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

Jianying Zhang, Song Ren, Dongfeng Sun, Garry X. Shen

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 flotation ultracentrifugation. Lp(a) was removed from LDL by lysine–Sepharose CL 4B affinity chromatography as previously described. Lp(a)-free LDLs were isolated by sequential flotation ultracentrifugation. LDL fractions (d=1.024 to 1.063 g/mL) were isolated from fresh human plasma of healthy donors by sequential flotation ultracentrifugation. Lp(a) was removed from LDL by lysine–Sepharose CL 4B affinity chromatography as previously described. Lp(a)-free LDLs were isolated by sequential flotation ultracentrifugation.
Selected Abbreviations and Acronyms

AGE = advanced glycation end product
CD = conjugated diene
(HUVEC) = human umbilical vein endothelial cell
PAGE = polyacrylamide gel electrophoresis
PAI-1 = plasminogen activator inhibitor-1
TBARS = thiobarbituric acid–reactive substances
tPA = tissue plasminogen activator

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 endotoxin in lipoproteins were monitored by the
Limulus amebocyte lysate test using commercially available kits
(E-TOXATE, Sigma Chemical Co). No detectable amount of endo-
toxin 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 0.01% EDTA and
0.01% NaN₃ 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.

Preparation of Glucitollysine

- L-Lysine (Sigma) was incubated with 27 mmol/L glucose for
24 hours in 0.1 mol/L sodium phosphate buffer (pH 7.4) containing
0.1% NaCl, 50 mmol/L Tris, 0.1% NP-40, 2.5% gelatin, and 0.5% BSA.

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 In-
mstruments Inc) and monitored at 245 nm.¹⁹

Preparation of Glucitollysine

α-N-Ac-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.²⁰ 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 de-
scribed.²¹ 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 μg/mL
of penicillin, and 200 μg/mL of streptomycin in a humidified incubator
under 95% air–5% CO₂ at 37°C. Confluent cells were incubated in
heparin-free medium 199 with or without LDL.

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 microtest
plate spectrophotometer at 490 nm and were expressed in micro-
grams of antigen per milligram of cellular proteins.¹³

Northern Blotting Analysis

Total cellular RNA was extracted from cells at the end of incubation by
the guanidine isothiocyanate–CsCl method.²² RNA was dena-
tured, 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-1,²³ 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.²⁴ 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.

Metabolic Labeling and Immunoprecipitation

Confluent cells in 60-mm dishes were treated with 100 μCi/mL of
³H-Tran²⁵ label (38 TBq/mmol/L, 85% methionine, and 15% cystine,
ICN) in methionine- and cystine-free supplemented with 2 mmol/L
glutamine and 10% serum with or without lipoproteins as previously
described.²⁶ Cells and media were harvested at the end of incubation
and dialyzed with a buffer containing 0.5 mmol/L NaCl, 1 mmol/L EDTA,
50 mmol/L Tris, 0.1% NP-40, 2.5% gelatin, and 0.5% BSA. Dila-
ded media and cell lysates were first incubated with 30 μg/mL of rabbit IgG
at 25°C for 1 hour and then with 40 μL of a 50% slurry of protein
A–Sepharose for 30 minutes. Sepharose beads in the mixtures were
removed by centrifugation. Resultant supernatant was incubated at 25°C
for 2 hours with goat anti-human tPA IgG or rabbit anti-human PAI-1
IgG (American Diagnostica Inc). Immune complexes of tPA or PAI-1
were recovered by using protein A–Sepharose. The beads were washed
sequential ly, as described above, the buffer dilution supplemented
with 0.5% sodium decylxolate followed by 10 mmol/L Tris (pH 7.5)
containing 0.1% NP-40. Proteins were recovered from the beads 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.¹³

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. Cellular proteins were
precipitated with 5% trichloroacetic acid. Radioactivity incorporated
into cellular precipitates was analyzed by scintillation counting. No
detectable reduction in the incorporation of radioactive leucine was
found in HUVeCs treated with the indicated amounts of native or
glycated lipoproteins (data not shown).

Analysis of CDs

Lipids in modified or native lipoproteins were extracted in chloro-
form/methanol, 2:1, vol/vol. The organic phase of lipid extracts was
dried under N₂ at room temperature. The lipid residue was resus-
pended in absolute ethanol. Absorbance of lipid extracts was
measured from 220 to 330 nm against an ethanol blank by using a
UV spectrophotometer and was expressed in arbitrary units.²² The
extent of peroxidation was estimated from absorbance minima at 242
and 233 nm for the quantitative analysis of CDs as previously
described.²³²⁶

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.²⁷²⁸

Measurement of Protein Concentrations in Lipoproteins and Cells

Protein contents in lipoproteins were measured by using a modified
Lowry method.²⁹ For analyzing total proteins in cultured cells, cells
were lysed in PBS containing 0.5% Triton X-100 and 0.1% SDS.
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 [14C]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 μg/mL of glycated LDL modified with 25 to 200 mmol/L glucose for 1 week. Glycated LDL (100 μg/mL) modified by ≥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 μg/mg by 50, 100, or 200 mmol/L, respectively, of glucose-modified LDL) compared with native LDL (5.37±0.37 μg/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 μg/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-

Figure 1. Amino acid profiles of native and glycated LDL. A, Native LDL; B, glycated LDL (modified by 200 mmol/L glucose for 1 week); C, synthesized glucitollysine; and D, aminoguanidine (25 mmol/L)-treated glycated LDL (200 mmol/L glucose for 1 week). Derivatized amino acids in LDL were analyzed on reverse-phase high-performance liquid chromatography columns with a gradient solvent system composed of 100% to 70% of 70 mmol/L sodium acetate at 0% to 30% of acetonitrile. I indicates isoleucine; L, leucine; *, glucitollysine; F, phenylalanine; and K, lysine.
The ratio between the areas of the peaks corresponding to the two compounds.

Treatment with equal amounts of glycated LDL, the levels of PAI-1 (4.52 ± 0.6 g/mg) compared with native LDL (0.32 ± 0.31 μg/mg after 48 hours) 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 compared with cells treated with native LDL (4.34 ± 0.51 μg/mg versus native LDL + actinomycin D, 4.28 ± 0.32 μg/mg, n = 4, P < 0.01 versus native LDL.

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

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).

Table 2. Comparison of CDs in Glycated and Oxidized LDL

<table>
<thead>
<tr>
<th>Lipoproteins</th>
<th>Absorbance Minima*</th>
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<tbody>
<tr>
<td></td>
<td>233 nm</td>
</tr>
<tr>
<td></td>
<td>242 nm</td>
</tr>
<tr>
<td>Native LDL</td>
<td>0.0</td>
</tr>
<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.

Figure 2. CDs in native, glycated, and oxidized LDL. Glycated LDL was modified by 50 mmol/L glucose for 2 weeks and oxidized LDL was modified by 5 μmol/L CuSO4 for 24 hours. Lipids were extracted from native or modified lipoproteins with chloroform/methanol. Lipid extracts resuspended in ethanol were analyzed by spectrophotometry from 220 to 330 nm against ethanol blank. Absorbance was expressed in arbitrary units. A, Native LDL; B, glycated LDL; and C, oxidized LDL.

Figure 3. Effects of glycated LDL modified by various concentrations of glucose on the secretion of PAI-1 and tPA antigen from HUVECs. Cells were incubated with 100 μg/mL native LDL or glycated LDL (modified by 25 to 200 mmol/L glucose for 1 week) for 48 hours (PAI-1, top) or 24 hours (tPA, bottom). PAI-1 or tPA antigens in the media were analyzed by ELISA as described in Methods. Values are mean ± SD in μg of PAI-1 or tPA/mg cellular protein (n = 4). *P < 0.05, **P < 0.01 versus native 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 versus controls; §P<0.01 versus native LDL incubated at 37°C for the same length of time; †P<0.01 versus glycated LDL modified by 25 mmol/L glucose for 1 week.

P<0.01: glycated LDL, 6.74±0.42 μg/mg versus glycated LDL+actinomycin D, 4.43±0.40 μg/mg, P<0.01; actinomycin D alone, 4.50±0.23 μg/mg) close to those of controls (4.10±0.10 μg/mg, Figure 5).

The levels of tPA antigen in the media of HUVECs were elevated with the elongation of incubation time. The secretion 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).

Figure 4. Time and dose dependence of glycated LDL on PAI-1 secretion from cultured HUVECs. Cells were treated without addition (control), with 100 μg/mL native LDL (n-LDL) or glycated LDL (gly-LDL, modified by 50 mmol/L glucose for 2 weeks) for 8 to 48 hours (top), or with 100 μg/mL native LDL or glycated LDL for 48 hours (bottom). Levels of PAI-1 antigen in the media were determined by ELISA. Values are mean±SD (n=4). *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.

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 cells and 51% higher than controls (P<0.001) and 17% less than in glycated LDL–treated ECs (P<0.05, Figure 11).

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 (Figure 11). Additionally, aminoguanidine treatment normalized the decrease in the secretion of PAI-1 and tPA from vascular 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 activity in diabetic and hyperglycemic states.
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 [1H]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 is native LDL. Although the TBARS assay did not reveal a significant increase in peroxidation in glycated LDL, CD40L 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

23. Wun TC, Kretzmer KK. cDNA cloning and expression in E. coli of a plasminogen activator inhibitor (PAI) related to a PAI produced by HepG2 hepatoma cell. FERBS Lett. 1987;210:11–16.
Influence of Glycation on LDL-Induced Generation of Fibrinolytic Regulators in Vascular Endothelial Cells
Jianying Zhang, Song Ren, Dongfeng Sun and Garry X. Shen

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