Influence of Glycation on LDL-Induced Generation of Fibrinolytic Regulators in Vascular Endothelial Cells

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Abstract—Hyperglycemia and dyslipidemia are two biochemical markers of diabetes mellitus. Increased incidence of cardiovascular disease and impaired fibrinolytic activity have been found in diabetic subjects. Previous studies have demonstrated that low density lipoproteins (LDLs) stimulate the production of plasminogen activator inhibitor-1 (PAI-1) and reduce the generation of tissue plasminogen activator (tPA) in vascular endothelial cells (ECs). The present study investigated the effect of glycated LDL on the production of PAI-1 and tPA in cultured human umbilical vein ECs (HUVECs). Glycation increased the abundance of glucitollysine and conjugated dienes in LDL and amplified the overproduction of PAI-1 and the reduction in tPA generation from HUVECs induced by LDL. The steady-state levels of PAI-1 mRNA in glycated LDL–treated ECs were significantly higher than those in native LDL–treated cells. Actinomycin D blocked the increase in PAI-1 generation induced by glycated LDL. Glycated LDL did not significantly reduce the levels of tPA mRNA but attenuated de novo synthesis of tPA in ECs. Treatment with 25 mmol/L aminoguanidine, an antioxidant and inhibitor of the formation of advanced glycation end products, during glycation normalized glycated LDL–induced generation of PAI-1 and tPA in ECs. The results of the present study indicate that glycation enhances the production of PAI-1 and attenuates tPA synthesis in ECs induced by LDL, which may contribute to the increased incidence of cardiovascular complications in diabetes. Formation of advanced glycation end products or peroxidation may be involved in glycated LDL–induced alterations in the generation of fibrinolytic regulators from ECs. (Arterioscler Thromb Vasc Biol. 1998;18:1140-1148.)

Key Words: glycated LDL ■ plasminogen activator inhibitor-1 ■ tissue plasminogen activator ■ endothelial cells ■ aminoguanidine

Diabetes mellitus is the most common metabolic disorder in worldwide populations. The risk for developing cardiovascular complications is increased by 2- to 6-fold in diabetic subjects. Disorders in lipid metabolism, coagulation, and fibrinolysis have been implicated in the development of diabetic vascular complications. It is generally accepted that hemostasis is determined by a balance between coagulation and fibrinolysis. Imbalance between the two antagonistic systems may result in thrombosis or hemorrhage. Plasmin, the active product of the fibrinolytic system, functions by dissolving fibrin clots and maintains the fluidity of blood. The generation of plasmin is regulated by plasminogen activators and their inhibitors. tPA is the main activator for plasminogen in the blood circulation. The activity of tPA is modulated by its major physiological inhibitor, PAI-1. The levels of PAI-1 mRNA are significantly increased in atherosclerotic vessels and are correlated with the severity of atherosclerotic lesions. An increase in PAI-1 production or a decrease in tPA generation from vascular ECs may reduce fibrinolytic activity in the blood. Attenuated fibrinolytic activity or reduced fibrinolytic response to ischemic stimulus has been found in diabetic subjects.

Vascular ECs synthesize both PAI-1 and tPA. The generation of PAI-1 or tPA from ECs is regulated by a variety of biological agonists, including plasma lipoproteins. The release of PAI-1 from ECs has been increased by treatments with VLDLs isolated from hypertriglyceridemic individuals. LDLs and their oxidized forms, modified by acetylation, UV radiation, or CuSO₄, stimulate the production of PAI-1 in ECs. Lp(a), an LDL-like lipoprotein, stimulates PAI-1 production in ECs, and oxidization amplifies the effect of Lp(a). Treatment with native LDL reduces the generation of tPA in ECs. Glycated LDLs are susceptible to oxidization in vitro. Glycated LDLs are susceptible to oxidization in vitro. Increased levels of glycated LDLs have been detected in subjects with poorly controlled diabetes. The influence of glycated lipoproteins on the production of fibrinolytic regulators in vascular ECs has not been documented. The present study examined the effect of glycation on LDL-induced generation of PAI-1 and tPA from cultured human vascular ECs.

Methods

Isolation of Lipoproteins

LDL fractions (d=1.024 to 1.063 g/mL) were isolated from fresh human plasma of healthy donors by sequential floating ultracentrifugation. Lp(a) was removed from LDL by lysine–Sepharose CL 4B affinity chromatography as previously described. Lp(a)-free LDLs...
were dialyzed at 4°C against 150 mmol/L NaCl and 0.01% EDTA (pH 7.4) and stored in sealed tubes overlaid with N2 at 4°C in the dark. The levels of endothelin in lipoproteins were monitored by the Limulus amebocyte lysate test using commercially available kits (E-TOXATE, Sigma Chemical Co). No detectable amount of endothelin was found in lipoprotein preparations used in this study.

**Modification of Lipoproteins**

LDL preparations were diluted to 2 mg of protein per milliliter with 0.1 mol/L phosphate buffer (pH 7.4) containing 0.1% Na2EDTA and then incubated with 5 to 200 mmol/L glucose and equimolar amounts of NaBH3CN for 1 to 3 weeks at 37°C in the dark. Residues were resuspended in 1000 μL of the coupling buffer containing 0.45 mol/L phenylisothiocyanate, incubated at room temperature for 5 minutes, and then dried. Glycated and native LDLs were processed identically except without the addition of glucose. At the end of glycation, lipoproteins were dialyzed to remove free glucose. Radiolabeled [35S]glucose (10 μCi/mL) was mixed with unlabeled glucose during glycation to monitor the incorporation of glucose. Oxidized LDLs were modified by 5 μmol/L CuSO4 at 22°C for 24 hours.

**Analysis of Amino Acid Profile of Lipoproteins**

Glycated and native LDLs were hydrolyzed in 2N HCl for 24 hours at 100°C, washed with coupling buffer (acetonitrile/ethanol/triethylamine/water, 10:5:2:3, vol/vol/vol/vol), and dried by evaporation. The samples were resuspended in 100 μL of the coupling buffer containing 0.45 mol/L phenylisothiocyanate, incubated at room temperature for 5 minutes, and then dried. Residues were resuspended in 400 μL of 70 mmol/L sodium acetate (pH 6.5) and analyzed on an Ultrasphere ODS C18 column (4.6 mm × 25 cm) using a gradient solvent system (0% to 30% acetonitrile, 100% to 70% of 70 mmol/L sodium acetate) on a high-performance liquid chromatography (Gold System, Beckman Instruments Inc) and monitored at 245 nm.

**Preparation of Glucitollysine**

α-N-Z-L-Lysine (Sigma) was incubated with 27 mmol/L glucose for 17 days in 0.2 mol/L sodium phosphate buffer (pH 8.0) as previously described. The mixture was passed through a Dowex 50 Wx8 column followed by a Sephadex G-15 column. The protecting group was removed by catalytic transfer hydrogenation.

**Cell Culture and Experimental Incubation**

HUVECs were obtained by collagenase digestion as previously described. Cell type was verified by morphology and the presence of factor VIII antigen. Cells were grown in medium 199 supplemented with 10% heat-inactivated FBS, 30 μg/mL of EC growth stimulator, 100 μg/mL of heparin, 0.1 mmol/L nonessential amino acids, 200 μmol of penicillin, and 200 μg/mL of streptomycin in a humidified incubator as nanomoles of malondialdehyde per milligram of protein in LDL as previously described.

**Measurement of PAI-1 and tPA Antigen**

Conditioned media of HUVECs were collected at the end of incubation. Cells were harvested in PBS (pH 7.4) containing 0.1% SDS and 0.5% Triton X-100. Total amounts of PAI-1 and tPA antigen (free and complex forms) in the media were estimated by using IMUBIND PAI-1 or tPA ELISA kits (American Diagnostica Inc). The levels of PAI-1 and tPA antigen were read on a microtiter plate spectrophotometer at 490 nm and were expressed in micrograms of antigen per milligram of cellular proteins.

**Northern Blotting Analysis**

Total cellular RNA was extracted from cells at the end of incubation by the guanidine isothiocyanate–CsCl method. DNA was denatured, subjected to electrophoresis on a 1% agarose-formaldehyde gel, and then transferred to Zeta- Probe GT blotting membranes (Bio-Rad). Plasmid containing the cDNA fragment–encoded human tPA, PAI-1, or β-actin was labeled with [32P]dCTP (>111 TBq/ mmol/L, New England Nuclear) by using random-primer labeling kits. Blots were prehybridized in 0.25 mol/L Na2HPO4 (pH 7.2) and 7% SDS for 10 minutes at 42°C and then hybridized with denatured probe for 16 hours at 42°C. After hybridization, blots were washed and subjected to autoradiography. The levels of PAI-1 and tPA mRNA were quantified from autoradiogram by density scanning and then adjusted with β-actin mRNA on rehybridized blots.

**Estimation of Cytotoxicity of Lipoproteins**

Cytotoxicity of lipoproteins was examined by incubating cells with 5 × 105 disintegrations per minute per well of [3H]leucine (54 Ci/mmol/L, ICN Radiochemicals) in leucine-free medium for 2 hours after treatment with lipoproteins. Cells were washed sequentially with, as described above, the buffer dilution supplemented with 0.5% sodium deoxycholate followed by 100 mmol/L Tris (pH 7.5) containing 0.1% NP-40. Proteins were recovered from the cells with 125 mmol/L Tris buffer (pH 6.8) containing 20% glycerol and 4.6% SDS and then analyzed by 12% SDS-PAGE. De novo synthesized tPA or PAI-1 was detected on dried gels by autoradiography and quantified by density scanning.

**Measurement of TBARS**

Lipid peroxidation in lipoproteins and the postculture media of ECs was determined by measuring the amount of TBARS and expressed as nanomoles of malondialdehyde per milligram of protein in LDL as previously described.
Statistical Analysis
Data are presented as mean±SD. Probability between paired data was estimated with Student’s \( t \) test. Comparisons among multiple groups were achieved by one-way ANOVA followed by Duncan’s test. The level of significance was defined as \( P<0.05 \).

Results
Biochemical Characterization of Glycated LDL
The abundance of lysine in glycated LDL (Figure 1B) was greatly reduced compared with native LDL (Figure 1A). An extra amino acid peak (Figure 1B) was detected in glycated LDL but not in native LDL. This product comigrated with synthesized glucitollysine (Figure 1C), a glycation product of lysine. The glucitollysine-to-lysine ratio was elevated 3-fold in LDL glycated by 25 mmol/L glucose compared with nonglycated LDL (Table 1). Radioactivity of \[^{14}C\]glucose in glucitollysine was 5 to 6 times higher than in background, lysine, or phenylalanine. The glucitollysine-to-lysine ratio is only one of several potential markers for the pathogenic alterations in glycated LDL. Glycated LDL migrated apparently faster than did native LDL toward the positively charged anode on 1% agarose electrophoresis gel, as expected (data not shown). The levels of TBARS in glycated LDL were very low or undetectable (0.10±0.09 nmol malondialdehyde per milligram protein, mean±SD, \( n=4 \)), which was slightly higher than but not significantly different from that in native LDL (0.06±0.06 nmol/mg). The levels of TBARS in oxidized LDL were 46.16±3.85 nmol/mg. In the lipid extracts of native LDL, no CDs were detected. Oxidized LDL contained CDs at 233 and 242 nm (Figure 2). The absorbance minimum of CDs at 233 nm in glycated LDL was significantly higher than that in oxidized LDL, but no CDs were found at 242 nm in glycated LDL (Table 2, Figure 2).

Effect of Glycated LDL on PAI-1 and tPA Secretion
The influence of the extent of glycation of LDL on the generation of PAI-1 and tPA was examined in HUVECs. Cells were treated with 100 \( \mu \)g/mL of glycated LDL modified with 25 to 200 mmol/L glucose for 1 week. Glycated LDL (100 \( \mu \)g/mL) modified by \( \geq 50 \) mmol/L glucose significantly increased the levels of PAI-1 antigen in the media of HUVECs after 48 hours of treatment (6.43±0.39, 6.58±0.27, or 6.76±0.14 \( \mu \)g/mg by 50, 100, or 200 mmol/L, respectively, of glucose-modified LDL) compared with native LDL (5.37±0.37 \( \mu \)g/mg, \( n=4 \), \( P<0.01 \), Figure 3, top). To evaluate the influence of the length of glycation on glycated LDL–induced PAI-1 generation, ECs were treated with 100 \( \mu \)g/mL of glycated LDL modified by a lower concentration of glucose for a longer time (25 mmol/L glucose for 3 weeks) for 48 hours compared with glycated LDL modified by 25 mmol/L glucose for 1 week or 50 mmol/L glucose for 2 weeks. The generation of PAI-1 in ECs treated with glycated LDL modified by 25 mmol/L glucose for 1 week or 50 mmol/L glucose for 2 weeks was significantly higher than in ECs treated with matching native LDL (\( P<0.01 \)). Native LDL proceeded in parallel with glycated LDL for 3 weeks without exposure to glucose-stimulated PAI-1 production compared with control cultures (\( P<0.05 \)), which was possi-
more evident in ECs treated with LDL modified by P, the ratio between the areas of the peaks corresponding to the two compounds. by high-performance liquid chromatography. Glucitollysine/lysine represents 37°C. Glucitollysine and lysine in nonglycated and glycated LDLs were analyzed P0.01. Treatment with equal amounts of glycated LDL, the levels of PAI-1 (4.52±0.6 units. A, Native LDL; B, glycated LDL; and C, oxidized LDL. The generation of tPA from HUVECs was significantly m reduced by a 24-hour treatment with 100 μg/mL of glycated LDL modified by 25 mmol/L glucose for 1 week (0.75±0.03 μg/mg) compared with native LDL (0.84±0.06 μg/mg, n=4, P<0.05, Figure 3, bottom). The decrease in tPA generation was more evident in ECs treated with LDL modified by ≥50 mmol/L glucose for 1 week (0.48±0.06, 0.51±0.05, or 0.56±0.06 μg/mg by 50, 100, or 200 mmol/L, respectively, of glucose-modified LDL) compared with native LDL (P<0.01).

After ≥24 hours of incubation with 100 μg/mL of native LDL, the levels of PAI-1 (4.52±0.26 μg/mg after 24 hours, 5.31±0.31 μg/mg after 48 hours) in the media were significantly higher than in time-matched controls (3.71±0.17 μg/mg after 24 hours, 4.09±0.26 μg/mg after 48 hours, n=4, P<0.01). Treatment with equal amounts of glycated LDL (modified by 50 mmol/L glucose for 2 weeks) induced significantly greater increases of PAI-1 released from HUVECs (5.35±0.37 μg/mg after 24 hours, 6.28±0.19 μg/mg after 48 hours) compared with cells treated with native LDL for matching periods (Figure 4, top). Dose-response curves for the effect of glycated LDL on PAI-1 generation were observed in HUVECs treated with 10 to 100 μg/mL of glycated or native LDL. The levels of PAI-1 antigen in the medium of HUVECs treated with ≥50 μg/mL of glycated LDL (4.66±0.25 μg/mg for 50 μg/mL, 5.41±0.35 μg/mg for 100 μg/mL) were significantly higher than those in cells treated with equal amounts of native LDL (3.37±0.22 μg/mg for 50 μg/mL, 4.28±0.16 μg/mg for 100 μg/mL, n=4, P<0.01, Figure 4, bottom).

To answer the question whether the transcription of PAI-1 was stimulated during this process, HUVECs were treated with 0.2 μg/mL of actinomycin D in addition to 100 μg/mg of native or glycated LDL.13 Actinomycin D treatment inhibited native and glycated LDL–induced PAI-1 generation from HUVECs to levels (native LDL, 5.34±0.51 μg/mg versus native LDL+actinomycin D, 4.28±0.32 μg/mg, n=4, **P<0.01 versus native LDL.}

### Table 1. Effect of Glucose Concentrations on Glucitollysine/Lysine Ratio in Glycated LDL

<table>
<thead>
<tr>
<th>Glucitollysine/Lysine</th>
<th>Glucose, mmol/L</th>
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<tbody>
<tr>
<td>0.18</td>
<td>0</td>
</tr>
<tr>
<td>0.18</td>
<td>5</td>
</tr>
<tr>
<td>0.19</td>
<td>10</td>
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<tr>
<td>0.57</td>
<td>25</td>
</tr>
<tr>
<td>0.98</td>
<td>50</td>
</tr>
<tr>
<td>1.12</td>
<td>100</td>
</tr>
<tr>
<td>1.19</td>
<td>200</td>
</tr>
</tbody>
</table>

Values are mean of two preparations. LDL (2 mg/mL) was glycated with 5 to 200 mmol/L glucose for 1 week at 37°C. Glucitollysine and lysine in nonglycated and glycated LDLs were analyzed by high-performance liquid chromatography. Glucitollysine/lysine represents the ratio between the areas of the peaks corresponding to the two compounds.

### Table 2. Comparison of CDs in Glycated and Oxidized LDL

<table>
<thead>
<tr>
<th>Lipoproteins</th>
<th>Absorbance Minima*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>233 nm</td>
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<tr>
<td>Native LDL</td>
<td>0.0</td>
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<tr>
<td>Glycated LDL</td>
<td>17.4±0.9</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>8.1±0.5§</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=4).

*Absorbance minima were expressed in arbitrary units.
†LDL was glycated with 50 mmol/L glucose for 2 weeks.
‡LDL was oxidized with 5 μmol/L CuSO4.
§P<0.001 versus glycated LDL.
TABLE 3. Comparison of PAI-1 Generation From HUVECs Treated With LDL Modified by Glucose for Various Lengths of Time

<table>
<thead>
<tr>
<th>Lipoproteins</th>
<th>Glucose, mmol/L</th>
<th>Time, Weeks*</th>
<th>PAI-1 Antigen, µg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>...</td>
<td>...</td>
<td>4.32±0.36</td>
</tr>
<tr>
<td>Native LDL</td>
<td>...</td>
<td>1</td>
<td>5.54±0.35†</td>
</tr>
<tr>
<td>Glycated LDL</td>
<td>25</td>
<td>1</td>
<td>5.42±0.28†</td>
</tr>
<tr>
<td>Native LDL</td>
<td>...</td>
<td>3</td>
<td>6.73±0.96†</td>
</tr>
<tr>
<td>Glycated LDL</td>
<td>25</td>
<td>3</td>
<td>10.07±1.25‡§</td>
</tr>
<tr>
<td>Native LDL</td>
<td>...</td>
<td>2</td>
<td>5.31±0.27†</td>
</tr>
<tr>
<td>Glycated LDL</td>
<td>50</td>
<td>2</td>
<td>6.55±0.24‡§</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=4).
*The period of time that LDL was incubated at 37°C with or without exposure to glucose.
†PAI-1 antigen in postculture media of ECs was measured by ELISA after 48 hours of incubation with 100 µg/mL native or glycated LDL.
‡P<0.05, §P<0.01 versus controls; ‡P<0.01 versus native LDL.

Figure 5. Effects of actinomycin D on PAI-1 generation induced by glycated and native LDL in HUVECs. Confluent HUVECs were incubated without addition (control) or with addition of 100 µg/mL native LDL (n-LDL), glycated LDL (gly-LDL, modified by 50 mmol/L glucose for 2 weeks), 0.2 µg/mL actinomycin D (Act.D), native LDL+actinomycin D, or glycated LDL+actinomycin D for 48 hours. Levels of PAI-1 antigen in the media were determined using ELISA. Values are mean±SD (n=4); *P<0.01 versus controls; †P<0.01 versus native LDL; and #P<0.01 versus glycated LDL.

of tPA from ECs was significantly reduced by treatment with 100 µg/mL of native LDL for ≥16 hours (0.91±0.05, 1.17±0.12, or 2.07±0.13 µg/mg for 16, 24, or 48 hours, respectively) compared with time-matched controls (1.12±0.07, 1.62±0.10, or 2.47±0.07 µg/mg for 16, 24, or 48 hours, P<0.05). Glycated LDL (modified by 50 mmol/L glucose for 2 weeks) further attenuated tPA release from ECs (0.69±0.07, 0.76±0.07, or 1.64±0.12 µg/mg for 16, 24, or 48 hours) compared with native LDL incubated for matching periods (P<0.05, Figure 6, top). Treatment with ≥25 µg/mL of native or glycated LDL for 24 hours significantly reduced tPA secretion from HUVECs compared with no-addition controls (1.15±0.09 µg/mg, P<0.01). The amounts of tPA secreted from ECs treated with 25 to 100 µg/mL of glycated LDL (0.75±0.05, 0.58±0.02, or 0.57±0.05 µg/mg by 25, 50, or 100 µg/mL) were significantly less than those from cells treated with corresponding amounts of native LDL (0.90±0.04, 0.71±0.04, or 0.67±0.04 µg/mg by 25, 50, or 100 µg/mL, P<0.05, Figure 6, bottom).

Effect of Glycated LDL on PAI-1 and tPA mRNA
PAI-1 mRNA in HUVECs was present as two distinguishable species, ∼3.4 and 2.4 kb, as previously described. Treatment with 100 µg/mL of native LDL for 48 hours increased the level of 2.4-kb PAI-1 mRNA by 2-fold (P<0.001) but slightly reduced the level of 3.4-kb PAI-1 mRNA. Treatment with an equal amount of glycated LDL evidently augmented the level of 2.4-kb PAI-1 mRNA compared with controls and native LDL–treated cells (Figure 7, top). The levels of 2.4-kb PAI-1 mRNA in glycated LDL–treated ECs were >3.25±0.21-fold higher than the mean of controls and were significantly greater than that in native LDL–treated cells (2.13±0.16-fold, n=3, P<0.001, Figure 7, bottom). This finding is consistent with the inhibitory effect of actinomycin D on glycated LDL–induced PAI-1 generation (Figure 5).
However, the level of tPA mRNA in HUVECs treated with 100 μg/mL of glycated or native LDL did not significantly differ from controls (Figure 8).

**Effect of Glycated LDL on De Novo Synthesis of tPA and PAI-1**

To determine whether the synthesis of fibrinolytic regulators was altered by glycated LDL treatment, HUVECs were metabolically labeled with [35 S]methionine/cystine in the presence of native or glycated LDL. De novo synthesized PAI-1 was mainly detected in the cell-associated pool of HUVECs. Radioactivity of PAI-1 was weak in the medium and not visualized by using autoradiography. Native LDL (100 μg/mL for 48 hours) moderately increased the level of de novo synthesized PAI-1 in the cell-associated compartment (3.47±0.9-fold) compared with controls (1.00±0.07-fold of the mean, n=4, P<0.05). Treatment with glycated LDL significantly increased the level of de novo synthesized PAI-1 (13.95±4.2-fold) compared with native LDL (n=4, P<0.05, Figure 9). De novo synthesized tPA was mainly found in the media of HUVECs. Treatment with 100 μg/mL of native LDL reduced tPA synthesis after 24 hours of incubation. Glycated LDL at the same concentration further attenuated tPA synthesis in HUVECs compared with native LDL (P<0.01, Figure 10).

**Effects of Oxidized and Aminoguanidine-Treated Glycated LDL on the Generation of PAI-1 and tPA**

In the media of HUVECs treated with 100 μg/mL of oxidized LDL for 48 hours, the levels of PAI-1 were 23% higher than in...
native LDL–treated cells and 51% higher than controls (P<0.001) but did not significantly differ from those of cells treated with glycated LDL. The generation of tPA in oxidized LDL–treated ECs was 35% lower than that from native LDL–treated with glycated LDL. The generation of tPA in oxidized LDL–treated cultures was 1.00±0.07%, 3.47±0.94%, 13.95±4.2%, and 3.40±1.35-fold increase of the mean of controls. *P<0.05, **P<0.01 versus controls; +P<0.05 versus native LDL; and #P<0.05 versus glycated LDL.

Aminoguanidine is an antioxidant and an inhibitor for the formation of AGEs. Addition of 25 mmol/L aminoguanidine during LDL glycation effectively inhibited the glycation of lysine (Figure 1C). The inhibitory effect of aminoguanidine on the glycation of lysine was evident in LDLs treated with ≥25 mmol/L aminoguanidine (data not shown). Glycated LDL–induced changes in the secretion of PAI-1 and tPA antigen (PAI-1, 148.5±5.3%; tPA, 52.8±5.2% of control) were effectively reduced by treatment with 25 mmol/L aminoguanidine during glycation (PAI-1, 122.7±7.9%; tPA, 72.0±5.3% of control, P<0.01) to levels that were not significantly different from those of native LDL–treated cells (PAI-1, 122.5±3.6%; tPA, 66.4±3.5% of control, Figure 11). Additionally, aminoguanidine treatment normalized the de novo synthesis of PAI-1 and tPA in ECs induced by glycated LDL (Figures 9 and 10).

**Discussion**

Attenuation of fibrinolytic activity has been frequently found in diabetic patients and has been implicated in the development of cardiovascular complications in diabetes. Alternations in the generation of PAI-1 and tPA from vascular ECs may directly affect fibrinolytic activity in the blood circulation. The results of the present studies demonstrate that glycation enhances the generation of PAI-1 and reduces the generation of tPA induced by LDL in cultured vascular ECs. Increased generation of PAI-1 accompanied by a decreased release of tPA from vascular ECs may result in hypofibrinolysis and lead to intravascular thrombogenesis. The findings of the present study may help investigators understand the mechanism for attenuated fibrinolytic

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**Figure 9.** Synthesis of PAI-1 in HUVECs treated with native or glycated LDL. Cells were metabolically labeled with 100 μCi/mL of [35S]methionine/cysteine in methionine/cysteine-free medium without or with addition of 100 μg/mL native LDL (n-LDL), glycated LDL (gly-LDL), or aminoguanidine-treated glycated LDL (AG-gly-LDL) for 48 hours. Cell-associated radioactive PAI-1 was immunoprecipitated by anti-human PAI-1 IgG and analyzed by 12% SDS-PAGE. Radioactivity of PAI-1 on dried gels was visualized by autoradiography and quantified by densitometry. Values are fold increase of the mean of controls (mean±SD, n=4). Levels of PAI-1 synthesis in control, native LDL–, glycated LDL–, or aminoguanidine plus glycated LDL–treated cultures were 1.00±0.07%, 3.47±0.94%, 13.95±4.2%, and 3.40±1.35-fold increase of the mean of controls. *P<0.05, **P<0.01 versus controls; +P<0.05 versus native LDL; and #P<0.05 versus glycated LDL.

**Figure 10.** Synthesis of tPA in HUVECs treated with native or glycated LDL. Cells were metabolically labeled with 100 μCi/mL of [35S]methionine/cysteine/cystine-free medium without or with addition of 100 μg/mL native LDL (n-LDL), glycated LDL (gly-LDL), or aminoguanidine-treated glycated LDL (AG-gly-LDL) for 24 hours. Immunoprecipitation was performed as described in the legend to Figure 9. Values are percent of the mean of controls (mean±SD, n=4). Levels of tPA in control, n-LDL–, gly-LDL–, or AG-gly-LDL–treated cultures were 100.0±7.0%, 88.0±3.4%, 72.0±6.0%, and 91.0±3.0% of the mean of controls. *P<0.05, **P<0.01 versus control; ++P<0.01 versus native LDL; and ###P<0.01 versus glycated LDL.

**Figure 11.** Effects of oxidized and aminoguanidine-modified LDL on PAI-1 and tPA secretion from HUVECs. Confluent cells were incubated with medium without addition (control) or with 100 μg/mL native LDL (n-LDL), glycated LDL (gly-LDL, modified by 50 mmol/L glucose for 2 weeks), glycated LDL treated with 25 mmol/L aminoguanidine (AG-gly-LDL), or oxidized LDL (ox-LDL) for 48 hours (PAI-1, top) or 24 hours (tPA, bottom). Conditioned media were collected for measuring PAI-1 or tPA antigen by ELISA. Values are mean±SD (n=4). *P<0.01, **P<0.001 versus controls; +P<0.01, + +P<0.001 versus native LDL; and #P<0.05, ##P<0.01 versus glycated LDL.
activity and increased thrombotic vascular complications in diabetes. It should be pointed out that the present study only examined the effects of LDL and its glycated form from healthy individuals. The composition of LDL in diabetes may differ from that from nondiabetic subjects with respect to factors other than glycation products. Investigation of the effects of LDL from various types of diabetic individuals may provide additional information on the modulation of EC-derived fibrinolytic regulators in diabetes.

The results of the present study indicate that the generation of PAI-1 and tPA in ECs is significantly altered by glycated LDL that has been modified by 25 to 50 mmol/L glucose for extended periods. This range of blood glucose occurs in uncontrolled diabetes. Previous studies reported that the levels of glucose may be elevated to a range of 200 to 2000 mg/dL (11 to 111 mmol/L) in patients with diabetic ketoacidosis. The extent of glycation of plasma proteins is correlated with the length of glycation time as well as glucose concentrations. This concept is supported by the results of the present study that LDL glycated by 25 mmol/L glucose for 3 weeks significantly increased PAI-1 generation compared with LDL glycated by the same concentration of glucose for a shorter period (1 week). We speculate that the generation of PAI-1 may be increased by LDL modified by even lower concentrations of glucose for longer periods in vivo.

Levin et al described that the generation of tPA from HUVECs was reduced by treatment with native LDL. The effect of native LDL on the secretion of tPA from HUVECs observed in the present study supports their observation. The level of tPA mRNA was inhibited by LDL in HUVECs in medium with the addition of 100 µg/mL of heparin in the previous study. It has been shown that ≥50 µg/mL of heparin effectively inhibits the levels of tPA mRNA in baboon aortic smooth muscle cells. In the present study, heparin was supplemented only in the growth medium but not in the stimulation medium to avoid interference with the regulation of tPA generation. Pilot studies in our laboratory have found that treatment with ≥200 µg/mL of native or glycated LDL in heparin-free medium evidently impairs [3H]leucine incorporation into HUVECs (data not shown). Glycated LDL did not reduce the mRNA level of tPA mRNA but did inhibit the synthesis of tPA in ECs. The difference between the effects of LDL on tPA mRNA in the present and the previous study is likely due to the variation in stimulation conditions.

The present study demonstrates that glycated LDL stimulates the steady-state levels of PAI-1 mRNA in ECs that are associated with increases in de novo synthesis and secretion of PAI-1. Actinomycin D, a known inhibitor of transcription, blocked glycated and native LDL–induced generation of PAI-1. The combination of these findings strongly suggests that both glycated and native LDL regulate PAI-1 production in ECs at the transcriptional level. In contrast, the steady-state level of tPA mRNA in ECs was not affected by glycated or native LDL. De novo synthesis of tPA was reduced in native and glycated LDL–treated ECs. Therefore, the reduction in tPA generation from ECs induced by native and glycated LDL may result from the decrease in tPA synthesis or secretion.

Previous studies have demonstrated that oxidized LDL stimulates the generation of PAI-1 in ECs. The results of the present study indicate that oxidized LDL also reduces tPA generation in HUVECs. Glycated LDL may be more susceptible to oxidation than native LDL. Although the TBARS assay did not reveal a significant increase in peroxidation in glycated LDL, CDs were detected at 233 nm in lipid extracts of glycated LDL in our studies. The pattern of CDs in glycated LDL apparently differs from that in oxidized LDL (Figure 2). The findings suggest the presence of excess lipid peroxidation products in glycated LDL, and glycoxidation may be a major contributing factor for glycated LDL–induced alterations in PAI-1 and tPA generation in ECs.

Extended glycation induces the generation of AGEs in blood components and tissues. AGEs promote cross-linking between glycation products and arterial wall proteins, which may be involved in atherogenesis. AGE complexes also enable the generation of reactive oxygen intermediates. Receptors for AGEs have been identified in several types of cells, including ECs. Aminoguanidine inhibits the formation of AGEs by forming a complex with early glycation products. Treatment with aminoguanidine has prevented the formation of AGEs in the aortic wall of diabetic rats. The results of the present study for the first time demonstrate that treatment with aminoguanidine during glycation normalized glycated LDL–induced overproduction of PAI-1 and the reduction of tPA synthesis in HUVECs. Aminoguanidine is also an inhibitor of NO synthesis and diamine oxidase. Previous studies indicated that treatment with aminoguanidine prevented the oxidation of LDL. Both AGEs and oxidation may contribute to glycated LDL–induced generation of fibrinolytic regulators in vascular ECs, and the levels of AGEs are higher in both apoB and the lipid moiety of LDLs isolated from diabetic patients than from nondiabetic subjects. Determination of interactions between AGEs, LDL–specific structure, and the EC surface may help investigators define the regulatory mechanism for glycated LDL–induced alterations in the generation of fibrinolytic regulators from ECs.

The present study was performed in ECs isolated from umbilical veins. Previous studies have demonstrated that HUVECs are functionally closer to arterial ECs than to ECs isolated from other veins. This is possibly due to the unique circumstance of the umbilical vein, which carries oxygenated and nutrient-bearing blood instead of regular venous blood from the placenta to fetus. It is reasonable, therefore, to predict that HUVECs may respond to glycated LDL in a way similar to arterial ECs than do other venous ECs.

In summary, glycation enhances the overproduction of PAI-1 and further reduces the generation of tPA induced by LDL in vascular ECs. The effect of glycated LDL on the generation of PAI-1 and tPA was effectively prevented by treatment with aminoguanidine. Our findings suggest that increased levels of glycated LDL in the blood circulation may attenuate fibrinolytic activity. Management of hyperglycemia and hyperbetalipoproteinemia or the reduction of glycation of lipoproteins through pharmacological intervention potentially prevents the development of thrombotic vascular complications in diabetic subjects.

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References
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