10) consisting of never-obese subjects (BMI 24.3 ± 1.9 kg/m^2, 6 females and 4 males). The groups were matched for age and smoking habits. All subjects were Caucasian and born in Sweden. Except for obesity, they were healthy and took no medication. For methodological experiments abdominal subcutaneous adipose tissue was obtained during elective surgery on subjects operated upon because of non-malignant disorders. These subjects were not selected for BMI. The study was approved by the ethics committee of the Karolinska Institute, Stockholm, Sweden. All individuals gave their informed consent to participate.

The waist-to-hip ratio and BMI were measured on the day before surgery after an overnight fast. Blood pressure was measured and venous blood samples were taken for metabolic and hormone tests after 15 min rest in the recumbent position. PAI-1 was assayed as activity in plasma using a commercially available kit (Chromolize PAI-1; Biopool, Umea< , Sweden). Plasma PAI-1 activity was measured in all of the non-obese individuals, but not in 10 of the obese individuals due to lack of unthawed plasma. Serum insulin was determined by radioimmunoassay (InsulinRIA; Pharmacia, Uppsala, Sweden).

Adipose tissue biopsies. Abdominal subcutaneous adipose tissue was obtained by biopsy under local anaesthesia with lidocaine [16]. The adipose tissue specimens, the weight of which ranged from 0.5 to 1 g, were divided into two parts. One part was immediately used for secretion studies. The other part was used for mRNA analysis and determination of fat cell size. The latter measurements were performed on isolated fat cells (see below).

Isolation of fat cells and determination of fat cell size and number. Isolated fat cells were prepared by collagenase treatment according to the method of Rodbell [17]. The method has been described in detail and has been evaluated previously [18]. The cells were kept in an albumin solution, composed as described below. Direct microscopic determination of the fat cell diameter was based on the mean of 100 cells from each subject. The mean fat cell volume and weight were calculated, taking into account the skewness in the distribution of the cell diameter. The total lipid content in each incubation was determined by organic extraction [19] and subsequent gravimetric measurement. Assuming that lipids constitute over 95% of the fat cell weight, the number of fat cells was then calculated by dividing the total lipid weight by the mean cell weight.

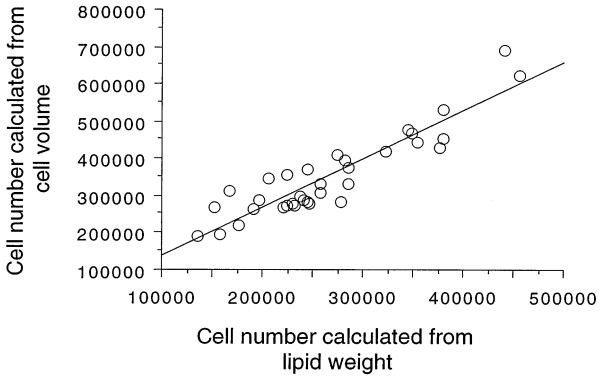

Fig. 1. Results of two methods of calculating cell number in 200 µl aliquots of packed fat cells from abdominal subcutaneous adipose tissue of 34 subjects spanning a wide range in body weight and cell size. Cell number was calculated from cell volume (y-axis) or lipid weight (x-axis) as described in Methods. The linear relationship is shown comparing the two methods in subcutaneous fat (r = 0.92, $p < 0.0001$)

The remaining part of the fat cell suspension was centrifuged for 1 min at 8 g in order to obtain a packed cell sample. Exactly 200 µl of this sample was frozen at --70 °C for subsequent mRNA analysis.

Two ways of determining the number of fat cells homogenized for analysis of PAI-1 mRNA were compared in separate methodological experiments on samples of subcutaneous adipose tissue from 34 subjects with a wide range of BMI (22.0--50.0 kg/m^2). First, the number of cells was calculated as described above by extraction of the lipids of a 25 µl aliquot of packed cells. Second, the number of fat cells was estimated by dividing the amount of packed cells used in the mRNA determination (200 µl) by the mean fat cell volume. In simple regression analysis (Fig. 1), there was a close correlation between these two methods (r = 0.92, p = 0.0001), and analysis of covariance did not reveal any significant difference between obese (BMI > 27 kg/m^2) and non-obese (BMI < 27 kg/m^2) subjects. Although the latter method gave a higher estimate of the cell number by 24 ± 2%, the slope of the regression line did not differ significantly from the former, indicating only a minor effect of variations in cell size and the procedure of packing the fat cells on this relationship. Since only small amounts of adipose tissue can be obtained by biopsy in clinical studies, the latter mode of determination was subsequently used as it consumed less fat tissue.

PAI-1 secretion. PAI-1 secretion from adipose tissue was measured in all subjects. Adipose tissue was cut into small pieces (each weighing about 10 mg). All visible vessels and coagulation particles were removed. The remaining tissue was rinsed in 37 °C physiological saline and then incubated (300 mg tissue in 3 ml medium) in a Krebs Ringer phosphate buffer (pH 7.4) supplemented with 40 g/l of defatted bovine serum albumin and 1 g/l of glucose. The incubation was carried out for 2 h at 37 °C in a shaking water bath with air as the gas phase. After incubation, 2 ml of the medium was removed, frozen in liquid nitrogen and stored at --70 °C. The tissue was immediately homogenized and the total lipid extracted as described [20]. Both the lipid weight and the number of fat cells of the incubated tissue were used as a denominator for calculating PAI-1 release. At the time of analysis, all the frozen medium samples were freeze-dried simultaneously, and the material was re-dissolved in 150 ml of distilled water. It was necessary to concentrate

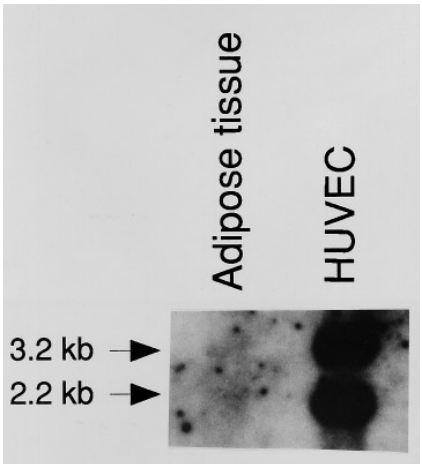

Fig. 2. Demonstration of PAI-1 mRNA expression in adipose tissue. Northern blot analysis of 3 μg total RNA derived from human umbilical vein endothelial cells (HUVEC) and 15 μg human subcutaneous adipose tissue. The autoradiogram was exposed for 1 week

the medium in this way in order to obtain detectable PAI-1 values. The recovery of the freeze-drying step was tested and constantly found to be 75 %. The PAI-1 protein concentration in the medium was quantified using an ELISA (TintELIZE PAI-1; Biopool, Umea< , Sweden).

mRNA analysis. To obtain RNA probes for hybridization, a 1.1 kb Pst I-fragment of PAI-1 cDNA [21] was subcloned in the pGEM-3 vector (Promega, Madison, Wis., USA). In vitro RNA synthesis was performed according to Melton et al. [22]. Antisense RNA labelled with [^{35}S]UTP and unlabelled sense RNA was synthesized for RNA-RNA hybridization in solution. Total nucleic acid (TNA) from the 200 μl packed isolated fat cells sample was extracted by phenol-chloroform after digestion with proteinase K [23]. In some experiments, total RNA was prepared using the acid guanidinium thiocyanate-phenol-chloroform method [24]. The integrity of the RNA was confirmed by electrophoresis in ethidium bromide containing agarose gels, and the RNA concentration was determined spectrophotometrically. Quantitative mRNA analysis of the TNA sample was performed using hybridization in solution [23]. A series of aliquots ranging from 2 to 20 μg of TNA from the tissue specimens was hybridized to ^{35}S-labelled antisense RNA for 24 h at 70 °C in a solution containing 0.6 mol/l NaCl, 20 mmol/l Tris-HCl, pH 7.5, 4 mmol/l EDTA, 0.1 % sodium dodecyl sulfate (SDS), 1 mmol/l dithiothreitol (DTT) and 25 % formamide. After hybridization the samples were treated with RNase (40 μg/ml RNase A and 100 U/ml RNase T_1) for 45 min at 37 °C. Trichloroacetic acid precipitated material was collected on Whatman GF/C filters (Whatman International Ltd., Maidstone, UK) and counted in a liquid scintillation counter. A standard curve was constructed by hybridizing ^{35}S-labelled antisense RNA with known amounts of in vitro synthesized unlabelled sense RNA.

Northern blotting and hybridization on DuPont GeneScreen Plus nylon membranes (NEN Research Products, Boston, Mass., USA) were performed according to the manufacturer's protocol. Blots were hybridized with 10^6 cpm/ml of a [α32P]-dCTP labelled Sfi I and Bgl II fragment (1255 bp) of the cDNA for PAI-1 (courtesy of Dr. T. Ny, Department of Biochemistry and Biophysics, University of Umea< , Sweden).

Table 1. Characteristics of the obese and non-obese subjects

	Obese (n = 32)	Non-obese (n = 10)	p value
Body mass index (kg/m^2)	42.6 ± 1.2	24.3 ± 0.6	< 0.001
Waist-to-hip ratio	0.95 ± 0.01	0.85 ± 0.02	< 0.001
(all female)	0.93 ± 0.01 (n = 23)	0.82 ± 0.03 (n = 6)	< 0.001
Glucose (mmol/l)	6.0 ± 0.3	5.0 ± 0.1	< 0.05
Insulin (pmol/l)	125 ± 11	43 ± 7	< 0.001
Plasma triglycerides (mmol/l)	2.0 ± 0.2	0.9 ± 0.1	< 0.01
Plasma cholesterol (mmol/l)	5.6 ± 0.2	4.9 ± 0.3	NS
Fat cell volume (pl)	786 ± 24	548 ± 30	< 0.001
Age (years)	39.3 ± 2.1	42.2 ± 2.5	NS

NS, Not significant. Values are mean ± SEM. Statistical significance was determined using a Student's unpaired t-test

Statistical analysis. Differences in continuous variables between obese and non-obese subjects were tested by Student's unpaired t-test. Correlations were analysed by a linear regression method. All values are given as mean ± SEM.

Results

Adipose tissue in humans can produce PAI-1. Human abdominal subcutaneous adipose tissue was obtained by biopsy, incubated in a physiological buffer, and the PAI-1 antigen was subsequently measured using a PAI-1 specific ELISA in order to investigate whether human adipose tissue can secrete PAI-1. PAI-1 antigen was detected in the incubation buffer, and the amount of secreted PAI-1 was proportional to the length of the incubation (1--6 h) and to the amount of adipose tissue used in the incubation (data not shown). A 2-h incubation period was used in all subsequent studies. In methodological experiments, PAI-1 secretion from isolated fat cells and tissue fragments were compared. Almost identical values were obtained. We preferred to use fragments, as isolation of fat cells is tissue consuming and the amount of available tissue was a limiting factor.

PAI-1 mRNA was detected in the freshly isolated fat cells. Figure 2 shows a Northern blot analysis using either RNA extracted from human endothelial cells or RNA extracted from human subcutaneous adipose tissue. Both lanes were hybridized with a probe constituting 1255 bp of the cDNA for PAI-1. As shown in Figure 2, both the 3.2 and the 2.2 PAI-1 transcripts were visualized using both RNA preparations. In addition, PAI-1 mRNA was detected by a solution hybridization procedure. Thus, these data show that human adipose tissue has the ability both to produce PAI-1 mRNA and secrete PAI-1 protein.

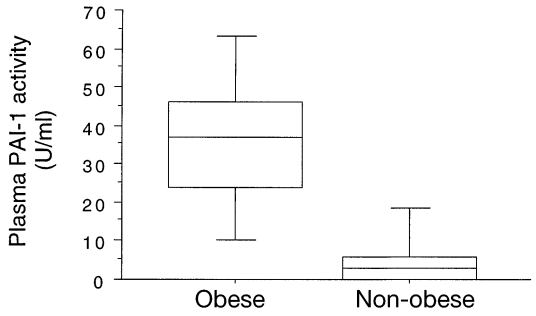

Fig. 3. Box plot displaying 10th--90th percentiles of plasma PAI-1 activity in obese as compared to non-obese subjects (36.0 ± 4.0 vs 5.6 ± 2.3 U/ml, mean ± SEM, $p < 0.0001$)

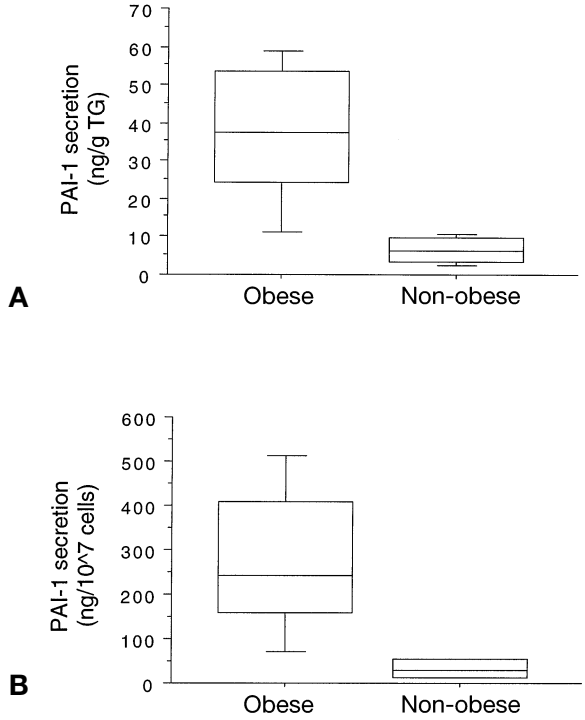

Fig. 4A, B. PAI-1 secretion from human subcutaneous adipose tissue. Box plot displaying 10th--90th percentiles of PAI-1 antigen levels. **A**. Adipose tissue secretion rate of PAI-1 per g lipid in obese individuals as compared to non-obese individuals (37.7 ± 3.4 vs 6.5 ± 1.1 ng PAI-1/g triglyceride, mean ± SEM, $p < 0.0001$). **B**. PAI-1 secretion per 10^7 adipose cells (281.0 ± 29.6 vs 32.8 ± 5.7 ng PAI-1/10^7 cells, $p < 0.0001$)

Adipose tissue secretion of PAI-1 in obese and non-obese individuals. To study potential differences in PAI-1 secretion from adipose tissue of obese and non-obese individuals, biopsies of subcutaneous adipose tissue were collected from 10 non-obese and 32 obese individuals. Basic characteristics of the two groups are shown in Table 1. The obese subjects showed a number of abnormalities known to be associated with overweight such as enlarged fat cell volume and high fasting plasma levels of triglycerides, insulin and glucose. Plasma PAI-1 activity was increased sevenfold in the obese subjects as compared

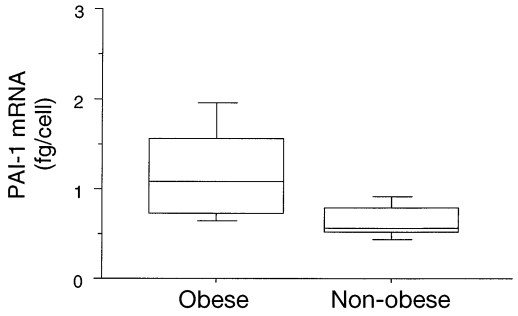

Fig. 5. PAI-1 mRNA levels in adipose tissue from obese and non-obese individuals. Box plot displaying 10th--90th percentiles of PAI-1 mRNA levels per adipose cell. Adipose tissue from the obese individuals showed an increased content of PAI-1 mRNA per fat cell (1.18 ± 0.10 vs 0.64 ± 0.07 fg/cell, mean ± SEM, $p < 0.05$)

to the non-obese group (36.0 ± 4.0 vs 5.6 ± 2.3 U/ml, $p < 0.0001$) (Fig. 3).

Adipose tissue secretion rates of PAI-1 per g lipid weight was increased sixfold in the obese individuals (37.7 ± 3.4 vs 6.5 ± 1.1 ng PAI-1/g triglycerides, $p < 0.0001$) (Fig. 4A). Expressing PAI-1 secretion per adipose tissue fat cell made the difference between obese and non-obese individuals even larger, with an eightfold higher secretion in obese individuals (281.0 ± 29.6 vs 32.8 ± 5.7 ng PAI-1/10^7 cells, $p < 0.0001$) (Fig. 4B). In agreement with the secretion data, the adipose tissue from the obese individuals showed an increased content of PAI-1 mRNA per fat cell (1.18 ± 0.10 vs 0.64 ± 0.07 pg/10^3 cells, $p < 0.05$) (Fig. 5). We expressed the amount of PAI-1 mRNA per fat cell instead of relating it to ubiquitous mRNA, as the fat cells varied in size. It has been shown that the amount of total and ubiquitous RNA is cell size dependent [25]. Accordingly, the γ-actin mRNA correlated with cell size in our samples (data not shown). In all subjects taken together, the PAI-1 mRNA levels correlated significantly with the amount of PAI-1 secreted ($r = 0.47$, $p < 0.01$). As two distinct groups had been chosen for the study, lean and grossly obese individuals, this relation was also examined among the obese subjects, in whom a moderately strong correlation was found between PAI-1 mRNA levels and PAI-1 secretion ($r = 31$, $p = 0.09$). Group size rendered the corresponding analysis inappropriate in the non-obese group. There were also clear associations between adipose tissue secretion of PAI-1 and PAI-1 mRNA levels on the one hand and fat cell volume on the other ($r = 0.78$, $p < 0.0001$ and $r = 0.59$, $p < 0.0001$, respectively, in all samples analysed together and $r = 0.68$, $p < 0.0001$ and $r = 0.51$, $p < 0.01$, respectively, in the obese group) (Fig. 6A and B). Around 40% (adjusted r^2) of the variation in PAI-1 secretion could be attributed to variation in fat cell volume. In addition, adipose tissue secretion of PAI-1 correlated significantly with

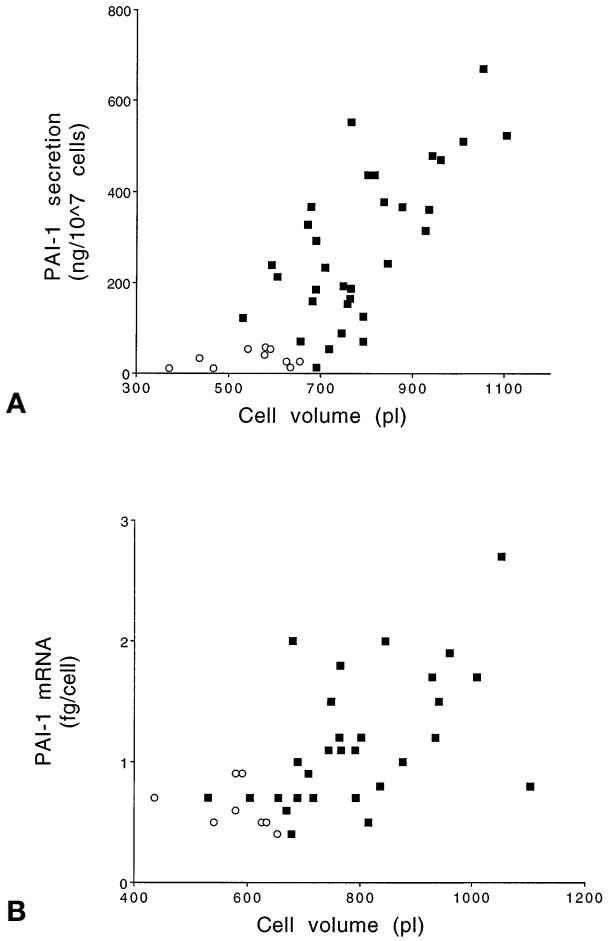

Fig. 6A, B. Associations between adipose tissue secretion of PAI-1 and PAI-1 mRNA levels on the one hand and fat cell volume on the other. **A**. Relationship between adipose tissue secretion of PAI-1 and fat cell volume ($r = 0.68$, $p < 0.0001$, in obese individuals). **B**. Relationship beween PAI-1 mRNA levels and fat cell volume ($r = 0.51$, $p < 0.0001$, in obese individuals). ■ and ○ denote obese and non-obese individuals, respectively

BMI ($r = 0.66$, $p < 0.0001$ for the entire group and $r = 0.39$, $p < 0.05$ for obese individuals). No sex differences were found for PAI-1 secretion (data not shown).

Discussion

This study examined the role of adipose tissue synthesis of PAI-1. The results clearly show that human adipose tissue releases significant amounts of PAI-1 protein in vitro and that this release is increased in obesity.

Furthermore, the elevated plasma PAI-1 activity and increased PAI-1 secretion from adipose tissue of obese individuals were accompanied by an increased steady-state expression of the PAI-1 gene. Both adipocyte PAI-1 mRNA and adipocyte PAI-1 secretion rate were related to adipocyte volume. It is therefore tempting to speculate that overexpression of the PAI-1 gene in adipose tissue is the basic disturbance underlying the increased plasma PAI-1 activity in obesity. When the data on PAI-1, BMI and fat cell volume are considered together, it appears that accumulation of lipids in the fat cells of adipose tissue has a strong influence on PAI-1 secretion. Thus, the increased adipose tissue secretion of PAI-1 and increased plasma PAI-1 activity in obesity might be explained by a combination of an increased fat cell volume and an increased total fat mass. It has been demonstrated that triglyceride-rich lipoproteins can stimulate PAI-1 secretion [26--28] and enhance PAI-1 transcriptional activity (Eriksson et al. unpublished) and mRNA stability [29] in vitro. It can be envisioned that fat cells filled with triglycerides show an increased PAI-1 expression as a result of similar mechanisms.

Not much is known about the regulation of gene expression in fat cells. Recent data suggest that cytokines, in particular tumour necrosis factor-α (TNFα), are of importance. Incubation of mice fat cells with TNFα increases the PAI-1 mRNA concentration in these cells [13]. In a recent study on a similar cohort to the present one, we observed increased expression of the TNFα gene and enhanced secretion of TNFα from subcutaneous abdominal fat tissue [30, 31]. It is likely that the present obese subjects also had elevated adipose tissue expression and secretion of TNFα which could have been of importance for increased PAI-1 expression in the same tissue. However, in a recent study, transforming growth factor-β1 significantly increased PAI-1 antigen production by adipocytes in culture, whereas TNFα did not have any effect [15]. Another regulatory factor could be insulin. It has previously been demonstrated that incubation of hepatocytes with this hormone stimulates PAI-1 gene expression [32--35]. The marked hyperinsulinaemia of the obese individuals studied might have stimulated expression and secretion of adipose tissue PAI-1.

Subjects of either sex were investigated in this study. A separation of the material according to gender did not reveal any significant differences between men and women. This is in contrast to the findings with leptin, another adipose tissue derived protein. The gene expression and circulating plasma levels of leptin are higher in women than in men [31]. This gender difference is more apparent in obese than in non-obese subjects. In other words, different mechanisms may be responsible for the increase in adipose tissue leptin and PAI-1 in obesity, involving gender-specific factors for leptin but not for PAI-1.

It is well established that adipose tissue is a heterogeneous metabolic organ [36]. Also the protein secretory function may be subjected to regional variations. Interestingly, rat PAI-1 mRNA expression has been shown to be lower in subcutaneous than in visceral

fat [14]. Furthermore, plasma PAI-1 levels correlated with visceral fat area, but not with subcutaneous fat area, in a study on Japanese subjects [14]. These data may seem at odds with the present study. However, ethnic differences could play a role in the divergent findings. In addition, in contrast to our study, adipose tissue PAI-1 secretion and mRNA levels were not measured in the former study. Unfortunately, we have no information about visceral fat in our subjects.

What then is the clinical relevance of the present findings? CHD is frequently accompanied by abdominal obesity [2] and elevated plasma levels of PAI-1 [5--8], the latter being associated with recurrent cardiovascular events [10--12]. Insulin resistance, which is intimately related to obesity, is a predictor of CHD [37] and a major determinant of plasma PAI-1 elevation [1]. Furthermore, the prognostic role of PAI-1 in CHD is related principally to insulin resistance [12]. However, the source of the excess plasma PAI-1 in obese, insulin-resistance patients at high risk of CHD is unknown. Our data suggest that the elevation in plasma PAI-1 associated with obesity results at least in part from increased synthesis of PAI-1 by adipose tissue itself. Other potential sources of PAI-1, such as hepatocytes and endothelial cells, may also contribute to the elevated plasma PAI-1 seen in obese subjects. Obesity is also a major cause of pulmonary embolism and deep vein thrombosis, thrombotic conditions for which increased plasma PAI-1 activity could be of major importance.

In summary, this study shows that adipose tissue secretes significant amounts of PAI-1 and that PAI-1 secretion from adipose tissue is increased in obese individuals with elevated plasma PAI-1 activity. The secretion and mRNA expression of PAI-1 is associated with the cell size and the lipid content of fat cells. Elevated plasma PAI-1 in obesity may be caused by increased expression of adipocyte PAI-1, which, in turn, leads to increased secretion of the protein from adipose tissue.

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