Adiponectin Mediates the Suppressive Effect of Rosiglitazone on Plasminogen Activator Inhibitor-1 Production

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Objective—The purpose of this study was to examine the effects of PPAR-γ agonist rosiglitazone, relative to sulfonylureas, on circulating levels of adiponectin and the prothrombotic factor, plasminogen activator inhibitor (PAI)-1, in type 2 diabetic patients, and to investigate, in animal models, whether the antithrombotic action of rosiglitazone was mediated through adiponectin.

Methods and Results—Our clinical study (n=64) showed that after 24-week add-on therapy, the rosiglitazone group had a greater mean reduction in plasma PAI-1 levels (25%, versus 12% in sulfonylurea group, P=0.002). Stepwise multiple linear regression analysis identified the reduction in plasma fasting glucose and the rise in adiponectin levels to be independently associated with the reduction in PAI-1 concentration in the rosiglitazone-treated patients. Rosiglitazone (20 mg/kg/d) reduced adipose tissue PAI-1 mRNA expression and its plasma levels in wild-type C57 mice with diet-induced obesity (P<0.001), but this suppressive effect was attenuated in adiponectin knockout mice. Adenovirus-mediated overexpression of adiponectin led to a significant suppression of adipose tissue PAI-1 expression and its circulating concentrations in db/db diabetic mice. Our in vitro study demonstrated that recombinant adiponectin directly inhibited PAI-1 production in 3T3-L1 adipocytes.

Conclusions—The antithrombotic effect of rosiglitazone is mediated, at least in part, through the suppressive effect of adiponectin on PAI-1 production. (Arterioscler Thromb Vasc Biol. 2007;27:2777-2782.)

Key Words: obesity ■ hyperglycemia ■ adipokines ■ thrombotic diseases

Plasminogen activator inhibitor (PAI)-1, a member of the serine protease inhibitor family, regulates thrombus formation through the inhibition of tissue plasminogen activator, an anticoagulant factor, and plays a key role in the pathogenesis of cardiovascular events. In apolipoprotein E-deficient mice, an accelerated atherosclerosis animal model, PAI-1 deficiency protects against atherosclerosis progression after photochemical injury to carotid atherosclerotic plaques, and reduces neointima formation after oxidative, and copper induced arterial injuries. Likewise, increased PAI-1 expression, mediated by an adenoviral vector, has been shown to promote neointima growth in balloon-injured rat carotid arteries. In clinical studies, elevated plasma PAI-1 levels are found in subjects with atherosclerotic diseases and have been shown to predict the development of cardiovascular events. In middle-aged men, a high baseline plasma PAI-1 level increases the prediction of coronary heart disease and ischemic stroke, in addition to conventional risk factors. Subjects with obesity, impaired glucose tolerance, and type 2 diabetes, conditions known to be associated with insulin resistance, have elevated PAI-1 levels, which correlate with peripheral insulin resistance, assessed by hyperinsulinemic euglycemic clamp study. In addition, pharmacological therapies with insulin sensitizers such as biguanide and thiazolidinediones have been shown to reduce circulating PAI-1 levels in subjects with type 2 diabetes. In cultured human preadipocytes, a direct attenuation of PAI-1 mRNA and protein expression by thiazolidinediones, possibly mediated through PPAR-γ activation, has been demonstrated. It should be noted that within the adipose tissue, preadipocytes, dominating the stromal cell fraction, is an important source of PAI-1 production, especially in subjects with visceral obesity.

The mechanism whereby PPAR-γ agonists reduce PAI-1 expression in adipose tissue is unclear. One possibility is that this may be mediated through other adipokines, such as

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2777
adiponectin and tumor necrosis factor (TNF)-α, the dysregulation of which in type 2 diabetes and obesity is also ameliorated by PPAR-γ agonists. An inverse relationship between circulating adiponectin and PAI-1 levels has been previously reported in patients with coronary artery disease. In contrast to PAI-1, serum adiponectin levels are reduced in type 2 diabetes, obesity, and coronary artery disease, and high levels are protective against cardiovascular diseases in humans and animals. We hypothesize that adiponectin, an insulin-sensitizing and antiatherogenic adipokine which exhibits a 2-fold increase on thiazolidinedione therapy, mediates the suppressive effect of PPAR-γ agonists on circulating PAI-1 levels. Here we report our findings from clinical, animal, and in vitro studies which have provided support for this hypothesis.

Materials and Methods

Clinical Study

Subjects

The effects of rosiglitazone versus sulfonylurea on circulating PAI-1 and adiponectin levels, were investigated in a randomized, open-label, parallel group study. The study involved 64 Chinese subjects with type 2 diabetes, treated with diet alone, metformin, or sulfonylurea <half-maximum dose for at least 6 months, with suboptimal glycemic control, as defined by HbA1c ≥7.5%. Patients were randomly assigned to receive add-on therapy with either rosiglitzone 4 mg or sulfonylurea (glibenclamide 5 mg or glimepiride 80 mg) daily for 4 weeks, while keeping the doses of their usual antidiabetic agents constant. The doses of the add-on therapy were doubled after 4 weeks in subjects with fasting blood glucose level >8.0 mmol/L and without symptomatic or asymptomatic hypoglycemia, defined as blood glucose level <3.0 mmol/L. The final doses of all antidiabetic agents were then kept constant for another 20 weeks. The study protocol was approved by the local Institutional Review Board, and written informed consent was obtained from all subjects.

Biochemical Measurements

Circulating PAI-1 level was measured in fasting blood samples taken at 9 to 10 AM, by using an in-house human PAI-1-ELISA kit, as described in the supplemental materials (available online at http://atvb.ahajournals.org). Serum adiponectin level was also determined with an in-house ELISA assay. Plasma high-sensitivity C-reactive protein (hs-CRP) was measured with a particle-enhanced immunoturbidimetric assay (Roche). Serum malondialdehyde (MDA) and total plasma 8-isoprostane were measured using OXI-TEK TBARS assay kit (Alexis Biochemicals) and a specific enzyme immunoassay kit (Cayman Chemical), respectively. Serum insulin measurement and other biochemical assays were performed as previously described.

Animal Studies

Mice

C57BL/6N mice and C57BL/KsJ db/db diabetic mice were purchased from the Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. The adiponectin-knockout (adiponectin-KO) mice with C57BL/6J background were generated at the laboratory of Lawrence Chan (Baylor College, University of Texas, Houston). C57 BL/6N (wild-type) mice and adiponectin-KO mice with diet-induced obesity were generated by allowing free access of a high-fat diet (45 kcal% from fat, D12451, Research Diet), from the age of 4 weeks.

Study Protocol

Mice used in this experiment were 10 to 12 weeks of age, housed in a room at controlled temperature [23 ± 1°C] with a 12 hour light-dark cycle, and had free access to water and standard mouse diet (db/db and control C57 BL/6N) or high-fat diet. Rosiglitazone (20 mg/kg) (GSK) was daily administrated to wild-type and adiponectin-KO mice with diet-induced obesity by intragastric gavage for 2 weeks. Recombinant adenoviruses expressing murine adiponectin or luciferase (as a control) were produced as we previously described, and 10^7 pfu of the recombinant adenovirus were introduced into db/db diabetic mice through tail-vein injection. Mice were fasted for 6 hours (steady-state) and blood glucose levels were measured using Accu-Check Advantage II Glucometer (Roche) before euthanized at day 14 after adenovirus injection. Circulating levels of mouse PAI-1 and adiponectin were measured using the mouse adipokine assay kit from Linco Research and an in-house adiponectin ELISA assay, respectively. The mRNA abundance of PAI-1 in adipose tissue was quantified using real-time polymerase chain reaction (PCR) as we described elsewhere. All of the experiments were conducted in accordance with our institutional guidelines for the humane treatment of laboratory animals.

Statistical Analysis

All continuous variables are expressed as mean±SEM or median and interquartile range, if the data were not normally distributed. Statistical analyses were performed using the SPSS 13.0 software package (SPSS). Comparison of variables between 2 groups was performed using independent sample t test or paired t test as appropriate. Skewed data were logarithmically transformed before analysis. The log-change ratio was obtained by taking the log of the ratio of the postmeasurement to the premeasurement. Stepwise multiple linear regression analyses were used to examine the association of PAI-1 reduction with the changes in other parameters which showed a significant change in the rosiglitazone group, but not in the sulfonylurea group, after Bonferroni correction for multiple testing. A 2-sided value of P<0.05 was considered significant.

Results

Changes in Serum Adiponectin and Fasting Blood Glucose Were Independently Associated With the PAI-1 Reduction in Rosiglitazone-Treated Patients

The study subjects received add-on therapy with either rosiglitazone (21 M+11 F; aged 55.4±9.8 years) or sulfonylurea (15 M+17 F; aged 57.1±10.1 years). Baseline clinical characteristics, metabolic profiles and concomitant medications were similar between the 2 groups except that current smoking was more prevalent in the rosiglitazone group (22 versus 9%, P=0.05). The difference in baseline PAI-1 levels between the 2 groups was not statistically significant (P=0.087). The changes in cardiometabolic parameters, versus baseline values, after 24-week of add-on therapy are summarized in Table 1: both rosiglitazone and sulfonylurea resulted in significant reductions in HbA1c, fasting plasma glucose, and MDA. Both treatment groups also had a significant reduction in PAI-1 (−25.2%, P<0.001 and −11.9%, P=0.001, respectively), with a greater reduction being seen in the rosiglitazone group (P=0.002 versus sulfonylurea group). Plasma adiponectin showed a marked rise in the rosiglitazone group (P<0.001), whereas only a slight, albeit statistically significant increase (P=0.036) was seen in the sulfonylurea group. Rosiglitazone, but not sulfonylurea, significantly reduced HOMA-IR and free fatty acid (FFA). Neither group showed a significant change in fasting insulin. A significant change in BMI was seen only in the rosiglitazone group.

After Bonferroni’s correction for multiple comparisons, the parameters with significant change in the rosiglitazone group, but not in the sulfonylurea group, included fasting glucose (and hence HOMA-IR), BMI, adiponectin, and FFA. On
stepwise multiple linear regression analysis, only the changes in fasting glucose and adiponectin were significantly and independently associated with the reduction in PAI-1 in the rosiglitazone group. These 2 parameters accounted for 43.8% of the total variation in PAI-1, with 15.6% being explained by the change in adiponectin level (Table 2).

The Suppressive Effects of Rosiglitazone on PAI-1 Expression Was Attenuated in Adiponectin-KO Mice

To investigate whether rosiglitazone reduced circulating PAI-1 levels through its effect on adiponectin expression, both adiponectin-KO mice and the wild-type littermates (controls) were fed with a high-fat diet and treated with daily high dose rosiglitazone for 2 weeks. In wild type mice with diet-induced-obesity (DIO), rosiglitazone treatment significantly increased serum adiponectin concentrations (vehicle: 12.4±3.7 µg/mL; Rosiglitazone: 21.6±5.9 µg/mL, P=0.016), and also markedly reduced the PAI-1 expression level in their adipose tissue (Figure 1a). On the other hand, there was only a small but statistically significant reduction of PAI-1 expression in adiponectin-KO mice with DIO. After rosiglitazone treatment in the mice with DIO, plasma PAI-1 concentrations were also significantly decreased in control mice (Vehicle: 47.7±8.3 ng/mL, Rosiglitazone: 24.1±5.2 ng/mL, P=0.013, n=10), whereas the reduction was not statistically significant in the adiponectin-KO mice (Vehicle: 54.5±7.9; Rosiglitazone: 42.3±6.7; P=0.074; n=10; Figure 1b). Furthermore, the magnitude of rosiglitazone-mediated decrease in PAI-1 concentration in control mice was significantly greater than that in adiponectin-KO mice (control with DIO: 49.7±6.6%; KO mice with DIO: 22.6±3.5%; p=0.018). Rosiglitazone treatment reduced plasma glucose levels at steady states (6-hour fasting) to a similar extent in KO and control mice (Figure 1c).

PAI-1 Expression Was Increased in db/db Diabetic Mice and Suppressed by Adenovirus-Mediated Overexpression of Adiponectin

Serum adiponectin levels at baseline were 12.7±2.6 µg/mL. After injection of adiponectin-expressing adenoviruses, serum adiponectin started to rise on day 2 (18.9±3.2 µg/mL), peaked on day 7 (40.1±6.7 µg/mL), and then gradually decreased to the basal level by day 21. Injection of luciferase-expressing adenovirus did not affect serum adiponectin levels in db/db mice. PAI-1 expression level in db/db diabetic mice was significantly higher than that in the lean controls. In both db/db diabetic mice and lean

### Table 1. Clinical Characteristics and Metabolic Profile of the Study Groups Before and After Treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rosiglitazone</th>
<th>Sulfonylurea</th>
<th>P</th>
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<tr>
<td>SBP, mm Hg</td>
<td>129.2±2.6</td>
<td>124.5±2.2</td>
<td>NS</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>79.1±1.3</td>
<td>75.5±1.0</td>
<td>NS</td>
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<tr>
<td>BMI, kg/m²</td>
<td>25.4±0.7</td>
<td>26.0±0.7</td>
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<tr>
<td>WC, cm</td>
<td>86.8±1.9</td>
<td>87.4±1.9</td>
<td>NS</td>
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<tr>
<td>WHR</td>
<td>0.90±0.01</td>
<td>0.91±0.01</td>
<td>NS</td>
</tr>
<tr>
<td>FPG, mmol/L</td>
<td>8.7±0.3</td>
<td>7.0±0.3†</td>
<td>&lt;0.001</td>
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<td>HbA1c, %</td>
<td>8.7±0.1</td>
<td>7.6±0.2</td>
<td>&lt;0.001</td>
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<tr>
<td>TC, mmol/L</td>
<td>5.1±0.2</td>
<td>5.2±0.2†</td>
<td>NS</td>
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<tr>
<td>TG, mmol/L</td>
<td>1.6 (1.1–2.3)‡</td>
<td>1.5 (1.1–2.4)‡</td>
<td>NS</td>
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<tr>
<td>LDL, mmol/L</td>
<td>3.1±0.2</td>
<td>3.2±0.2†</td>
<td>NS</td>
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<tr>
<td>HDL, mmol/L</td>
<td>1.21±0.05</td>
<td>1.27±0.06</td>
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<tr>
<td>FFA, mmol/L</td>
<td>0.44±0.03</td>
<td>0.35±0.03</td>
<td>0.003</td>
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<td>HOMA-IR</td>
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<td>2.8±0.3</td>
<td>&lt;0.001</td>
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<tr>
<td>Fasting insulin, mIU/L</td>
<td>9.2 (6.1–13.8)</td>
<td>7.3 (6.0–11.0)</td>
<td>NS</td>
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<tr>
<td>Adiponectin, µg/mL</td>
<td>6.0 (4.2–10.7)</td>
<td>10.6 (6.3–23.5)‡‡</td>
<td>&lt;0.001</td>
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<tr>
<td>MDA, nmol/mL</td>
<td>6.6±0.5</td>
<td>5.7±0.3</td>
<td>0.002</td>
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<tr>
<td>8-Isoprostane, pg/mL</td>
<td>492.1±16.4</td>
<td>479.1±14.6</td>
<td>NS</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>1.5 (0.6–2.7)</td>
<td>1.0 (0.5–2.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>PAI-1, ng/mL</td>
<td>63.1±3.4</td>
<td>47.2±2.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 2. Stepwise Multiple Linear Regression Analysis Showing the Significant Independent Determinants of the Change in PAI-1 in the Rosiglitazone Group

<table>
<thead>
<tr>
<th>Parameters*</th>
<th>Adjusted R²</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG</td>
<td>0.282</td>
<td>0.002</td>
</tr>
<tr>
<td>FPG+Adiponectin†</td>
<td>0.438</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Parameters are log-change ratio before analysis. †Model included adiponectin. FPG (fasting plasma glucose); FFA (free fatty acid) and body mass index.
controls, overexpression of adiponectin alone significantly reduced PAI-1 expression in adipose tissues, and concomitantly decreased circulating PAI-1 concentrations (Figure 2; wild-type/luciferase: 24.1±1.08 ng/mL, wild-type/adiponectin: 16.96±0.44 ng/mL, P=0.042; db/db/luciferase: 62.5±3.12 ng/mL; db/db/adiponectin:34.8±2.39 ng/mL, P=0.001).

Recombinant Adiponectin Directly Inhibited PAI-1 in 3T3-L1 Adipocytes
The direct effect of adiponectin on PAI-1 expression in 3T3-L1 adipocytes was investigated. Chronic treatment of mature adipocytes with recombinant full-length adiponectin caused a marked reduction of PAI-1 mRNA expression in a dose-dependent manner (Figure 3). PAI-1 concentrations in the conditioned medium decreased by ≈33% and 62% after treatment with adiponectin at the concentrations of 5 μg/mL and 10 μg/mL, respectively.

Discussion
Increased PAI-1 expression has been suggested to contribute to the enhanced atherothrombotic risk in type 2 diabetes.25,26 In this study, we had confirmed that treatment with rosiglitazone, a thiazolidinedione antidiabetic drug, could reduce circulating levels of PAI-1 in diabetic patients.27 We further showed that changes in glycemia and serum adiponectin levels were independently associated with changes in PAI-1 levels in rosiglitazone-treated patients. The greater reduction in fasting glucose induced by rosiglitazone (mean dose 6 mg/d), compared with that achieved by sulfonylurea add-on therapy, suggested that a greater improvement in glycemia could have contributed to a greater reduction in PAI-1 levels in the rosiglitazone group. Our data also suggested that the almost 2-fold increase in circulating adiponectin levels in the rosiglitazone-treated patients could have contributed independently to the reduction in PAI-1 levels in these patients.

Our findings were at variance with another study which showed that pioglitazone did not reduce significantly plasma PAI-1 levels, despite elevations in adiponectin,28 although a trend of reduction was apparent. It should be noted that the previous study involved half our sample size and treatment duration, and a relatively lower treatment dose, and the authors conceded that the baseline PAI-1 levels of their patients may not be high enough to accurately assess the lowering effect of pioglitazone.

Our clinical study suggested that adiponectin might be involved in the regulation of PAI-1 expression. Previous studies demonstrated that PAI-1 expression could be upregulated by hyperglycemia,29 FFA, triglycerides, insulin, transforming growth factor (TGF)-β, angiotensin II, CRP, and TNF-α.30 However, the regulation of PAI-1 expression by adiponectin had not been previously reported. We therefore investigated this possibility in 2 sets of animal experiments.

Previous studies reported that a high dose of pioglitazone could reduce insulin resistance even in ob/ob mice with adiponectin deficiency, possibly through PPAR-γ induced reductions in TNF-α and resistin expression, and FFA levels.31 In this study, high-dose rosiglitazone could also reduce...
By the suppressive effects of rosiglitazone on PAI-1, rosiglitazone demonstrated the important role of adiponectin in mediating partly via adiponectin. Although our data clearly expressed in their adipose tissue, compared with lean controls, analogous to increased PAI-1 expression in epididymal fat, a visceral depot, in both diabetic and lean mice. Our in vitro study showed that adiponectin can directly suppress PAI-1 production in 3T3-L1 adipocytes. In addition to its direct effects on adipocytes, adiponectin might also suppress PAI-1 production through indirect mechanisms. For example, adiponectin has insulin-sensitizing and anti-inflammatory properties.19 It also increases fatty acid oxidation and reduces the synthesis of fatty acids and triglyceride.33 Furthermore, adiponectin may reduce PAI-1 production through suppressing the production of other adipokines, such as TNF-α, which stimulates PAI-1 gene expression in human adipocytes.34

PPARγ is predominantly expressed in adipose tissue, and adiponectin is almost exclusively secreted from adipocytes. Thiazolidinediones stimulate adiponectin production via enhancing adiponectin secretion from adipocytes.39 It is conceivable that the reported direct effect of PPARγ agonists on suppressing PAI-1 expression in human adipose tissue is mediated partly via adiponectin. Although our data clearly demonstrated the important role of adiponectin in mediating the suppressive effects of rosiglitazone on PAI-1, rosiglitazone can still reduce PAI-1 expression in adiponectin-deficient mice (albeit to a much less extent), suggesting the involvement of other adiponectin-independent mechanisms. Indeed, recent studies suggest that macrophage-adipocyte interaction plays a key role in initiating the inflammatory response associated with obesity.36 The PPARγ agonists can directly act on macrophages to decrease the production of proinflammatory cytokines, including IL1β, TNFα, and IL6,37 which in turn will alleviate their stimulatory effect on adipocyte PAI-1 production. Consistent with our findings, a recent study suggests that pioglitazone ameliorates insulin resistance and diabetes in both adiponectin-dependent and -independent pathways.31

In conclusion, our studies have provided, for the first time, evidence to support a beneficial effect of adiponectin on PAI-1 expression. Thus, in addition to its pleiotropic metabolic, antiatherogenic, antiinflammatory, and antioxidant actions,19 this antithrombotic property of adiponectin may contribute to the potential vasoprotective effects of drugs which can increase adiponectin production, such as the thiazolidinediones.

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Disclosures
None.

References


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Methods:

**Immunoassay for quantification of total PAI-1 in human serum**

The monoclonal antibody PAI-3E529 (2 μg/ml) (Molecular Innovation Inc.) was used for coating a 96-well microtiter plate overnight at 4 C. Human serum was diluted (1:100) and 100 μl of the diluted samples or standards were applied to each well, incubated at room temperature for 1 hour, washed three times, then incubated with 100 μl of the in-house detection antibody for 1 hour. After three washes, the wells were incubated with streptavidin-conjugated horseradish peroxidase (PIERCE) (1:10000) for 0.5 hr and subsequently reacted with tetramethyl-benzidine reagent (PIERCE) for 15 min. A total of 50 μl 2M H₂SO₄ was added to each well to stop the reaction, and the absorbance at 450 nm was measured. The intra- and interassay coefficients at high, medium and low levels were 3.6 and 4.4 %, 2.9 and 3.9%, and 3.1 and 1.6%, respectively.

**In vitro analysis to evaluate the direct effects of recombinant adiponectin on PAI-1 production in mouse 3T3-L1 adipocyte cells**

Mouse 3T3-L1 fibroblast cells were grown in DMEM culture medium containing 25 mM glucose and 10% fetal bovine serum, and were differentiated into mature adipocytes as we previously described (ref 1). 3T3-L1 adipocytes at day 8 after differentiation were seeded into six-well plates and treated with various concentrations of full-length recombinant adiponectin generated from mammalian cells (refs 1 and 2) in a serum-free condition. Conditioned medium were collected at 48 h after treatment for
determination of PAI-I concentration as above. Total RNA was extracted for quantitative RT PCR analysis of PAI-1 expression.

References:
