Increased plasma levels of plasminogen activator inhibitor-1 and soluble vascular cell adhesion molecule after triacylglycerol infusion in man

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Summary
Increased plasma plasminogen activator inhibitor-1 (PAI-1) has been implicated in the development of vascular disease. In type 2 diabetes mellitus high PAI-1 levels are associated with increased plasma concentrations of free fatty acids (FFA) and triacylglycerol indicating an association or a causal relationship. To answer that question, the effect of FFA/triacylglycerol on plasma PAI-1 was examined. Ten healthy male volunteers were studied for 6 h during infusion of triacylglycerol [1.5 ml/min]/heparin [0.2 IU/(kg·min)] (LIP; n=10), saline only (SAL; n=10), and saline/heparin (HEP; n=5). Plasma insulin concentrations were kept constant at ~35 pmol/l by intravenous somatostatin-insulin infusions and there was no significant change in plasma glucose levels during any of the study protocols. LIP increased plasma triacylglycerol and FFA ~3- (p<0.001) and ~8- (p<0.000001) fold, respectively, within 90 min. Baseline plasma PAI-1 measured by a bio-immunoassay was similar in HEP (11.4±2.8 ng/ml), SAL (16.6±3.6 ng/ml), and LIP studies (15.2±3.4 ng/ml). Since studies were initiated in the morning, PAI-1 decreased (p<0.025) over time following its normal diurnal variation to 6.4±2.0 ng/ml and 4.0±2.4 ng/ml at 360 min in SAL and HEP, respectively. During LIP, however, PAI-1 increased to ~2.6 fold higher levels than during SAL at 360 min (16.4±4.0 ng/ml, p<0.01). While tissue plasminogen activator (tPA) and adipsin, an adipocyte derived protease, were unaffected by LIP, changes in soluble vascular cell adhesion molecule-1 (sVCAM-1) were significantly correlated (p=0.02) with those seen for PAI-1. This suggests that hyperlipidemia independent of insulin and plasma glucose levels stimulates vascular tissue and in turn might induce an increase in plasma PAI-1. PAI-1 then could contribute to the development of atherothrombotic vascular disease.

Keywords
Fibrinolysis, PAI-1, VCAM-1, adhesion molecules, vascular disease, insulin resistance, free fatty acids

Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Introduction
Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator in vivo (1). Elevation of plasma PAI-1 activity found in patients with cardiovascular disease (2, 3) strongly suggests a relationship between PAI-1 and the development of thrombosis and atherosclerosis. A recent study showing that PAI-1 deficient mice are protected against atherosclerosis progression (4) supports the hypothesis that PAI-1 contributes...
to the pathogenesis of atherosclerosis by inhibiting local fibrin clearance (5). Plasma PAI-1 is also closely correlated to serum triacylglycerol concentrations and insulin resistance (6). Therefore, PAI-1 might represent a link between insulin resistance and coronary artery disease (7). Plasma concentrations of free fatty acids (FFA), triacylglycerol, and insulin, all elevated in insulin resistance and diabetes mellitus type 2 (8, 9), might be the mediators of PAI-1 elevation. (Pro)insulin (10, 11), VLDL-triacylglycerol, and FFA (12, 13) stimulate PAI-1 production in hepatocytes, whereas VLDL (14, 15), and FFA (16, 17) enhance PAI-1 expression in cultured human endothelial cells. However, cells might behave differently under in vitro or in vivo conditions. Although insulin infusion induced an increase in PAI-1 in rabbits (18), no elevation of plasma PAI-1 was observed upon infusion of insulin in man (19, 20). In a recent study combined hyperinsulinemia, hyperglycemia, and hypertriacylglycerolemia resulted in a rise of plasma PAI-1 in healthy men (19). This study, however, did not discriminate which of these variables was primarily responsible for induction of PAI-1.

To answer that question we determined the effect of an increase in plasma FFA/triacylglycerol on PAI-1 plasma levels in the presence of constant plasma insulin concentrations. To obtain information on possible effects of FFA/triacylglycerol elevation on tissues and organs potentially involved in the regulation of PAI-1 plasma levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) as a marker for vascular cells (21-23) and tissue plasminogen activator (tPA) were determined simultaneously. Since tPA is rapidly cleared by the liver, altered liver perfusion and/or tPA clearance by the liver would affect tPA plasma levels (24-26). Furthermore, adipsin, an adipocyte derived protease (27), which has been shown previously by others and us to correlate with PAI-1 in obese, insulin resistant subjects (28, 29) was also determined.

We found that infusion of a triacylglycerol emulsion together with heparin resulted in short term increase of plasma FFA/triacylglycerol concomitantly with plasma PAI-1 and sVCAM levels, while no specific effect on plasma concentrations of tPA and adipsin was seen. This suggests that FFA/triacylglycerol increase plasma PAI-1 levels rather via an effect on vascular cells.

**Methods**

**Subjects**

Ten healthy male volunteers (25±1 yrs; body mass index: 22.5±0.5 kg/m²) without family history of diabetes mellitus or dyslipidemia were included in this study. They were neither glucose intolerant, nor suffering from conditions related to insulin resistance, nor taking any medications on a regular basis. The different study days were separated by an interval of 1 to 25 weeks, during which body weight, diet, lifestyle, and basal metabolic parameters remained unchanged. The protocol was reviewed and approved by the Human Ethics Committee of the University of Vienna Medical School, and informed consent was obtained from all subjects after the nature of the procedure has been explained to them.

**Study protocol**

Each subject was studied at least twice. They were assigned either to triacylglycerol emulsion/heparin or saline infusion and subsequently to the other regimen in a formally randomized order. Five of the subjects participated in an additional protocol that employed saline/heparin infusion to evaluate effects of heparin itself. All studies were started at 9:00 a.m. (-60 min timepoint) after an overnight fast of 8 h with the insertion of one teflon catheter for blood sampling into a hand vein and two catheters for infusions into antecubital veins. Through one catheter insulin (Humulin; Lilly, Fegersheim, France) together with D-glucose (20% (w/v) Leopold Pharma, Linz, Austria) and somatostatin (UCB Pharma, Vienna, Austria) were infused. Triacylglycerol emulsion (Intralipid 20%; Pharmacia & Upjohn, Vienna, Austria), heparin (Baxter AG, Vienna, Austria), or normal saline were administered via the other catheter.

To examine the effects of acute hyperlipidemia on the fibrinolytic system, plasma FFA concentrations were raised as described previously (30) by combined infusion (0 - 360 min) of a triacylglycerol emulsion (Intralipid 20%) (1.5 ml/min) and heparin [200 IU bolus, continuous infusion 0.2 IU/(kg·min)]. Heparin was used to stimulate lipoprotein lipase and in turn to catalyze the hydrolysis of triacylglycerols (31). Somatostatin [0.1 μU/(kg·min)] was administered from -10 min until 360 min to inhibit endogenous secretion of insulin, glucagon, and growth hormone (32). Plasma insulin was maintained at fasting peripheral concentrations (~35 pmol/l) by continuous insulin [0.1 mU/(kg·min)]- glucose [variable] infusion (0 - 360 min).

**Table 1: Metabolic data.**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lipid/Hep. (n=10)</th>
<th>Saline/Hep. (n=5)</th>
<th>Saline (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.9±0.2</td>
<td>4.8±0.2</td>
<td>5.1±0.1</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>35±5</td>
<td>37±5</td>
<td>39±5</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>2.2±0.1</td>
<td>3.1±0.1</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
<td>0.8±0.1</td>
</tr>
</tbody>
</table>

* p<0.0001 vs saline, † p<0.005 vs respective subjects during lipid/heparin infusion, ‡ p<0.001 vs saline, †µ p<0.01 vs respective subjects during lipid/heparin infusion.
During control studies normal saline (0.9% NaCl) [1.5 ml/min], or saline and heparin [200 IU bolus, continuous infusion 0.2 IU/(kg min)] were infused instead of triacylglycerol/heparin. All infused solutions were free of endotoxin.

Blood was collected through the third catheter, into vials containing EDTA (0.18% final concentration) at 0 min (baseline), 45 min, 180 min, and 360 min and immediately placed on ice. Platelet-poor plasma was obtained by centrifugation for 15 min at 2500 g at 4°C within 1 h after blood collection, immediately frozen in aliquots, and stored at -70°C until analyzed.

**Analytical methods**

Plasma glucose concentrations were measured by the glucose oxidase method (Glucose analyzer II; Beckman Instr., Inc., Fullerton, CA). Plasma FFA concentrations were determined using a microfluorimetric method (Wako Chem USA Inc., Richmond, VA). Plasma triacylglycerol was hydrolyzed by lipase and the released glycerol was measured by a peroxidase coupled colorimetric assay (34). PAI-1 was determined using the Actibind®-PAI-1 assay (Technoclone, Vienna, Austria) according to the manufacturer’s protocol. tPA was determined in the same plasma samples by a specific solid phase ELISA based on monoclonal antibodies (Technoclone, Vienna, Austria). This assay recognizes all species of tPA including tPA in complex with PAI-1. Plasma adipin concentrations were measured by a specific ELISA using monoclonal antibodies as described previously (29). The inter- and intra-assay variations of the PAI-1 activity and adipin assays were less than 10% and 5%, respectively. The inter- and intra-assay variations of the tPA antigen assay were less than 15% and 10%, respectively. Soluble VCAM-1 (sVCAM-1) was analyzed by a commercially available ELISA (British Bio-Technology Product Ltd., Abdington, UK) following the manufacturer’s instructions with both inter- and intraassay coefficients of variation being less than 9%. In order to exclude a possible interference of high plasma triacylglycerol concentrations with the PAI-1, tPA or sVCAM-1 assays used, triacylglycerol concentrations in pooled normal EDTA-plasma were increased by addition of a triacylglycerol emulsion. No effect of high triacylglycerol concentrations on PAI-1, tPA, and sVCAM-1 measurements was observed (data not shown).

**Figure 1:** Panel A. Time course of plasma PAI-1 during infusion of triacylglycerol/heparin (●) or saline (○) in the same subjects (n=10). All data are expressed as means±SEM. *p<0.025 vs saline

Panel B. Time course of plasma PAI-1 during infusion of saline/heparin (●) or saline (○) in 5 of the 10 subjects shown in panel A. All data are expressed as means±SEM.

Panel C. Time course of the differences in PAI-1 between triacylglycerol/heparin and saline infusion studies (●; n=10) and between saline/heparin and saline infusion studies (○; n=5), respectively. All data are expressed as means±SEM. *p<0.025 vs baseline and vs saline/heparin-saline.
Data analysis

All data are given as means ± SEM. Normal distribution of the values within a group was tested by the Kolmogorov-Smirnov test. Statistical comparisons between normally distributed groups were performed using paired or unpaired student’s t-tests as appropriate. Data within a group were compared by repeated measurement analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc testing. Associations between two variables were tested with Spearman’s correlation coefficient. A p-value of less than 0.025 was considered statistically significant. Statistical analysis was performed using Prism (V.2.01) software.

Results

Fasting plasma concentrations of glucose, insulin, FFA, and triacylglycerol were comparable in saline, saline/heparin and lipid/heparin infusion studies (Table 1). During somatostatin-insulin infusion, plasma insulin concentrations remained at baseline and were similar during triacylglycerol and control studies. During triacylglycerol/heparin infusion, plasma triacylglycerol and FFA markedly increased as compared to controls (Table 1). Plasma glucose remained constant in the saline and saline/heparin studies, mean plasma glucose concentrations slightly, but not significantly increased in the absence of exogenous glucose administration during lipid infusion (Table 1).

Baseline plasma PAI-1 was similar in saline and lipid/heparin studies (Fig. 1A). Following its known diurnal variation PAI-1 decreased (p<0.01) during saline infusion. The extent of this decline in PAI-1 (~50%) is in line with previous studies (35-37). During triacylglycerol/heparin infusion, PAI-1 decreased however only initially (45-180 min), but then increased to ~2.6-fold higher levels than during saline infusion at 360 min (p=0.011) (Fig. 1A). To evaluate effects of heparin on plasma PAI-1, the time courses of PAI-1 during infusion of saline/heparin and of saline were compared in 5 subjects (Fig. 1B). Heparin administration had no effect on plasma PAI-1. As demonstrated in Figure 1C, the differences in PAI-1 between triacylglycerol/heparin and saline infusion constantly increased over time, while the difference between saline/heparin and saline was always negative, indicating a specific effect of triacylglycerol on PAI-1 plasma levels.

Baseline plasma tPA concentrations were comparable in saline (5.1±1.5 ng/ml), triacylglycerol/heparin (4.6±1.2 ng/ml), and saline/heparin (2.1±0.3 ng/ml) studies. tPA slightly decreased during saline control experiments (Fig. 2). However, during lipid/heparin and saline/heparin infusion plasma tPA antigen increased without a significant difference between these two latter protocols (Fig. 2).

Baseline plasma concentrations of sVCAM-1 were not different between the three protocols (Fig. 3). During lipid/heparin infusion, however, sVCAM-1 steadily increased (p<0.001) and was ~25% higher as compared to saline (p=0.001) infusion at the end of the studies.

Baseline plasma adipsin concentrations were comparable in lipid/heparin (1.47±0.09 µg/ml) and saline (1.39±0.07 µg/ml) studies and similarly decreased slightly, but not significantly

Figure 2: Time course of plasma concentrations of tPA antigen during infusion of triacylglycerol/heparin (●, n=10), saline (○, n=10), or saline/heparin (■, n=5). All data are given as mean±SEM. * p<0.025 vs respective subjects during saline infusion. ** p<0.005 vs saline.

Figure 3: Time course of plasma concentrations of sVCAM antigen during infusion of triacylglycerol/heparin (●, n=10), saline (○, n=10), or saline/heparin (■, n=5). All data are given as mean±SEM. * p<0.025 vs saline, ** p=0.001 vs saline.
to 1.22±0.1 µg/ml and 1.14±0.08 µg/ml at 360 min of lipid/heparin and saline infusions, respectively. This indicates that none of the infusion protocols had a significant effect on plasma adipsin levels.

During lipid infusion the changes from baseline in sVCAM-1 were significantly correlated to changes in PAI-1 at 360 min (r=0.75, p=0.02; Fig. 4). No significant correlation was observed between the changes in tPA or the small changes in adipsin and the changes in PAI-1 during lipid infusion (data not shown).

**Discussion**

This study demonstrates that short-term (6 h) elevation of plasma FFA/triacylglycerol by combined triacylglycerol/heparin infusion increased plasma PAI-1 in the presence of constant stable fasting insulin concentrations. Simultaneously lipid infusion induced a rise in the plasma concentrations of the soluble form of the endothelial adhesion molecule VCAM-1 (sVCAM-1) but had no specific effect on plasma tPA and no significant effect on plasma glucose levels. Heparin alone had neither an effect on plasma PAI-1, which is in accordance with previously published data (38-40), nor on sVCAM-1. Adipsin remained unaffected by all regimens used. The slight rise in plasma glucose concentrations during lipid infusion might indicate lipid-induced inhibition of glucose uptake (30).

So far the predominant cellular source of plasma PAI-1 is not known. The high expression of PAI-1 in cultured endothelial cells (41) suggests that these cells at least are capable to substantially contribute to plasma PAI-1. However, the presence of PAI-1 in various other cell types (e.g. hepatocytes, smooth muscle cells) and many tissues including liver and adipose tissue rather suggests that plasma PAI-1 may originate from a variety of tissues (42, 43). Platelets are also a rich source of PAI-1 (44); however, PAI-1 is released from platelets in its inactive latent form (1, 43) that is not recognized by the assay used. Therefore, changes in PAI-1 plasma levels observed in this study cannot be attributed to an effect of FFA/triacylglycerol on platelet PAI-1 release.

The results described in this report suggest that increased plasma PAI-1, associated with insulin resistance and type 2 diabetes, can at least in part be attributed to increased plasma concentrations of FFA and triacylglycerol. Previous in vivo studies demonstrated a strong positive correlation between plasma triacylglycerol and plasma PAI-1 activity (6, 45).

Increased secretion or decreased clearance of PAI-1 might be responsible for the rise in plasma PAI-1 observed during FFA/triacylglycerol infusion. Since tPA and tPA/PAI-1 complexes are rapidly cleared from the circulation by the liver, where they are taken up by receptor mediated mechanisms (1), changes in liver perfusion and/or tPA clearance will affect tPA plasma levels (24-26). In the present study tPA increased similarly during lipid/heparin and saline/heparin infusions indicating that this rise in plasma tPA can be attributed to heparin. Elevation of plasma tPA by heparin administration has been shown in several previous studies (38, 39, 46). A possible mechanism for this increase in tPA concentration could be a heparin induced release of tPA from binding sites on the surface of endothelial cells (47). Alternatively, heparin might also induce tPA synthesis (48). Plasma FFA/triacylglycerol infusion, however, had no specific effect on plasma tPA. Therefore, an effect of lipid infusion on clearance of tPA and in turn PAI-1 cleared by the liver as tPA/PAI-1 complex, is unlikely. This assumption is supported by a previous study that showed no influence of a comparable lipid infusion on liver blood flow, tPA synthesis and tPA clearance. Taken together, the observed increase in plasma PAI-1 is not likely due to a tPA dependent mechanism such as liver clearance.

On the other hand the present report demonstrates that lipid infusion increases plasma concentrations of sVCAM-1. Increased concentrations of circulating soluble adhesion molecules in hyperlipidemia (49) and diabetes mellitus (23) have been reported and may indicate stimulation of vascular cells. Endothelial cells release sVCAM-1 in relation to VCAM-1 expression on the cell surface (21, 22). Therefore, the observed increase in plasma sVCAM-1 concentrations during FFA/triacylglycerol elevation likely reflects vascular cell activation associated with selective upregulation and shedding of VCAM-1. Since the changes in sVCAM-1 and PAI-1 were significantly correlated and since vascular cells including endothelial cells (41) are also a possible source of plasma PAI-1, it can be speculated that vascular cells might contribute to the increase in plasma PAI-1 during triacylglycerol/heparin infusions.

![Figure 4: Correlation of the changes from baseline in sVCAM-1 to changes in PAI-1 at 360 min of triacylglycerol/heparin infusion. r=0.75, p=0.02](image-url)
Lipoproteins were shown to carry the cytokine transforming growth factor β (TGF-β) (50) as well as endotoxin (51). Both are known inducers of PAI-1 synthesis (43). Previous studies employing lipid infusions in the absence of heparin could not detect an effect on plasma PAI-1 (52, 53), indicating that the carrier function of plasma lipids for cytokines and endotoxin most likely did not contribute to the effects observed in our study. In these studies plasma FFA concentrations might not have been raised significantly because heparin, which stimulates the endothelial lipoprotein lipase to hydrolyze triacylglycerol (31), was not administered simultaneously with lipids. Recent in vitro data support the hypothesis that FFA-induced expression of PAI-1 might play an important role in the elevation of plasma PAI-1 activity in insulin resistance. It has been shown that triacylglycerol rich VLDL induce PAI-1 in cultured hepatocytes and endothelial cells (13-15, 54) an effect that might be mediated by released FFA; FFA increase PAI-1 expression in cultured endothelial cells and hepatocytes (12,13,16,17). These data indicate that FFA in fact might be the actual mediators responsible for the rise in plasma PAI-1 observed in our studies.

Plasma triacylglycerol concentrations reached during lipid infusion (~ 2.3 mmol/l) compare well with those measured in patients with premature vascular disease and insulin resistance (6, 55). The plasma FFA concentrations observed in the present study (~3 mmol/l) are also within the range of those [~0.75 mmol/l (56) to ~4.7 mmol/l (57, 58)] reported previously employing similar triglyceride/heparin infusion regimens. FFA concentrations of ~1 mmol/l (59, 60) up to ~3 mmol/l (61) may occur in vivo in association with insulin resistance and type 2 diabetes.

In conclusion, our results demonstrate that an acute rise in plasma FFA/triacylglycerol concentrations induces plasma PAI-1. The simultaneous rise in sVCAM-1 and its correlation to changes in plasma PAI-1 levels suggests activation of vascular cells, a possible source of increased plasma PAI-1. Elevated plasma PAI-1 is a predictor of coronary events (3) and has been implicated in the development of vascular disease (5). Therefore hyperlipidemia may contribute to increased PAI-1 and in turn accelerated atherothrombotic vascular disease associated with insulin resistance and type 2 diabetes mellitus.

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Addendum

The following authors significantly contributed to this study: Michael Krebs (idea, organization of the study, contribution in study design and laboratory analysis, wording of the manuscript), Margarethe Geiger (expertise in coagulation and fibrinolysis, funding and supervision of measurements of fibrinolytic variables), Katja Polak (coordination and performance of lipid and saline infusion studies), Anja Vales (contribution in laboratory analysis), Leopold Schmetterer (contribution in study design and infusion studies, logistic support), Oswald F. Wagner (determination of sVCAM-1, expertise in endothelial dysfunction associated with metabolic disorders), Werner Waldhäusl (laboratory analysis of metabolic parameters, pathophysiological expertise), Bernd R. Binder (logistic support, expertise in fibrinolysis and vascular biology, funding, supervision, wording of the manuscript) and Michael Roden (study design, funding, expertise in metabolic studies, wording of the manuscript).

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