THE EFFECTS OF GLYCATED LIPOPROTEINS ON PRODUCTION OF FIBRINOLYTIC REGULATORS IN VASCULAR ENDOTHELIAL CELLS

By

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ABBREVIATION

AGEs	Advanced glycation end products
ANOVA	Analysis of variance
ApoB-100	Apolipoprotein B-100
ApoB	Apolipoprotein B
Apo(a)	Apolipoprotein(a)
ACVD	Atherosclerotic cardiovascular diseases
BSA	Bovine serum albumin
CO_2	Carbon dioxide
°C	Degree celsius
cpm	Counts per minute
Ci	Curie
$CuSO_4$	Cupric sulfate
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DDW	Double-distilled water
DM	Diabetes mellitus
dCTP	Deoxycytidine triphosphate
DMEM	Dulbecco's modification of Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EC	Endothelial cells

EACA	6-amino hexanoic acid
FBS	Fetal bovine serum
h	Hour
HDL	High density lipoproteins
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
HbA_1c	Glycosylated form of hemoglobin
HCl	Hydrochloride
IDDM	Insulin-dependent diabetes mullitus
kb	Kilobase-pair
kDa	Kilodalton
L	Liter
LDL	Low density lipoproteins
Lp(a)	Lipoprotein(a)
LAL	Limulus amebocyte lysate
mg	Milligram
mRNA	Messenger Ribonucleic acid
mM	Millimolar
min	Minute
MI	Myocardial infarction
ml	Milliliter
MDA	Malondialdehyde

Mops	3-(N-morpholino) propanesulfonic acid
MBq	Megabecquerel
N_2	Nitrogen
nm	Nanometer
NIDDM	Non-insulin-dependent diabetes mellitus
NaCl	Sodium chloride
NaN_3	Sodium azide
$NaCNBH_3$	Sodium cyanoborohydride
O_2	Oxygen
p	Statistical probability
pH	Powers of hydrogen
PBS	Phosphate buffered saline
PAs	Plasminogen activators
PAIs	Plasminogen activator inhibitors
PAI-1	Plasminogen activator inhibitor-1
PITC	Phenylisothiocyanate
PVD	Peripheral vascular diseases
RAGE	Receptor for advanced glycation end products
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBARS	Thiobarbituric acid reactive substances

t-PA	Tissue-type plasminogen activator
TCA	Trichloroacetic acid
TBq	Terabecquerel
µg	Microgram
µm	Micrometer
µCi	Microcurie
U	Unit
u-PA	Urokinase-type plasminogen activator
VLDL	Very low density lipoproteins

ABSTRACT

Hyperglycemia and dyslipidemia are two biochemical markers of diabetes mellitus (DM). Increased incidence of cardiovascular diseases and impaired fibrinolytic activity have been found in diabetic subjects. Relationship between the levels of glycated lipoproteins and the regulation of fibrinolytic system in diabetes remains unclear. The present study investigated the effects of glycated low density lipoproteins (LDL) and lipoprotein(a) [Lp(a)] on the production of plasminogen activator inhibitor-1 (PAI-1) and tissue-type plasminogen activator (t-PA) from cultured human umbilical vein endothelial cells (HUVEC). The levels of PAI-1 antigen were increased in the post-cultural media of HUVEC treated with native LDL or Lp(a) for ≥ 24 h. Glycation amplified the increase in PAI-1 secretion induced by native lipoproteins from HUVEC. A significant increase in PAI-1 generation was found in cultures treated with ≥ 50 μg/ml of glycated LDL or with ≥ 5 μg/ml of glycated Lp(a) for ≥ 24 h. The level of 2.4 kb PAI-1 mRNA in HUVEC treated with glycated LDL or Lp(a) was significantly increased and that was associated with moderate reduction of 3.4 kb PAI-1 mRNA level. The *de novo* synthesis and secretion of t-PA in HUVEC treated with 100 μg/ml of native LDL or with 5 μg/ml of native Lp(a) were reduced by incubation for ≥ 16 h. Treatment with ≥ 25 μg/ml of native LDL or ≥ 2.5 μg/ml of native Lp(a) for 24 h significantly reduced the secretion of t-PA from HUVEC compared to control cultures. Treatments with glycated LDL or Lp(a) further reduced the secretion and *de novo* synthesis of t-PA from HUVEC compared to native LDL and Lp(a). Equimolar concentrations of native or glycated bovine serum albumin (BSA) did not alter the production of PAI-1 or t-PA in HUVEC. Treatment with ≥ 25 mM aminoguanidine, an inhibitor of the formation for advanced glycation end

products (AGEs), during the glycation of lipoproteins normalized the generation of PAI-1 and t-PA from HUVEC induced by glycated lipoproteins. The results of the present study indicate that glycation enhances the production of PAI-1 and attenuates the synthesis of t-PA in vascular endothelial cells (EC) induced by native lipoproteins. Increased formation of AGEs in glycated lipoproteins may be responsible for the changes in production of fibrinolytic regulators. The imbalance between the fibrinolytic activators and inhibitors induced by glycated LDL and Lp(a) may contribute to the increased incidence of thrombotic vascular diseases in diabetic subjects.

1. INTRODUCTION

Diabetes mellitus (DM) is by far the most common metabolic disorder, with a prevalence estimated to be 1-5 % of world wide populations [1]. Both type I and type II DM are characterized by hyperglycemia with an absolute or relative lack of insulin. The risk for developing atherosclerotic microvascular disease in diabetic subjects is two- to six-fold higher than that in normal subjects [1]. Duration and magnitude of hyperglycemia are both strongly correlated with the extent and the rate of progression of diabetic microvascular diseases [2]. Disorders in lipid metabolism, coagulation and fibrinolysis have been implicated as causal factors for developing vascular diseases in diabetes [3].

Under normal conditions, intact endothelium provides a non-thrombogenic surface that prevents the adhesion of platelets or other blood cells to the vessel wall. Endothelium encounters blood-born insults incessantly. These insults, notably lipids, immune complexes and microorganisms pose major threats to the integrity of blood vessels. In diabetic subjects, endothelium is directly exposed to increased levels of glucose, lipids and lipoproteins and may easily be damaged. Endothelial cells (EC) play a critical role in the penetration and accumulation of low density lipoproteins (LDL) in arterial wall. It has been suggested [4] that the integrity of the endothelium prevents lipid accumulation in the vascular wall. On the other hand, Minick *et al.* [5] found that, after deendothelialization of the aorta of rabbits, lipid accumulation was only found in reendothelialized areas. Smith & Staples [6, 7] found a nearly two-fold higher LDL concentration in intact aorta intima than in corresponding blood plasma. Intact endothelium was a prerequisite for this accumulation. Endothelial cells are one of the major sources for producing fibrinolytic

regulators. Decreased fibrinolytic activity in the circulating plasma may play an important role in the mechanisms of acute thrombus formation in subjects with coronary artery diseases. Net fibrinolytic activity is mainly the result of balance between the levels of plasminogen activator inhibitor-1 (PAI-1) and tissue type plasminogen activator (t-PA).

To understand better the pathogenesis of diabetic vascular diseases, the effects of glycated lipoproteins, LDL and lipoprotein(a) [Lp(a)], which are two strong risk factors of coronary heart disease [8, 9], on the production of fibrinolytic regulators in vascular EC were investigated.

1.1 Low Density Lipoproteins

Lipoproteins are a group of spherical particles containing a central core of non-polar lipids (primarily triglycerides and cholesteryl ester) with phospholipids and apolipoproteins on the surface. Apolipoproteins are inserted into the phospholipid bilayer (Figure 1). One of the basic functions of lipoproteins is to transport non-water-soluble cholesterol and triglycerides between liver and peripheral tissues. The metabolism of lipoproteins largely depends on apolipoproteins contained within the lipoprotein complex. Lipoproteins are generally divided into four broad categories: chylomicrons, very low density lipoproteins (VLDL), LDL and high density lipoproteins (HDL). LDL is the first type of lipoproteins which have been linked to the development of atherosclerosis [10]. The lipid component of LDL consists of a polar core of neutral lipid comprised mostly of esterified cholesterol. Surrounding this polar core is a lipid coat composed of phospholipids and free cholesterol. In general, lipids comprise 75% of the mass of the LDL particle and protein makes up the remaining 25%. The total lipids in the LDL

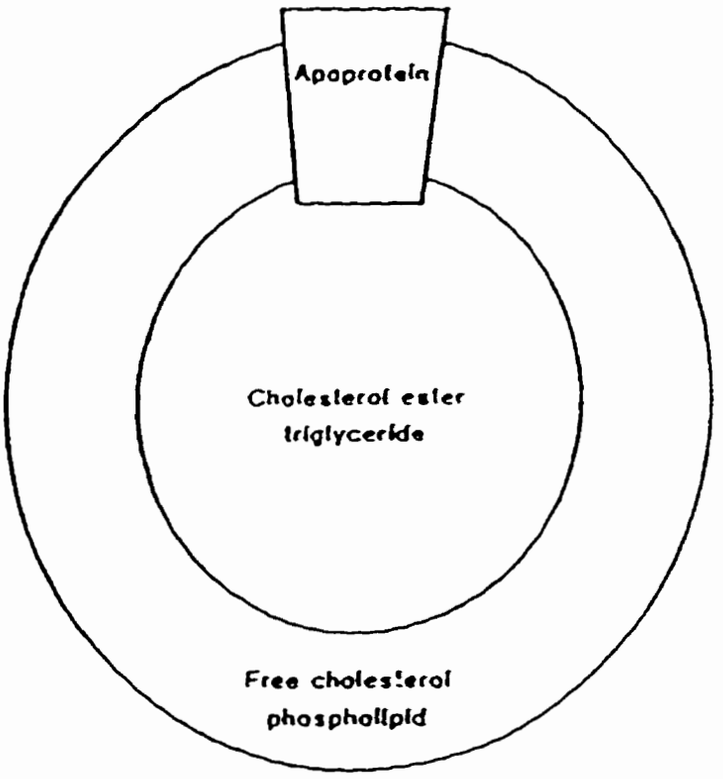


Figure 1. General structure of lipoproteins.

particles are comprised of 60% cholesterol and 30% phospholipids. Eighty percent of cholesterol is in the form of cholesteryl esters presented in the core of the particle. The phospholipids of LDL consist primarily of phosphatidylcholine (65%) and sphingomyelin (25%). LDL also contains at least five different types of glycosphingolipids [10]. The only apolipoprotein in LDL is apolipoprotein B-100 (apoB-100). Only one molecule of apoB-100 is present in each particle of LDL. Clearance of LDL is mainly mediated by the LDL receptor on the surfaces of liver and peripheral cells. LDL may also be cleared by phagocyte cells [11]. Modified LDL is cleared by different types of receptors. Various kinds of clinical and experimental studies have firmly established that elevated plasma concentrations of LDL are associated with accelerated atherogenesis [12].

1.2 Lipoprotein (a)

Lp(a) represents a distinct genetic form of LDL having an additional apolipoprotein, apolipoprotein(a) [apo(a)], which is a specific marker of Lp(a). Apo(a) is linked to apoB-100 by a disulfide bond [13, 14]. Lp(a) contains one mole of apoB-100 and one or two moles of apo(a) [15] (Figure 2). Lp(a) is heterogeneous both in size and density. This heterogeneity may result from variations in protein : lipid ratio, apoB-100 : apo(a) stoichiometry and the size of the apo(a) which varied widely between 300 and 800 kDa [14].

A number of epidemiological studies have shown that high plasma levels of Lp(a) are associated with an increased risk for premature atherosclerotic cardiovascular diseases (ACVD) [13-16]. Lp(a) particles have been found in atherosclerotic vascular wall [17, 18]. Lp(a) competes for the binding of plasminogen to fibrin or to specific receptors on plasma membrane of endothelial cells and platelets [19, 20]. Lp(a) regulates the synthesis of a major fibrinolytic protein, PAI-1, in EC [21] and oxidation enhances the effect of Lp(a) on PAI-1 production in EC [22].

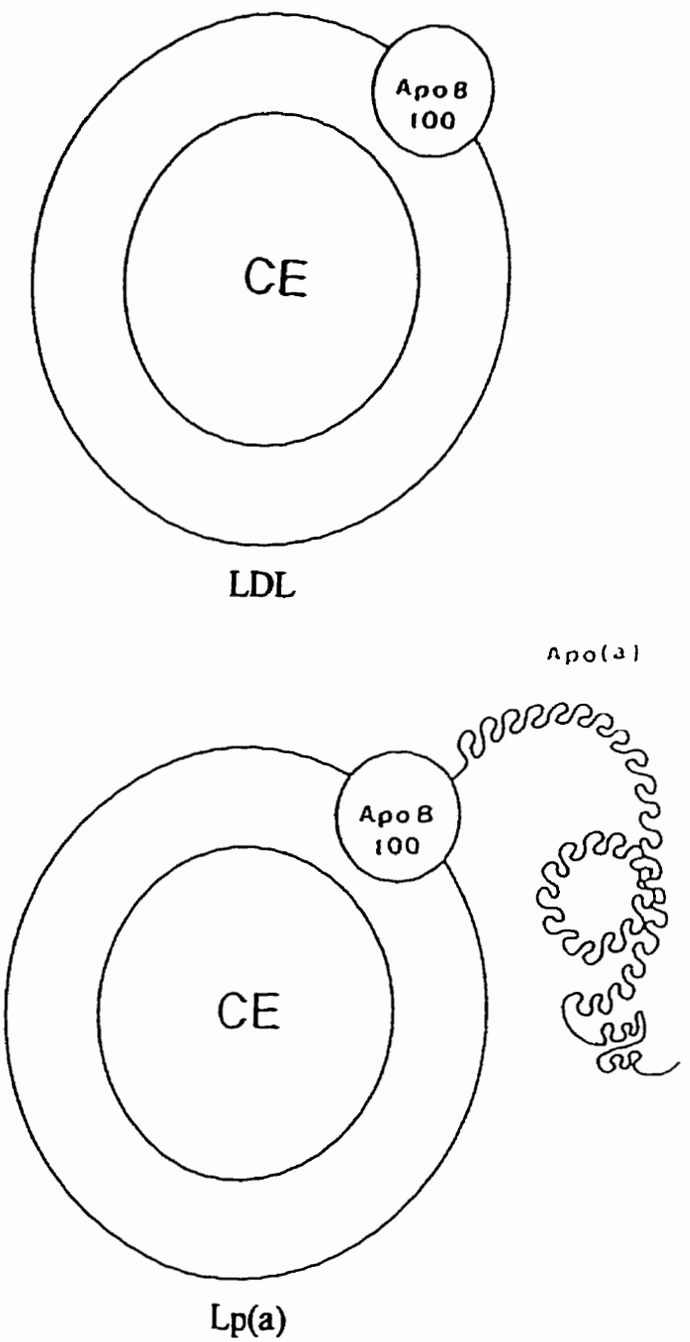


Figure 2. Comparison of LDL and Lp(a) structure.

1.3 Fibrinolytic system

It is generally assumed that vascular homeostasis results from the regulated interaction of coagulation and fibrinolysis systems. Imbalance of these systems may lead to hemorrhage or thrombosis. Degradation of fibrin, the key step of intravascular fibrinolysis, normally results from the action of plasmin. Plasmin is a serine protease conversed from the inactive proenzyme, plasminogen [23]. There are two types of plasminogen activators (PAs), t-PA and urokinase-type PA (u-PA). t-PA is the primary PA in blood circulation. u-PA appears to act in extravascular compartments. t-PA and u-PA are immunologically distinct and are encoded by different genes [24]. The fibrinolytic process usually is localized to fibrin clots because plasminogen is selectively incorporated into fibrin thrombi at the time of thrombus formation. In addition, t-PA and u-PA derived from cells in vascular wall near the site of thrombus formation will interact with plasminogen in thrombus. The activity of t-PA is greatly increased in the presence of fibrin clots. The activity of PAs is controlled by PA inhibitors (PAIs). PAI-1 is the primary physiological inhibitor of plasminogen activation [25]. PAI-1 is synthesized by a large variety of cultured cells, including EC and smooth muscle cells. The biosynthesis of PAI-1 *in vitro* can be induced by a diverse group of growth factors, cytokines, and hormones [26]. PAI-1 mRNA levels in severe atherosclerotic vessels were significantly increased compared to that in normal arteries [27]. Increased production of PAI-1 may decrease fibrinolytic activity in local circulation and leads to a prothrombotic state. This condition may promote mural thrombosis, which is frequently involved in the development of atherosclerotic lesions [25].

1.4 Changes of lipoproteins in DM

Uncontrolled diabetes is associated with multiple abnormalities in lipid and lipoprotein metabolism, and lipid levels are usually elevated in untreated and poorly controlled insulin-dependent diabetes mellitus (IDDM) [28, 29]. IDDM subjects who are receiving conventional insulin therapy usually have normal levels of total cholesterol in LDL and HDL in plasma [30]. In untreated or poorly controlled IDDM patients, the plasma concentrations of LDL are increased and that often associated with reduced levels of HDL cholesterol [31, 32]. LDL production rates were reported to be elevated in IDDM and returned to normal level after insulin treatment. This may reflect the increased synthesis of precursor of VLDL or the impaired removal of VLDL remnants [30]. Impaired receptor-mediated clearance of LDL may be caused by the glycation of apoB-100 [33, 34] or the deficient binding to the receptor [35].

In IDDM subjects with poor metabolic control and particularly in those with proteinuria, higher levels of Lp(a) have been found [36-38]. Glucose control is associated with reduced Lp(a) levels. The explanation for decreased Lp(a) concentrations after improved metabolic control is unknown. Hyperglycemia may facilitate the rate of apo(a) biosynthesis and which leads to increase serum Lp(a) levels [39, 40]. It is also possible that glycation impedes the clearance of Lp(a) from plasma.

The plasma concentrations of LDL cholesterol and LDL apoB-100 are usually within or close to normal limits in non-insulin-dependent diabetes mellitus (NIDDM) subjects with well controlled blood glucose [41, 42]. When blood glucose levels are moderately elevated, the defect in clearance becomes evident and LDL levels may be

increased [43]. The increase in the proportion of triglyceride in LDL has been consistently observed in NIDDM subjects. In some cases, LDL particles are in greater density than normal LDL [11].

The levels of Lp(a) are modestly higher in subjects with NIDDM. In contrast to IDDM, no association has been found between Lp(a) levels and glycemic control [44]. Glucose control in NIDDM was unable to reduce their Lp(a) levels in most studies [45, 46] with one exception [47]. The cause for the differences between improved metabolic control and consistently high Lp(a) concentrations in NIDDM subjects has not been known.

1.5 Changes of fibrinolytic activity in DM

In diabetic subjects, fibrinolytic activity can be normal, increased or decreased [48]. The release of fibrinolytic activity after vascular stimulation was impaired in subjects with diabetes [49]. Further analysis revealed that t-PA was present in normal or increased amounts in the plasma of diabetic subjects, but most t-PA were biologically inactive because they were bound in complexes with their inhibitors. The decreased fibrinolytic activity presented in NIDDM subjects was associated with elevated concentration of PAI-1 [50]. Fibrinolytic response to venous occlusion was lower than normal in diabetic subjects [3]. PAI-1 in blood is mainly (>90%) stored in platelets [51]. PAI-1 concentrations in fully lysed platelets isolated from diabetic subjects (NIDDM) were significantly higher compared to that from controls [52]. High PAI-1 plasma levels have been found in several disorders characterized by thrombotic events, such as acute myocardial infarction and deep venous thrombosis. Gray *et al.* [53] studied 155 patients

admitted for acute myocardial infarction. The levels of PAI-1 activity in blood of the acute myocardial infarction subjects with diabetes were significant higher than those without diabetes. In IDDM, the increase in PAI-1 activity was associated with retinopathy and microalbuminuria [54, 55].

The influence of metabolic parameters on fibrinolysis is still unclear. It has been found that insulin, proinsulin-like molecules, VLDL, LDL and glucose stimulated PAI-1 production *in vitro* [56]. LDL, HDL and Lp(a) inhibited the secretion of t-PA from EC. Oxidized LDL and Lp(a) enhanced the secretion of PAI-1 from vascular EC [22].

1.6 Glycation and oxidation of lipoproteins in DM

Hyperglycemia, the most obvious metabolic abnormality in DM, is the primary causal factor responsible for the development of diabetic vascular complications. One of the most acclaimed hypotheses on the pathogenesis of diabetic complications concerns the non-enzymatic glycation reactions which may be capable of altering the function of a molecule. Duration and magnitude of hyperglycemia are both strongly correlated with the extent and rate of progression of diabetic microvascular disease [1]. It is well documented that a glycosylated form of hemoglobin (HbA_1c) is elevated in diabetics and the levels of HbA_1c are parallel to blood glucose levels [57]. Glycation is not specific for hemoglobin, but also for collagen, erythrocyte membrane proteins, lens crystalline and lipoproteins.

Since lipoproteins are involved in the development of atherosclerosis, the major cause of morbidity and mortality in diabetic subjects, modification of lipoprotein particles occurring in diabetes is of considerable interest. LDL and Lp(a) have been considered as strong risk factors for atherosclerosis. The levels of glycated LDL in diabetic subjects

were significantly higher than those in control subjects, and were correlated with the levels of HbA_1c [58]. The implication of glycated LDL in atherogenesis has been suggested by the impaired recognition of glycated LDL by the LDL receptor [59] and the increased cholesteryl ester accumulation in monocyte/macrophages exposed to glycated LDL [60].

Previous studies did not reveal apparent differences between glycated LDL and Lp(a) in the terms of their physical and functional properties [61]. The major target of glycation appears to be apoB-100 rather than apo(a) in Lp(a) particle. Glycation of Lp(a) is greater in diabetic subjects than in non-diabetic subjects and the levels of glycated Lp(a) were positively correlated to that of HbA_1c in diabetic subjects [62].

When proteins or lipids are exposed to glucose, they undergo glycation and oxidation. Glycation involves the nonenzymatic binding of glucose to reactive amino groups located on lysine side chains and N-terminal amino acid residues of protein molecules, through the formation of ketoamine linkage (Figure 3). The early glycation products are in equilibrium with plasma glucose. When glucose levels fall, the early glycation products can dissociate from native proteins. Alternatively, if glycation continues, further molecular rearrangements may occur resulting in the formation of advanced glycation end products (AGEs). The latter are glucose-proteins or lipid heterogeneous structures containing yellow-brown fluorescent pigments. The formation of AGEs has been considered as irreversible. These products accumulate over the life-time of proteins and do not return to normal when hyperglycemia is corrected. AGEs have been hypothesized to contribute to the development of vascular diseases and it may,

at least in part, be through their binding to a novel integral membrane protein, the receptor for AGEs (RAGE) which are presented in a variety of cells [63]. RAGE and its mRNA have been identified in EC both *in vivo* and *in vitro* [64]. The interaction of AGEs with their receptors directly generates oxygen free radicals, which may result in a variety of changes in EC [65].

Because of the major role of AGEs in the development of diabetic complications, several compounds that inhibit their formation have been evaluated. Aminoguanidine is one of AGEs formation inhibitors. It binds to the carbonyl of Amadori-product-derived fragmentation products and forms unreactive 5- or 6-substituted triazines [66]. Aminoguanidine has been shown to inhibit vascular pathology in diabetic animals. Brownlee *et al.* [67] studied the effect of aminoguanidine on AGEs formation in alloxan-induced diabetic and non-diabetic rats. In the aminoguanidine-treated group, the levels of collagen cross-linked to lipoproteins through AGEs were similar to non-diabetic rats. The values of AGEs-linked collagen from the untreated diabetic group were 3-5 times higher than that from the aminoguanidine-treated diabetic group.

The presence of nonenzymatically bound glucose on lipoproteins may increase the likelihood of oxidative damage, which could affect both apolipoprotein and lipid core of lipoproteins [68]. Glycated LDL is more easily oxidized. It is possible that glycated LDL accumulates in the subendothelial space with decreased catabolism, allowing it to undergo oxidation [69].

An increased oxidative stress has been established in diabetes [70]. The increase in production of free radicals is linked to glucose auto-oxidation and to the release of free

radicals from glycated proteins. The production of free radicals is correlated to metabolic control and more directly to hyperglycemia [71]. It has been known that oxidative stress is accompanied by homeostatic alterations in diabetic subjects [72].

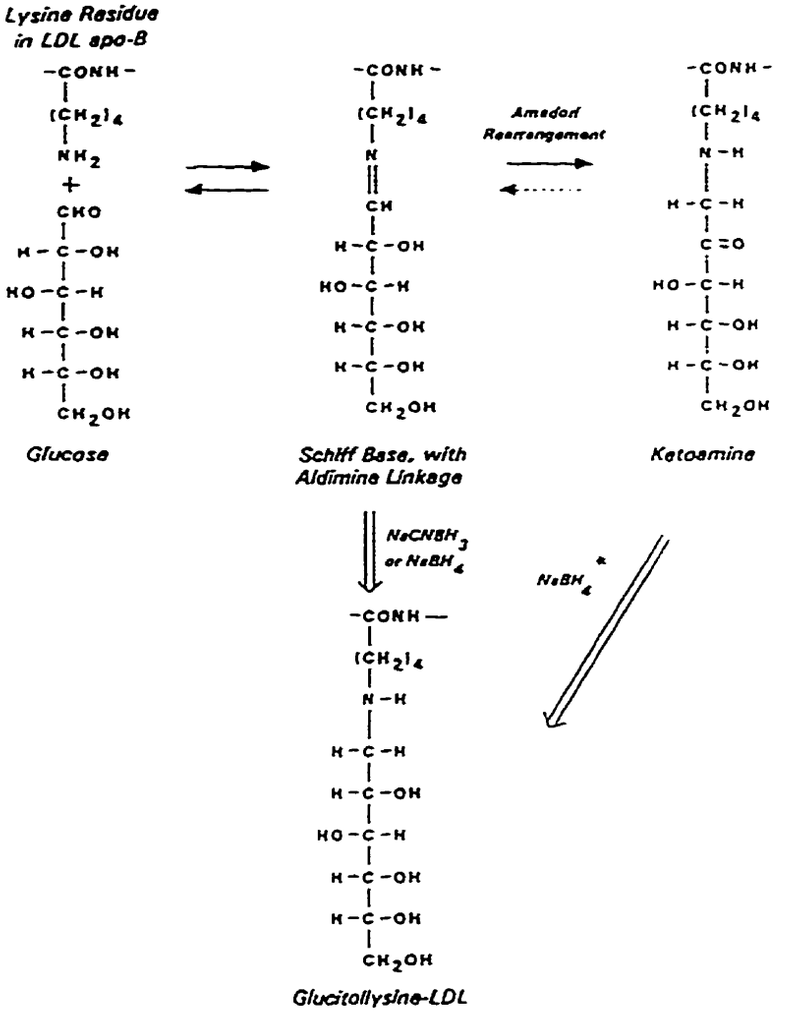


Figure 3. Reaction scheme for glycation of lipoproteins.

2. HYPOTHESIS

It has been known that glycation potentially enhances atherogenicity and thrombogenicity of plasma lipoproteins. Fibrinolytic system plays a critical role in the clearance of intravascular clots and is involved in the development of thrombosis and atherosclerosis. Fibrinolytic activity is usually attenuated in diabetic subjects. Previous studies in our laboratory demonstrated that oxidized LDL and Lp(a) enhanced production of PAI-1 in cultured vascular EC. An enhanced oxidative stress has been observed in subjects with diabetes, as indicated by diminished antioxidant status and increased blood levels of lipid peroxidation products. Glycation of proteins has been involved in this enhanced oxidative stress, through the generation of oxygen free radicals by glycated proteins. In this manner, glycation of apolipoprotein and lipid may result in oxidative damage to lipoproteins. The influence of glycated lipoproteins on the fibrinolytic system has not been well investigated.

2.1 Glycated LDL may alter the production of fibrinolytic regulators in EC

Glycation of LDL in diabetic subjects is significantly higher than that in control subjects, and is significantly correlated with HbA_1c [58]. Glycation may alter the conformation of lipoproteins. It has been known that the recognition by the LDL receptor on macrophages is impaired by glycation. LDL isolated from IDDM subjects stimulates the esterification of cholesterol in macrophages. Glycated LDL stimulated thrombin-induced thromboxane B_2 synthesis and platelet aggregation compared to native LDL. Oxidized LDL has been detected immunohistochemically in human atherosclerotic lesions. Native and oxidized LDL modified by $CuSO_4$, ultraviolet radiation or acetylation

increased PAI-1 secretion from HUVEC. We consider that glycated LDL may influence fibrinolytic activity by regulating the production of vascular cells-derived fibrinolytic regulators.

2.2 Glycated Lp(a) may alter the production of fibrinolytic regulators in EC

Lp(a) is a cholesterol-rich, LDL-like particle whose protein moiety contains apo(a), in addition to apoB-100. It has been demonstrated that increased plasma Lp(a) levels constitute an independent risk factor of cardiovascular disease. Increased circulating Lp(a) concentrations in diabetic subjects were found in some but not all of previous studies. The levels of glycated Lp(a) were higher in diabetic subjects than that in non-diabetic subjects and were positively correlated with the levels of HbA_1c. Native Lp(a) increased PAI-1 secretion from HUVEC and oxidation of Lp(a) modified by $CuSO_4$ enhanced this effect. Glycated Lp(a) may alter the production of fibrinolytic regulators in EC.

2.3 AGEs may contribute to the alternation of the production of fibrinolytic regulators-induced by glycated lipoproteins

AGEs are recognized by a class of receptors presented in a variety of cells [63]. AGEs receptor and its mRNA have been identified in endothelial cells both *in vivo* and *in vitro* [64]. The interaction of AGEs with their receptors directly generates oxygen free radicals, which may result in a variety of activation changes in EC [65]. Aminoguanidine effectively inhibits the formation of AGEs *in vitro* and *in vivo*. Studying the effects of aminoguanidine treated-glycated lipoproteins on the generation of PAI-1 and t-PA may

help to understand the mechanism by which glycated lipoproteins regulate the production of fibrinolytic regulators in EC.

The studies in this project will improve the understanding of the pathogenesis of thrombotic cardiovascular complications in diabetic subjects.

3. MATERIALS

3.1 Cells

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase digestion. EC were verified by nonoverlaping cobblestone culture morphology and the presence of factor VIII antigen.

3.2 Chemicals

[α^{32}P]-dCTP was obtained from Dupont, Mississauga, Ontario, Canada.

Diethylpyrocarbonate, sarkosyl, sodium citrate, 2-beta mercaptoethanol, cesium chloride, sodium acetate, sodium chloride, Tris-HCl, Tris-base, EDTA, SDS, MOPS, bromophenol blue, xylem cyan, glycerol, ethidium bromide, formaldehyde, formamide, benzamidine, EACA, sodium phosphate monobasic, sodium phosphate dibasic, sodium bicarbonate, anti-sheep IgG, albumin bovine, L-lysine, D-glucose, sodium azide, endothelial cell growth supplement, streptomycin, penicillin and PITC (Phenylisothiocyanate) were purchased from Sigma Chemical Co., St. Louis, MO.

Pyruvate, n-butanol, methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific, Edmonton, Alberta, Canada.

Fetal bovine serum (FBS), non-essential amino acid and guanidine isothiocyanate were obtained from Canadian Life Technologies Inc. Burlington, Ontario, Canada.

4. METHODS

4.1 Purification of Lp(a)

Density < 1.21 plasma fraction was separated from fresh human plasma by ultracentrifugation. Plasma was adjusted to a density of 1.21 g/ml with potassium bromide and centrifuged at 39,000 rpm for 40 h at 4 °C in a SW 60Ti rotor (Beckman Instrument Inc. Fullerton, CA). The top fraction was dialyzed against dialysis buffer consisting of 0.15 M NaCl, 0.01% EDTA and 0.01% NaN_3 (pH 7.4) at 4°C. The dialyzed plasma fraction was purified by using lysine-Sepharose-CL 4B affinity chromatography [73]. Lp(a) was eluted with 20 mM 6-amino hexanoic acid in 0.1 M phosphate buffer (pH 7.4) containing 1 mM benzamidine and 0.01% EDTA. Apo(a) phenotypes were determined by Western blotting analysis. Purified samples were run on a 2.75% SDS-polyacrylamide-agarose gel and transferred to a nitrocellulose membrane. Apo(a) isoforms were visualized by using anti-human Lp(a) antibodies (Immuo AG. Vienna, Austria).

4.2 Isolation of LDL

Native LDL was prepared from the unbound plasma fraction eluted from lysine-Sepharose-CL 4B affinity chromatography column which was used for Lp(a) purification. LDL (density 1.024 - 1.063) was isolated by sequential ultracentrifugation in a Ti 60 rotor (Beckman). LDL was dialyzed at 4°C against 150 mM NaCl solution supplement with 0.26 mM EDTA (pH 7.4). LDL as well as other lipoproteins described below were stored in nitrogen overlaid tubes with sealed caps at 4°C in the dark.

4.3 Modification of lipoproteins

4.3.1 Glycation

Lipoproteins [~2 mg/ml of LDL, ~200 μg/ml of Lp(a)] were incubated with indicated concentrations of glucose in 0.1 M phosphate buffer (pH 7.4) containing equimolar concentration of sodium cyanoborohydride ($NaCNBH_3$), 0.01% EDTA and 0.01% NaN_3 for 7 days. Incubations were carried out under N_2, in the dark, at 37°C. Control lipoproteins were prepared by incubating under identical conditions except glucose. Radiolabelled ^{14}C-glucose (10 μCi/ml) was added with unlabelled glucose during glycation for monitoring the incorporation of glucose into lipoproteins. At the end of incubation, samples were dialyzed 24 h at 4°C to remove free glucose.

Native and glycated lipoproteins were analyzed on a 1% agarose electrophoresis gel (Coring, Oneonta, N.Y.) at 90 V for 50 min in 75 mM barbital buffer (pH 8.6) to assess the change in electrophoretic mobility of modified lipoproteins.

4.3.2 Analyses of amino acid profile of glycated lipoproteins

Native lipoproteins and glycated lipoproteins were hydrolyzed in 2 N HCl for 24 h at 100°C. Hydrolyzed samples were subjected to three washes with coupling buffer (acetonitrile:ethanol:triethylamine:water, 10:5:2:3, v/v/v/v) and dried by evaporation. The samples were resuspended in 100 μl of coupling buffer containing 5 μl (0.42 μM) of phenylisothiocyanate (PITC) and incubated at room temperature for 5 min then dried. The products were resuspened in 400-600 μl of solvent A (70 mM sodium acetate, pH 6.5), filtered through a 0.2 μm pore size filter and then chromatographed at room temperature. All samples were assayed on a Beckman Ultrasphere ODS C_{18} column (4.6

mm × 25 cm) in a high performance liquid chromatography (HPLC) with Gold System (Beckman), principally as previously described [74]. A gradient of solvent A and solvent B (100% acetonitrile) system was developed as illustrated in Table 1 and 2.

Table 1. The gradient solvent system for glycated LDL analysis.

Elution time (min)	Flow rate (ml/min)	A%	B%	Duration (min)
initial	1	100	0	
0.00	1	75	25	10
10.00	1	75	25	13
23.00	1	100	0	1
24.00	1	100	0	2

Table 2. The gradient solvent system for glycated Lp(a) analysis.

Elution time (min)	Flow rate (ml/min)	A%	B%	Duration (min)
initial	1	100	0	
0.00	1	80	20	30
30.00	1	80	20	13
43.00	1	70	30	1
44.00	1	70	30	10

4.3.3 Oxidation

After isolation, lipoproteins [1 mg LDL protein/ml and 200 μg Lp(a) protein/ml] were first dialyzed against an EDTA-free phosphate buffer for 24 h and then 5μM $CuSO_4$ was added for oxidative modification at room temperature for 24 h. The oxidation of lipoprotein was terminated by the addition of 0.3 mM EDTA followed by dialysis for 24 h against of Krebs - Henseleit buffer containing 0.3 mM EDTA.

The degree of oxidative modification of lipoproteins was determined by measuring thiobarbituric acid reactive substances (TBARS). 1, 1, 3, 3-tetramethoxypropane was

used as a standard. Briefly, 2 ml of assay mixture (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl) was added to 200 μg (protein) of lipoproteins. The mixture was heated at 100 °C for 15 min. After cooling at room temperature, the samples were centrifuged at 200×g for 10 min. The absorbance of the supernatant was read at 535 nm wavelength by using a spectrophotometer [75]. The results were expressed as nM malondialdehyde (MDA)/mg protein of LDL or Lp(a).

4.4 Measurement of protein concentrations in lipoproteins and cells

Protein concentrations of lipoproteins were measured by the Lowry method which was modified for measuring proteins in lipoproteins [76]. BSA was used as a standard. For measuring the total amount of proteins in cultured cells, cells were harvested in PBS containing 0.5% Triton X-100 and 0.1% SDS. Aliquots of cell lysates were analyzed by the Lower method [76].

4.5 Cell culture and treatments

4.5.1 Cell culture

HUVEC were cultured in M-199 medium supplemented with 10% heat-inactivated FBS, 30 μg/ml of endothelial cell growth stimulator, 100 μg/ml of heparin, 0.1 mM nonessential amino acids and 200 U/ml of penicillin and 200 μg/ml of streptomycin in a humidified incubator under 95% air / 5% CO_2 at 37 °C.

4.5.2 Stimulation of cells

Confluent HUVEC were rinsed with heparin-free M-199 medium prior to conducting experiments then incubated in heparin-free M-199 medium with or without addition of native or modified lipoproteins for indicated doses and periods.

4.5.3 Measurement of TBARS in conditioned medium

At the end of incubation, the conditioned media of HUVEC were harvested and frozen at -20 °C until analysis. The samples were lyophilized by using a vacuum frozen dryer and the residues were dissolved in DDW. The degree of oxidation in the conditioned media was determined by measuring TBARS as described above.

4.6 Measurement of PAI-1 and t-PA antigen

Conditioned media of cultured HUVEC were collected at the end of incubation. Cells were harvested by using PBS (pH 7.4) containing 0.1% SDS and 0.5% Triton X-100. The total levels of PAI-1 and t-PA antigen in the conditioned media of HUVEC treated with modified lipoproteins were determined by using PAI-1 or t-PA enzyme-linked immunosorbant assay (ELISA) kit with a monoclonal antibody against human PAI-1 or t-PA (American Diagnostica Inc. Greenwich, CT) following the manufacturer's instructions. Absorbance at 490 nm wavelength was used to read on a micro-test plate spectrophotometer for the levels of PAI-1 or t-PA antigen. Total amounts of cellular protein in each well were measured by a modified Lowry Method [75].

4.7 Northern blotting analysis of PAI-1 and t-PA mRNA

Total cellular RNA was extracted from cells at the end of treatment, by the guanidine isothiocyanate-cesium chloride method [77]. The concentration of RNA was determined by the magnitude of sample absorbance read at 260 nm wavelength using a spectrophotometer. RNA was denatured and subjected to electrophoresis in a 1% agarose-formaldehyde gel and then transferred to Zeta-Probe GT blotting membranes (BioRad, Hercules, CA). Plasmids containing cDNA fragment encoded human PAI-1,

human t-PA or β-actin were labelled with [α^{32}P]-dCTP (>111 TBq/mM, Dupont NEN, Guelph, ON, CA) by using a random primer labeling kit. Blots were prehybridized in 0.25 M Na_2HPO_4 (pH 7.2) and 7% SDS for 5 min at 42°C and then hybridized with denatured probe for 16 h at 42°C. After hybridization, blots were washed and subjected to autoradiography. The levels of PAI-1 or t-PA mRNA were quantified from autoradiograms by using ultrascan XL laser scanning densitometry after correcting with the levels of β-actin mRNA on rehybridized blots.

4.8 Metabolic labelling and immunoprecipitation of t-PA

Confluent cells in 60-mm dishes were treated with 1 ml of methionine- and cystine-free DMEM (ICN, Radiochemicals, Irvine, CA) containing 100 μCi/ml of Tran ^{35}S-label (200 Ci/mM, 38 TBq/mM, 85% methionine and 15% cystine, ICN), supplemented with 2 mM glutamine and 10% serum with or without an addition of 100 μg/ml of native or glycated LDL for 8-48 h. Cells were harvested in 1 ml of PBS/well containing 0.5% Triton X-100 and 0.1% SDS as previously described. Cell lysates were centrifuged at 4°C for 2 min at 12,000 g and diluted with buffer containing 50 mM Tris, 500 mM NaCl, 0.1% NP-40, 1 mM EDTA, 2.5% gelatine and 0.5% BSA (NET buffer). The resulting supernatants were precleared by incubation with 30 μg/ml of control rabbit IgG at 25°C for 1 h, followed by addition of 40 μl of a 50% slurry of protein A-Sepharose (Sigma) and incubated for 30 min. The beads were removed by centrifugation. The supernatants were incubated at 25°C for 2 h with monoclonal antibody against human t-PA (American Diagnostica) followed by protein A-Sepharose as above and centrifugation to pellet the

immune complex. The beads were then washed sequentially with NET buffer supplemented with 0.5% sodium deoxycholate followed by 10 mM Tris (pH 7.5) with 0.1% NP-40. Immunoprecipitates were recovered from the beads by dissolving in 125 mM Tris buffer (pH 6.8) containing 20% glycerol and 4.6% SDS, then boiled for 5 min under nonreducing conditions and analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Radioactive t-PA was visualized from dried gels using autoradiography.

4.9 Evaluation of cytotoxicity of lipoproteins

Cytotoxicity of lipoproteins was estimated by the incorporation of ^{3}H-leucine. Following the stimulation with lipoproteins, cells were incubated with 5×10^{6} dpm/well of ^{3}H-leucine in leucine-free medium for 2 h. Unincorporated leucine was removed. The cells were washed three time with ice-cold PBS and dissolved in ice-cold 5% trichloroacetic acid (TCA). The mixtures were placed in boiling water for 20 min, and the particles were collected by using a glass filter. The radioactivity of particles was counted in a scintillation counter.

4.10 Detection of endotoxin

It has been known that endotoxin stimulates PAI-1 secretion in cultured HUVEC [78]. To investigate whether the lipoproteins used to stimulate the cells were contaminated with endotoxin, the levels of endotoxin were determined by limulus amebocyte lysate (LAL) test with a commercially available kit (E-TOXATE®, Sigma) following the manufacturer's recommendations. Lipoproteins were extracted with

chloroform (chloroform : lipoprotein = 1 : 4) for 4 h [79] to permit recovery of endotoxin. The emulsion was centrifuged for 10 min at 1100 × g, and the cloudy, middle layer was removed with a Pasteur pipette. The extracted lipoproteins were diluted 1/10 in endotoxin-free water and heated 5 min at 75°C and their pH value were adjusted to 6-8 with HCl. A positive test is indicated by the formation of a hard gel which permits complete inversion of the tubes without disruption of the gel. All other results, including soft gels, turgidity, increase in viscosity and clear liquid, were considered negative. No detectable endotoxin contamination was found in native or glycated lipoprotein preparations.

4.11 Statistical analysis

All results are presented as mean ± SD (number of experiments) unless otherwise indicated. Comparisons between multiple groups were achieved by using one-way ANOVA and followed by Duncan's new multiple range test. The level of significance is defined as $p<0.05$.

5. RESULTS

5.1 In vitro preparation and characterization of glycated lipoproteins

5.1.1 Electrophoretic mobility of lipoproteins

The increased electronegativity of glycated lipoproteins was detected by agarose gel electrophoresis. Glycated lipoproteins, both glycated LDL and glycated Lp(a), showed increased anodic migration in contrast to native lipoproteins that were incubated

under the same conditions as glycated lipoproteins omitting glucose. The result of agarose electrophoresis of glycated LDL is shown in Figure 4.

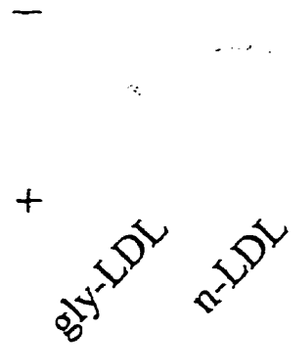


Figure 4. Electrophoresis of native and glycated LDL.
LDL was modified by 200 mM glucose at 37°C for 7 days. Glycated and native LDL were loaded on a 1% agarose gel and the gel was run at 90 V for 50 min in 75 mM barbital buffer (pH 8.6). Lipoproteins were visualized by staining with Sudan red B. Left: glycated LDL (gly-LDL); Right: native LDL (n-LDL).

5.1.2 Amino acid analysis for lipoproteins

Amino acid profiles in native LDL and Lp(a) [Figure 5 (A), Figure 6 (A)], or in glycated LDL and Lp(a) [Figure 5 (B), Figure 6 (B)] were analyzed by HPLC. A novel peak that appeared between isoleucine and phenylalanine in the amino acid profiles of glycated lipoproteins was considered as a glycated product of lysine, glucitollysine, based on the facts: 1) the abundance of lysine was reduced consistently in glycated lipoproteins; 2) the radioactivity of ^{14}C-glucose in putative glucitollysine was 5-6 times higher than that in background, phenylalanine or lysine individually as shown in Table 3.

Table 3. The radioactivity of amino acids in ^{14}C-glucose labelled glycated LDL.

Amino acid	Radioactivity (fold increase of background)
Methionine	1.82
Isoleucine+Leucine	1.82
Glucitollycine	5.34
Phenylalanine	1.06
Lysine	0.92

Native LDL was incubated with 200 mM glucose containing 10 μCi/ml of ^{14}C-glucose for 7 days as described in Methods. The amino acids of the reaction mixture were separated by HPLC. Each fraction of amino acid was collected and the radioactivity of the amino acids was counted in a scintillation counter. Values are generated from the results of one experiment.

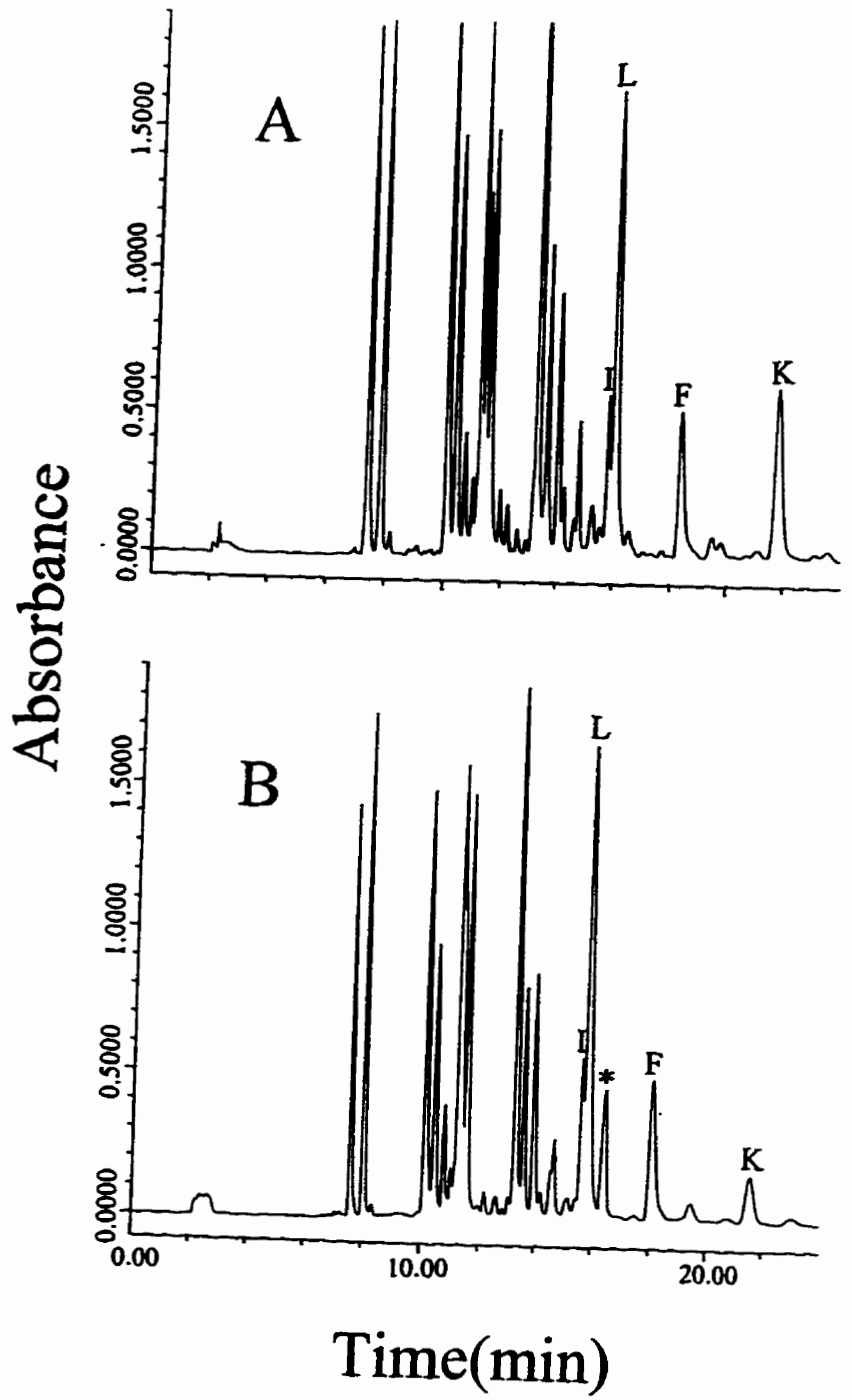


Figure 5. Amino acid profile of native and glycated LDL.

A: native LDL, LDL was unmodified; B: glycated LDL, LDL was modified by 200 mM glucose. Hydrolyzed LDL were derivertized with 0.42 μM of PITC and analyzed by a reverse phase HPLC with a solvent system of 100-70% of 70 mM sodium acetate and 0-30% of acetonitrile. I: isoleucine; L: leucine; *: putative glucitollysine; F: phenylalanine; K: lysine.

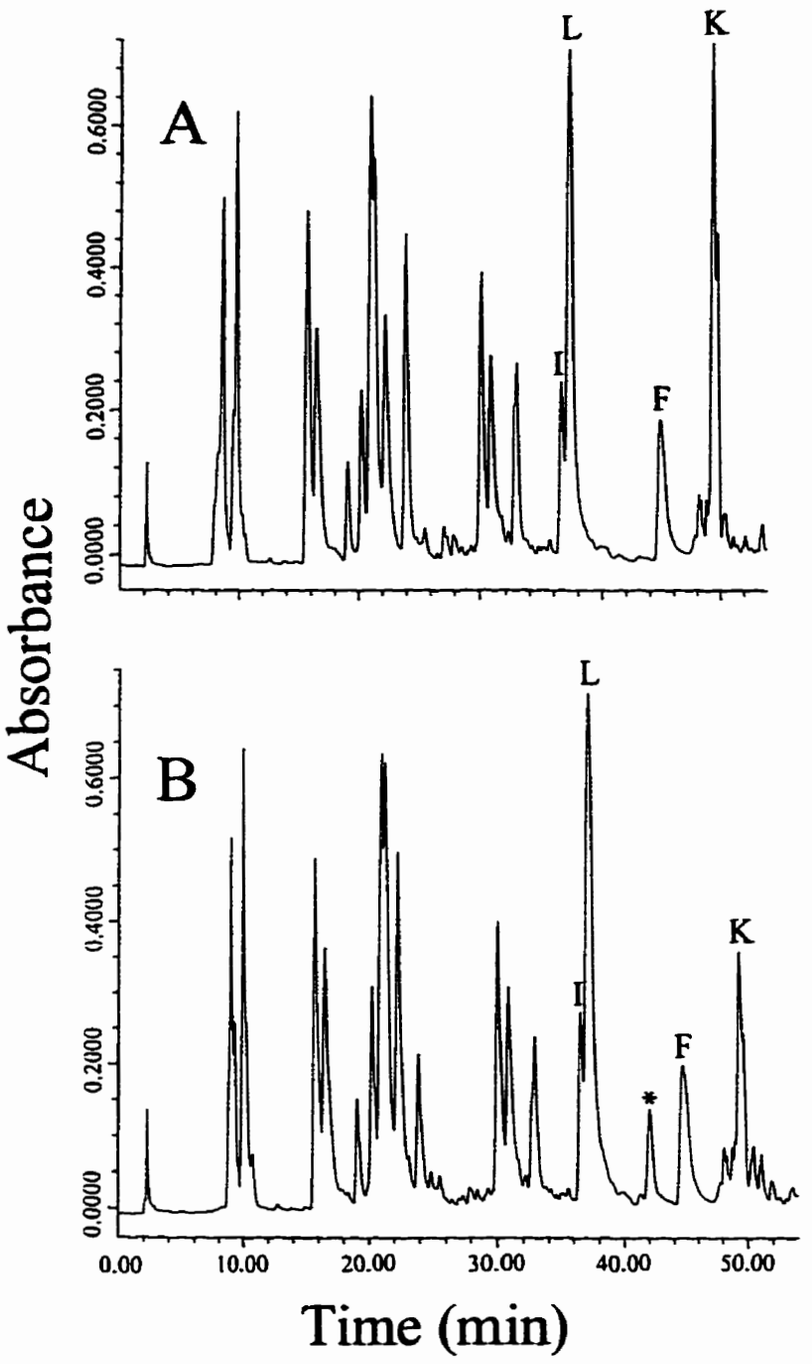


Figure 6. Amino acid profiles of native Lp(a) and glycated Lp(a).

Native Lp(a) and glycated Lp(a) was analyzed by reverse phase HPLC as described in methods. Lp(a) was incubated without glucose [native Lp(a), A] or with 200 mM glucose [glycated Lp(a), B] for 7 days at 37°C. Individual amino acids are identified by their single letter code, putative glucitollysine is identified by the asterisk.

The effect of increasing concentration of glucose on the glycation of lysine in LDL was observed. The ratio of peak area of glucitollysine to lysine was used to indicate the degree of incorporation of glucose to LDL as shown in Table 4. Detectable changes in glucitollysine/lysine ratio were found in LDL glycated by ≥ 25 mM glucose and these reached to a plateau in LDL treated with 100 mM glucose.

Table 4. Effect of glucose concentration on the formation of glucitollysine in glycated LDL.

Concentration of glucose (mM)	Ratio of glucitollysine/lysine (area of peak)
0	0.18
5	0.18
10	0.19
25	0.57
50	0.98
100	1.12
200	1.19

LDL (2 mg/ml) which was glycated with 0 to 200 mM glucose for 7 days at 37°C was analyzed by HPLC as described in Methods. The ratio of glucitollysine/lysine peak area was used to indicate the degree of incorporation of glucose into LDL. Values are generated from the results of one experiment.

5.2 Effect of glycated LDL on the production of fibrinolytic regulators in cultured HUVEC

5.2.1 Effect of glycated LDL on PAI-1 and t-PA secretion from EC

The levels of PAI-1 and t-PA antigen were determined in post-cultural media of HUVEC treated in heparin-free medium without (control) or with the addition of 10-100 μg/ml of native or glycated LDL for 24 or 48 h. In HUVEC treated with 100 μg/ml of LDL for ≥ 56 h or with ≥ 200 μg/ml of LDL for 48 h, visible morphological changes in cells were observed.

After ≥ 24 h of incubation, the levels of PAI-1 antigen in the media of HUVEC treated with 100 μg/ml of native LDL were significantly higher than those in time-matched controls ($p<0.001$). Treatment with glycated LDL for 24 or 48 h significantly increased the levels of PAI-1 in the media compared to native LDL for matching periods ($p<0.001$, Table 5 and Figure 7, upper panel). A significant increase in PAI-1 secretion was detected in EC treated with 100 μg/ml of native LDL for 48 h compared to that in control cultures, but not in cells treated with lower concentrations of native LDL. The levels of PAI-1 antigen were significantly higher in the media of HUVEC stimulated with ≥ 50 μg/ml of glycated LDL than those in cells treated with equal amounts of native LDL (Table 6 and Figure 7, lower panel).

The levels of t-PA antigen in the conditioned media of HUVEC treated with 100 μg/ml of native or glycated LDL were elevated progressively along with the increase of incubation time. The secretion of t-PA was significantly reduced by treatments with 100 μg/ml of native LDL for ≥ 16 h compared to that in control cultures (Table 7 and Figure

8, upper panel). The levels of t-PA antigen in the media of HUVEC treated with 100 μg/ml of glycated LDL for ≥ 16 h were significantly lower than those in cells treated with an equal amount of native LDL for matching periods ($p<0.001$). The maximal suppression of t-PA secretion induced by glycated LDL was detected in EC treated with 100 μg/ml of glycated LDL for 24 h. Treatments with ≥ 25 μg/ml of native or glycated LDL for 24 h significantly ($p<0.001$) reduced the secretion of t-PA antigen from HUVEC compared to control cultures ($p<0.001$). The inhibitory effect of glycated LDL on t-PA secretion was greater than that of native LDL (Table 8 and Figure 8, lower panel).

Table 5. Time-dependency of glycated LDL on PAI-1 secretion from HUVEC.

Time (hours)	PAI-1 antigen (μg/mg protein)		
	control	native LDL	glycated LDL
8	2.40 ± 0.26	2.41 ± 0.21	2.42 ± 0.15
16	3.36 ± 0.21	3.80 ± 0.21	3.73 ± 0.29
24	3.78 ± 0.23	4.36 ± 0.30*	5.19 ± 0.34*ˆ
48	4.18 ± 0.27	5.15 ± 0.33*	6.14 ± 0.14*ˆ

Confluent HUVEC were incubated in heparin-free medium without (control) or with the addition of 100 μg/ml of native or glycated LDL for 8 to 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; ˆp<0.001 compared to native LDL.

Table 6. Dose response of glycated LDL on the PAI-1 secretion from HUVEC.

LDL protein concentration	PAI-1 antigen (μg/mg protein)	
(μg/ml)	native LDL	glycated LDL
0 (control)	4.32 ± 0.36	4.32 ± 0.36
10	4.42 ± 0.34	4.25 ± 0.24
25	4.40 ± 0.28	4.32 ± 0.18
50	4.39 ± 0.22	5.24 ± 0.26*ˆ
100	5.47 ± 0.26*	6.27 ± 0.26*ˆ

Confluent HUVEC were incubated with 10-100 μg/ml of native or glycated LDL in heparin-free medium for 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; ˆp<0.001 compared to native LDL.

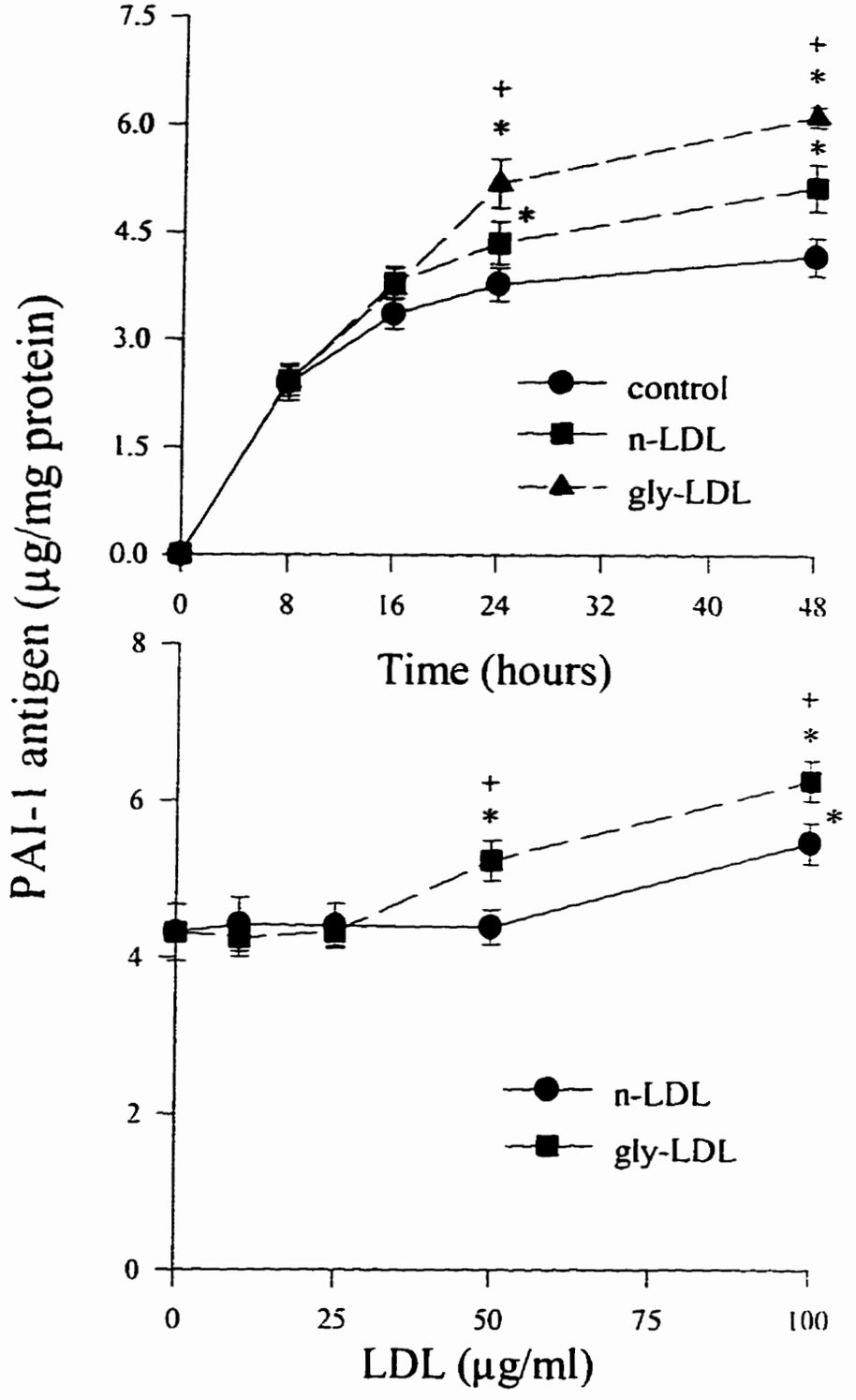


Figure 7. Time- and dose-dependence of glycated LDL on PAI-1 secretion from HUVEC. Cells were treated with 100 µg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) for 8-48 h (upper panel) or with 10-100 µg/ml of native or glycated LDL for 48 h (bottom panel). The levels of PAI-1 antigen in conditioned media were determined by ELISA as described in Methods. Values are presented as mean ± SD (n=4). *p<0.001 compared to control cultures; +p<0.001 compared to native LDL.

Table 7. Time dependency of glycated LDL on t-PA secretion from HUVEC.

Time (hours)	t-PA antigen (μg/mg protein)		
	control	native LDL	glycated LDL
8	0.59 ± 0.13	0.49 ± 0.10	0.43 ± 0.10
16	1.14 ± 0.07	0.91 ± 0.05*	0.70 ± 0.09*†
24	1.85 ± 0.24	1.14 ± 0.16*	0.80 ± 0.06*†
48	2.39 ± 0.11	2.10 ± 0.11*	1.71 ± 0.16*†

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 100 μg/ml of native LDL or glycated LDL for 8 to 48 h. The conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures: †p<0.001 compared to native LDL.

Table 8. Dose-response of glycated LDL on t-PA secretion from HUVEC.

LDL protein concentration	t-PA antigen (μg/mg protein)	
(μg/ml)	native LDL	glycated LDL
0 (control)	1.01 ± 0.12	1.01± 0.12
25	0.87 ± 0.04*	0.73 ± 0.04*†
50	0.66 ± 0.04*	0.59 ± 0.02*†
100	0.68 ± 0.06*	0.59 ± 0.02*†

Confluent HUVEC were incubated with 25-100 μg/ml of native or glycated LDL in heparin-free medium for 24 h. The conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4), *p<0.001 compared to control cultures: †p<0.001 compared to native LDL.

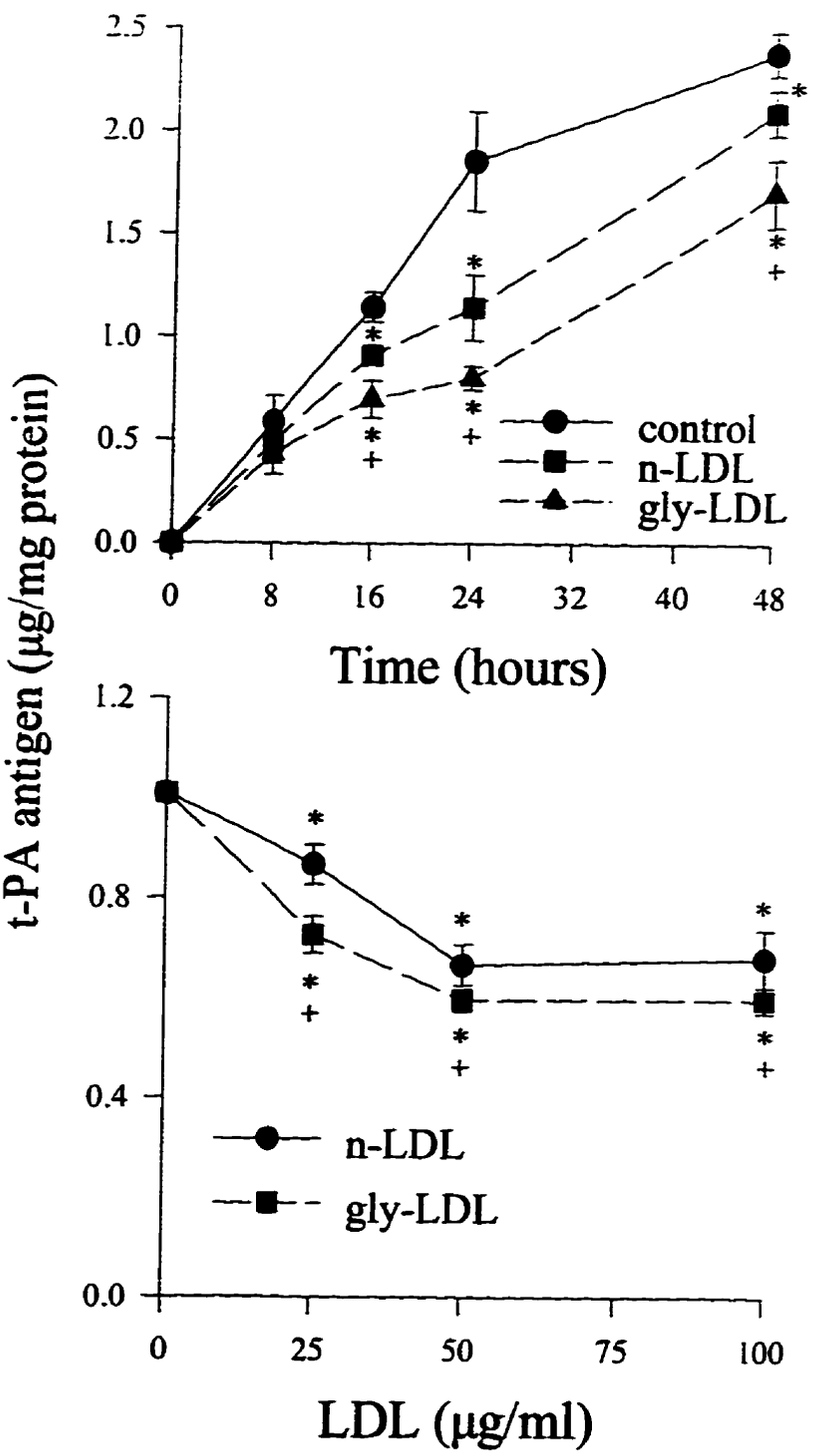


Figure 8. Time- and dose-dependence of glycated LDL on secretion of t-PA from HUVEC.

Cells were treated with 100 µg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) for 8-48 h (upper panel) or with 25-100 µg/ml of native or glycated LDL for 24 h (lower panel). The levels of t-PA antigen in conditioned media were determined by ELISA as described in Methods. Values were presented as µg/mg of cellular protein (mean ± SD, n=4). *p<0.001 compared to control cultures; +p<0.001 compared to native LDL.

Effects of glycated LDL on PAI-1 and t-PA antigen secretion were associated with the degree of glycation in LDL. Treatment with glycated LDL (100 μg/ml for 48 h) modified by ≥50 mM glucose significantly ($p<0.001$) increased the secretion of PAI-1 compared to cells treated with equal amounts of native LDL. The secretion of t-PA from HUVEC treated with glycated LDL (100 μg/ml for 24 h) modified by ≥ 25 mM glucose was significantly ($p<0.001$) reduced compared to that from cells treated with equal amounts of native LDL. The levels of PAI-1 and t-PA reached plateaus in HUVEC treated with LDL modified by ≥ 50 mM glucose (Table 9, 10 and Figure 9).

Table 9. Effects of glycated LDL modified by various concentrations of glucose on PAI-1 secretion from HUVEC.

Lipoproteins	Glucose (mM)	PAI-1 antigen (μg/mg protein
n-LDL	0	5.37 ± 0.37
gly-LDL	25	5.22 ± 0.28
gly-LDL	50	6.43 ± 0.39*
gly-LDL	100	6.58 ± 0.27*
gly-LDL	200	6.76 ± 0.14*

Confluent HUVEC were incubated in heparin-free medium without (control) or with the addition of 100 μg/ml of native or glycated LDL modified by 25 to 200 mM glucose for 48 h. The conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to native LDL.

Table 10. Effects of glycated LDL modified by various concentration of glucose on t-PA secretion from HUVEC.

lipoproteins	Glucose (mM)	t-PA antigen (μg/mg protein)
n-LDL	0	0.84 ± 0.06
gly-LDL	25	0.75 ± 0.07*
gly-LDL	50	0.48 ± 0.06*$^{+}$
gly-LDL	100	0.51 ± 0.05*$^{+}$
gly-LDL	200	0.56 ± 0.06*$^{+}$

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 100 μg/ml of native or glycated LDL modified by 25 to 200 mM glucose for 24 h and then the conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to native LDL; $^{+}$p<0.001 compared to glycated LDL modified by 25 mM glucose.

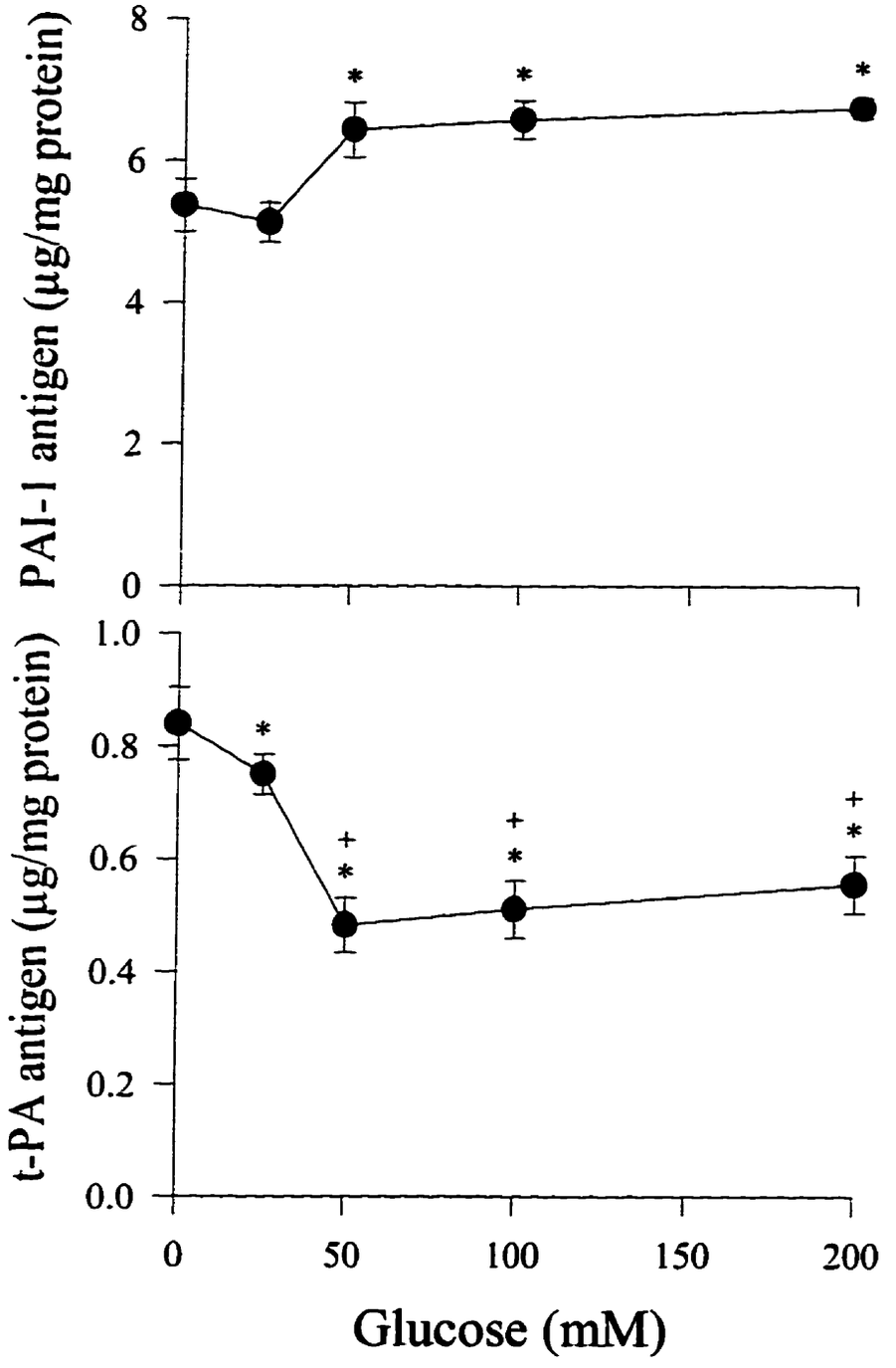


Figure 9. Effects of glycated LDL modified by various concentrations of glucose on PAI-1 and t-PA secretion from HUVEC.

Cells were incubated with the addition of 100 μg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) modified by 25 to 200 mM glucose for 48 h (PAI-1, upper panel) and 24 h (t-PA, bottom panel). The conditioned media were collected for measuring PAI-1 or t-PA antigen with ELISA. Values are presented as mean ± SD (n=4). *$p<0.001$ compared to native LDL; +$p<0.001$ compared to glycated LDL modified by 25 mM glucose.

5.2.2 Effect of glycated LDL on PAI-1 mRNA in EC

PAI-1 mRNA was presented as two distinguishable species, approximately 3.4 kb and 2.4 kb, in HUVEC as previously described [22]. Treatment with 100 μg/ml of native LDL for 48 h increased the levels of 2.4 kb PAI-1 mRNA and slightly decreased the levels of 3.4 kb PAI-1 mRNA. Treatment with an equal amount of glycated LDL greatly augmented the levels of 2.4 kb PAI-1 mRNA in HUVEC compared to control or native LDL (Figure 11).

A significant increase in 2.4 kb PAI-1 mRNA levels was found in HUVEC treated with 100 μg/ml of native LDL for 48 h compared to controls ($p<0.001$). Treatment with ≥ 50 μg/ml of glycated LDL for 48 h significantly increased the levels of 2.4 kb PAI-1 mRNA compared to equal amounts of native LDL (Table 11, 12 and Figure 10).

Table 11. Dose response of glycated LDL on PAI-1 mRNA levels in HUVEC.

LDL protein concentration	2.4 kb PAI-1 mRNA (fold of control)	
(μg/ml)	native LDL	glycated LDL
0 (control)	1	1
25	1.02[a]	1.02[a]
50	1.15 ± 0.21	2.05 ± 0.14*[+]
100	2.13 ± 0.16*	3.25 ± 0.21*[+]

Cells were incubated without (control) or with the addition of 25, 50, and 100 μg/ml of native LDL or glycated LDL for 48 h. Total RNA (20 μg/lane) was subjected to Northern blotting analysis. The levels of PAI-1 mRNA were quantified by using scanning densitometry. Values are expressed as the fold increase in 2.4 kb PAI-1 mRNA compared to control (mean ± SD, n=4). Results were adjusted with β-actin mRNA levels. a: values are generated from the results of one experiment. *$p<0.01$ compared to control cultures; [+]$p<0.01$ compared to native LDL.

Table 12. Time dependency of glycated LDL on PAI-1 mRNA levels in HUVEC.

Time (hours)	2.4 kb PAI-1 mRNA (fold increase of control)	
	native LDL	glycated LDL
8	0.93[a]	1.09[a]
16	1.02[a]	1.02[a]
24	1.11[a]	1.14[a]
48	2.13 ± 0.28*	3.25 ± 0.21*+

Cells were incubated without (control) or with the addition of 100 μg/ml of native LDL or glycated LDL for 8 to 48 h. Total RNA (20 μg/lane) was subjected to Northern blotting analysis. The levels of PAI-1 mRNA were quantified by using scanning densitometry. The results were adjusted with β-actin mRNA levels in the same lane. Values are expressed as fold increase in 2.4 kb PAI-1 mRNA compared to time-matched control (mean ± SD, n=4). a: values are generated from the results of one experiment. *$p<0.001$ compared to control cultures; +$p<0.001$ compared to native LDL.

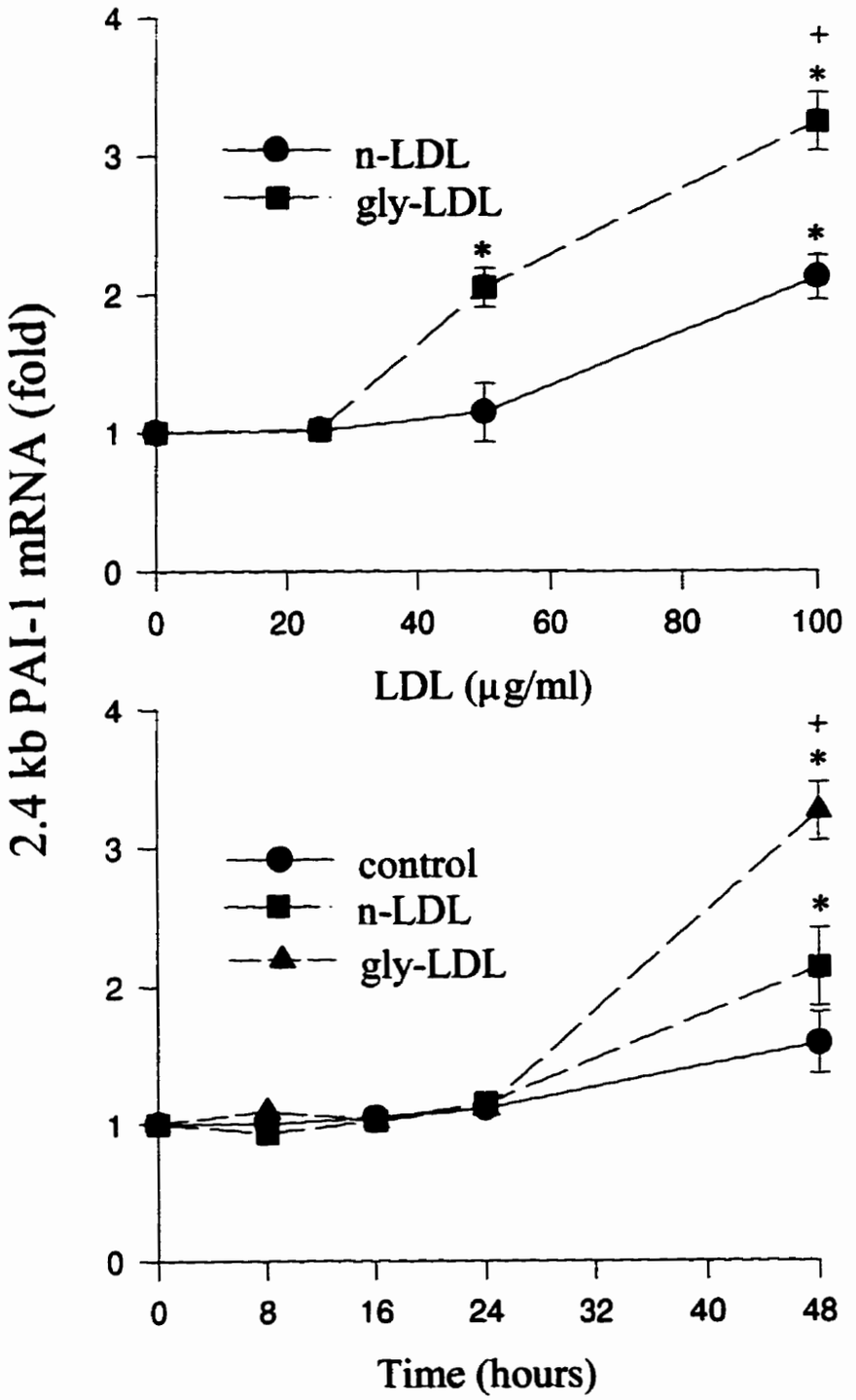


Figure 10. Dose- and time-dependence of glycated LDL on PAI-1 mRNA levels in HUVEC.

Upper panel: cells were incubated with 25, 50, 100 μg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) for 48 h. Lower panel: cells were incubated without (control) or with 100 μg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) for 8 to 48 h. Total RNA (20 μg/lane) was subjected to Northern blotting analysis. Values are expressed as fold increases in 2.4 kb PAI-1 mRNA levels compared to control. The levels of PAI-1 mRNA were quantified by using scanning densitometry and corrected with β-actin mRNA levels in the same lane. *$p<0.01$ compared to control cultures; +$p<0.01$ compared to native LDL.

5.2.3 Effect of glycated LDL on t-PA mRNA and synthesis in EC

The levels of t-PA mRNA in HUVEC treated with native or glycated LDL (100 μg/ml) for 24 h were not apparently altered after adjustment with β-actin mRNA levels in the same lane (Figure 12).

The effect of glycated LDL on *de novo* synthesis of t-PA was examined in HUVEC metabolically labelled with radioactive methionine and cystine. The molecular weight of t-PA generated from HUVEC appeared to be approximately 70 kDa. Radiolabelled t-PA was only found in the media but not in the cell-associated pool. Treatment with 100 μg/ml of native LDL for ≥16 h moderately reduced the levels of *de novo* synthesized t-PA which accumulated in the media. Glycated LDL at the same concentration evidently reduced t-PA synthesis in HUVEC compared to native LDL or control (Figure 13).

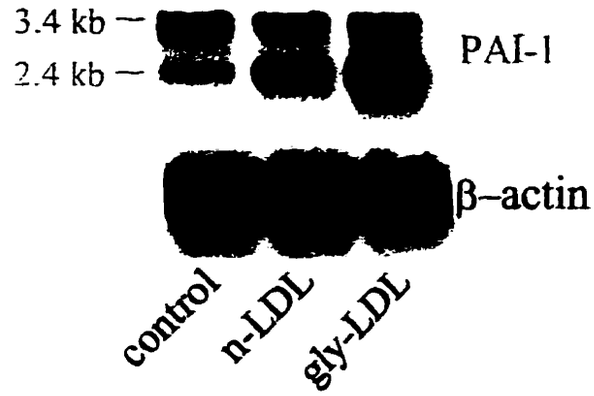


Figure 11. Northern blotting analysis of PAI-1 mRNA in cultured HUVEC.
Cells were treated with 100 μg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) for 48 h. Total RNA (20 μg/lane) extracted from the cells was analyzed by a 1% agarose-formaldehyde gel electrophoresis followed by Northern blotting analysis with human PAI-1 cDNA probe as described in text. The levels of ß-actin mRNA were estimated on stripped blots.

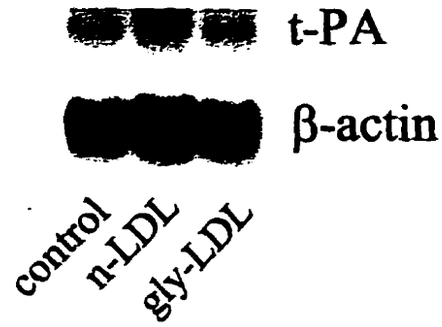


Figure 12. Northern blotting analysis of t-PA mRNA in cultured HUVEC.
Cells were treated with 100 μg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) for 24 h. Total RNA (20 μg/lane) extracted from the cells was analyzed by a 1% agarose-formaldehyde gel electrophoresis following by Northern blotting analysis with a human t-PA cDNA probe as described in text. The levels of ß-actin mRNA were estimated on stripped blots.

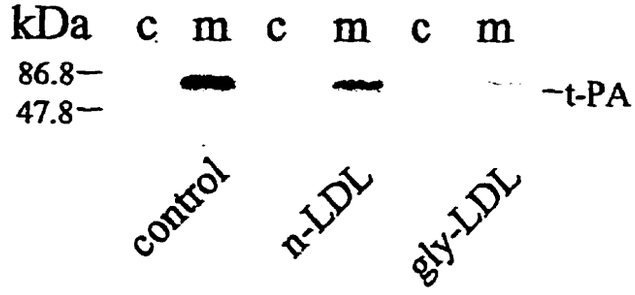


Figure 13. Synthesis of t-PA in HUVEC treated with native or glycated LDL.
Cells were metabolically labelled with 200 μCi/ml of ^{35}S-methionine in methionine and cystine-free medium without or with the addition of 100 μg/ml of native LDL (n-LDL), or glycated LDL (gly-LDL) for 24 h. Radioactive t-PA in the conditioned media (m) and the cell associated pool (c) was immunoprecipitated by goat anti-human t-PA IgG and was analyzed by a 12% SDS-PAGE. Molecular weight standards were analyzed in parallel lanes. Radioactive t-PA on dried gel was visualized by autoradiography.

Reduction in t-PA synthesis was detected in HUVEC treated with 100 μg/ml of native LDL for ≥ 16 h. The levels of t-PA synthesis in cells treated with 100 μg/ml of glycated LDL for 16-48 h were 16.8% to 25.0% lower than that in cells treated with native LDL for matching periods (Table 13).

Table 13. Time dependency of glycated LDL on t-PA synthesis in HUVEC.

Time (hours)	t-PA synthesis (% of control)	
	n-LDL	gly-LDL
0 (control)	100	100
8	91.47	83.45
16	79.64	66.25
24	86.80	65.08
48	86.52	71.74

Confluent HUVEC were incubated with 100 μg/ml of native LDL (n-LDL) or glycated LDL (gly-LDL) in methionine- and cystine-free medium containing 100 μCi/ml of Tran ^{35}S-label methionine (200 Ci/mM, 38 TBq/mM, 85% methionine and 15% cystine) for 8-48 h. The procedure for immunoprecipitation of t-PA is the same as previously described in text. The levels of *do novo* synthesized t-PA were quantified using scanning densitometry. Values are generated from the results of one experiment and presented as percentage of control.

5.3 Effect of glycated Lp(a) on the production of fibrinolytic regulators in cultured HUVEC

5.3.1 Effect of glycated Lp(a) on the secretion of PAI-1 and t-PA from EC

The levels of PAI-1 and t-PA antigen were determined in post-cultural media of HUVEC treated without (control) or with the addition of 1, 2.5, 5 and 10 μg/ml of native or glycated Lp(a) for 24 or 48 h in heparin-free medium.

The effect of native or glycated Lp(a) (5 μg/ml) on PAI-1 secretion was observed after 48 h treatment [Table 14 and Figure 14 (upper panel)]. The levels of PAI-1 secretion from HUVEC treated with ≥ 5 μg/ml of native or glycated Lp(a) were significantly increased compared to those from control cultures ($p<0.001$). PAI-1 antigen

level was 26.9% or 21.7% higher in the media of HUVEC treated with 5 or 10 μg/ml of glycated Lp(a) compared to that in cells treated with equal amounts of native Lp(a) respectively (Table 15 and Figure 14, lower panel).

Table 14. Time dependency of glycated Lp(a) on PAI-1 secretion from HUVEC.

Time (hours)	PAI-1 antigen (μg/mg protein)		
	control	native Lp(a)	glycated Lp(a)
8	1.89 ± 0.13	1.65 ± 0.07	1.68 ± 0.05
16	2.13 ± 0.18	2.04 ± 0.26	2.02 ± 0.18
24	3.00 ± 0.17	3.08 ± 0.24	3.19 ± 0.27
48	4.13 ± 0.43	5.57 ± 0.42*	6.36 ± 0.39*$^{+}$

Confluent HUVEC were treated in heparin-free medium without (control) or with the addition of 5 μg/ml of native or glycated Lp(a) for 8 to 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native Lp(a).

Table 15. Dose response of glycated Lp(a) on PAI-1 secretion from HUVEC.

Lipoprotein(a) (μg/ml)	PAI-1 antigen (μg/mg protein)	
	native Lp(a)	glycated Lp(a)
0 (control)	3.70 ± 0.10	3.70 ± 0.10
1	3.78 ± 0.17	3.65 ± 0.06
2.5	3.61 ± 0.17	3.72 ± 0.24
5	4.08 ± 0.24*	5.19 ± 0.24*$^{+}$
10	4.92 ± 0.40*	6.24 ± 0.67*$^{+}$

Confluent HUVEC were treated with 1-10 μg/ml of native or glycated Lp(a) in heparin-free medium for 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native Lp(a).

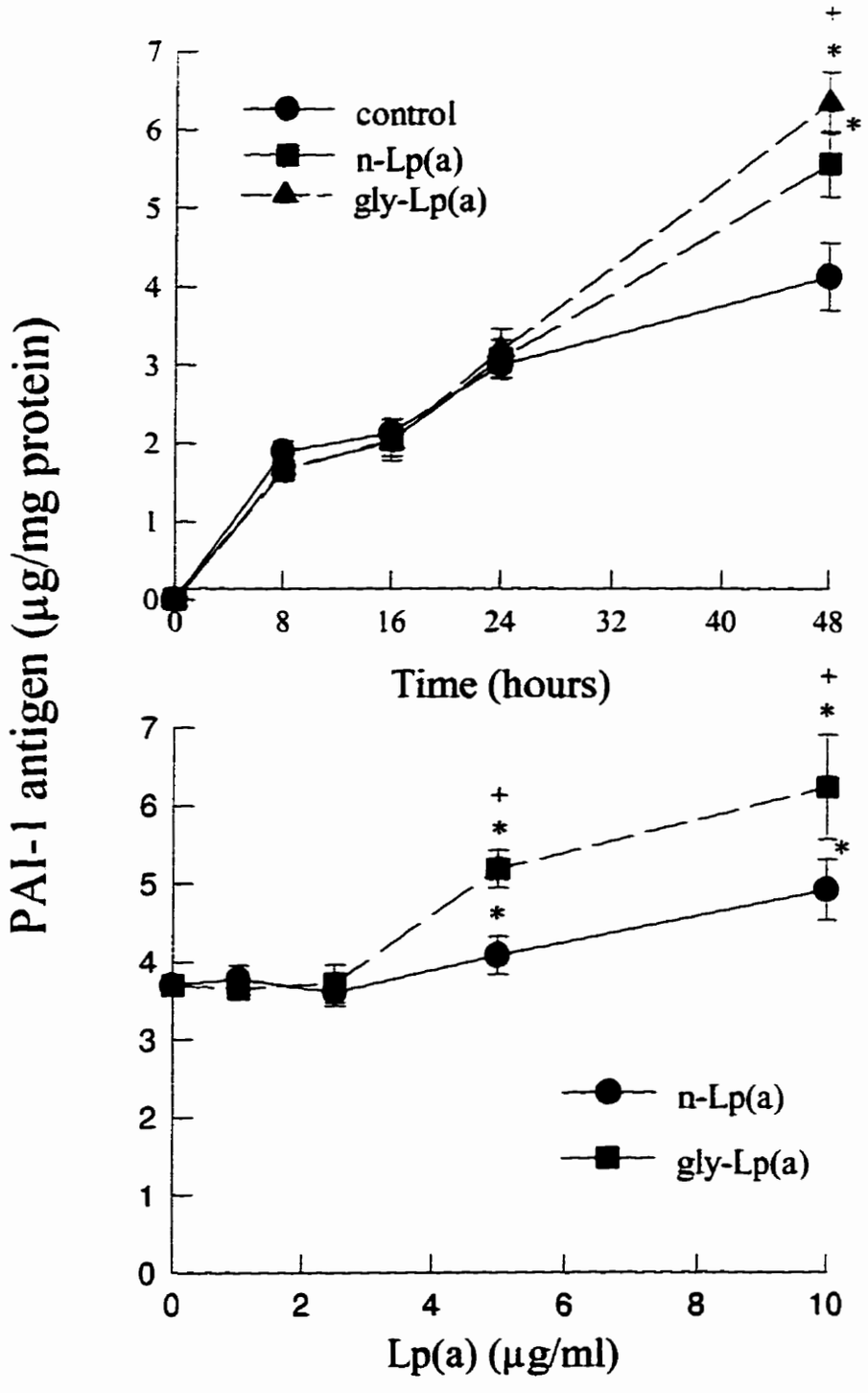


Figure 14. Time- and dose-dependence of glycated Lp(a) on secretion of PAI-1 from HUVEC.

Cells were treated with 5 µg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 8-48 h (upper panel) or 1-10 µg/ml of native or glycated Lp(a) for 48 h (lower panel). The levels of PAI-1 antigen in conditioned media were determined by ELISA as described in Methods. Values are presented as mean ± SD (n=4). *$p<0.001$ compared to control cultures; $^{+}p<0.001$ compared to native Lp(a).

After ≥ 16 h of incubation, the levels of t-PA in the media of HUVEC treated with 5 μg/ml of native Lp(a) were significantly lower than those in time-matched cultures ($p<0.001$). Treatment with glycated Lp(a) for ≥ 24 h significantly decreased the levels of t-PA in the media compared to native Lp(a) for matching periods ($p<0.001$, Table 16 and Figure 15, upper panel). A significant decrease in t-PA secretion was detected in EC treated with ≥ 2.5 μg/ml of native or glycated Lp(a) for 24 h. The levels of t-PA antigen were significantly lower in the medium of HUVEC treated with ≥ 2.5 μg/ml of glycated Lp(a) than those in the cells treated with the equal amounts of native Lp(a) (Table 17 and Figure 15, lower panel).

Table 16. Time dependency of glycated Lp(a) on t-PA secretion from HUVEC.

Time (hours)	t-PA antigen (μg/mg protein)		
	control	native Lp(a) (n=4)	glycated Lp(a) (n=4)
16	0.89 ± 0.11	0.66 ± 0.07*	0.62 ± 0.10*
24	1.33 ± 0.12	1.06 ± 0.06*	0.78 ± 0.05*˙
48	1.67 ± 0.13	1.27 ± 0.12*	0.83 ± 0.05*˙

Confluent HUVEC were treated in heparin-free medium without or with the addition of 5 μg/ml of native or glycated Lp(a) for 16 to 48 h. Conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; ˙p<0.001 compared to native Lp(a).

Table 17. Dose response of glycated Lp(a) on t-PA secretion from HUVEC.

Lp(a) protein concentration	t-PA antigen (μg/mg protein)	
(μg/ml)	native Lp(a)	glycated Lp(a)
0 (control)	1.25 ± 0.09	1.25 ± 0.09
2.5	1.00 ± 0.12*	0.89 ± 0.03*˙
5	0.78 ± 0.03*	0.58± 0.06*˙
10	0.73 ± 0.04*	0.64 ± 0.05*˙

Confluent HUVEC were treated with 2.5-10 μg/ml of native or glycated Lp(a) in heparin-free medium for 24 h. Conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; ˙p<0.001 compared to native Lp(a).

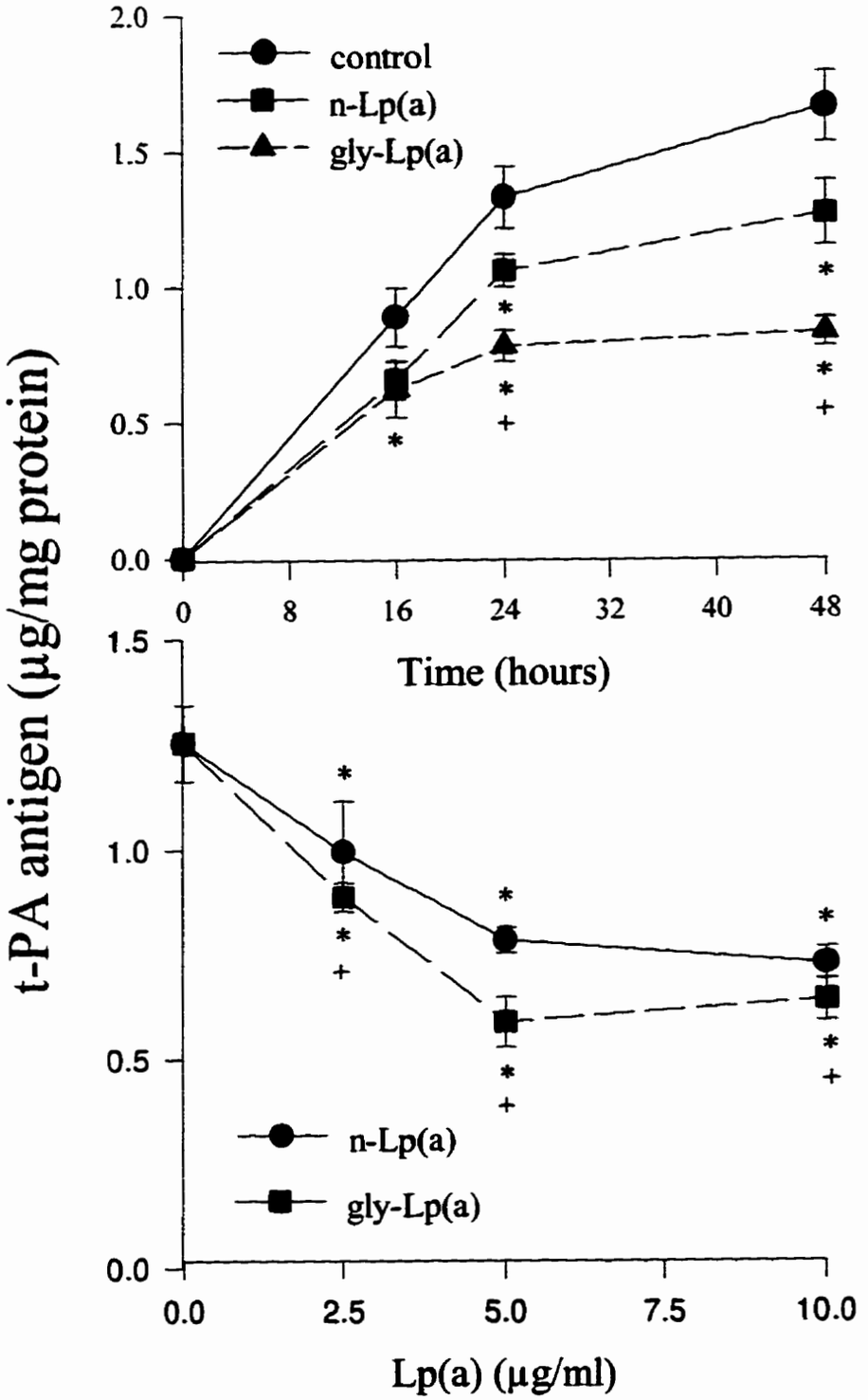


Figure 15. Time- and dose-dependence of glycated Lp(a) on secretion of t-PA from HUVEC.

Cells were treated with 5 µg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 16-48 h (upper panel) or 2.5-10 µg/ml of native or glycated Lp(a) for 24 h (lower panel). The levels of t-PA antigen in conditioned media were determined by ELISA as described in Methods. Values are presented as mean ± SD (n=4). *$p<0.001$ compared to control cultures; $^{+}p<0.001$ compared to native Lp(a).

5.3.2 Effect of glycated Lp(a) on PAI-1 mRNA in EC

The levels of PAI-1 mRNA were increased in HUVEC treated with 5 μg/ml of native or glycated Lp(a) for 48 h. The levels of 2.4 kb PAI-1 mRNA in HUVEC were greatly augmented by treatments with glycated Lp(a) compared to those in cells treated with equal amounts of native Lp(a). The levels of 3.4 kb PAI-1 mRNA were slightly decreased in HUVEC treated with native or glycated Lp(a) (Figure 17).

A significant increase in the levels of 2.4 kb PAI-1 mRNA was found in HUVEC treated with 5 μg/ml of native Lp(a) for 48 h compared to that in control cells ($p<0.001$, Table 18 and Figure 16, upper panel). Treatment with ≥ 2.5 μg/ml of glycated Lp(a) for 48 h increased the levels of 2.4 kb PAI-1 mRNA compared to treated with equal amounts of native Lp(a) (Table 19 and Figure 16, lower panel).

Table 18. Time dependency of glycated Lp(a) on PAI-1 mRNA levels in HUVEC.

Time (hours)	2.4 kb PAI-1 mRNA (fold of control)	
	native Lp(a)	glycated Lp(a)
8	1.01[a]	1.03[a]
16	1.06[a]	1.08[a]
24	1.14[a]	1.15[a]
48	2.23 ± 0.25*	3.52 ± 0.21*+

Cells were incubated without (control) or with the addition of 5 μg/ml of native Lp(a) or glycated Lp(a) for 8-48 h. Total RNA (20 μg/lane) was subjected to Northern blotting analysis. The levels of PAI-1 mRNA were quantified by using scanning densitometry. Results were adjusted with β-actin mRNA levels in the same lane. Values are expressed as the fold increase in 2.4 kb PAI-1 mRNA compared to control (mean ± SD, n=4). a: values are generated from the results of one experiment. *$p<0.01$ compared to control cultures; +$p<0.01$ compared to native Lp(a).

Table 19. Dose response of glycated Lp(a) on PAI-1 mRNA levels in HUVEC.

Lp(a) protein concentration	2.4 kb PAI-1 mRNA (fold of control)	
(μg/ml)	native Lp(a)	glycated Lp(a)
0 (control)	1	1
1	1.03[a]	1.03[a]
2.5	1.05[a]	2.23[a]
5	2.23 ± 0.25*	3.52 ± 0.21*+
10	3.46 ± 0.26*	3.53 ± 0.25*+

Cells were incubated without (control) or with the addition of 1, 2.5, 5 and 10 μg/ml of native or glycated Lp(a) for 48 h. Total RNA (20 μg/lane) was subjected to Northern blotting analysis. The levels of PAI-1 mRNA were quantified by using scanning densitometry. Results were adjusted with β-actin mRNA levels in the same lane. Values are expressed as the fold increase in 2.4 kb PAI-1 mRNA compared to control (mean ± SD, n=4). a: values are generated from the results of one experiment. *$p<0.01$ compared to control cultures; +$p<0.01$ compared to native Lp(a).

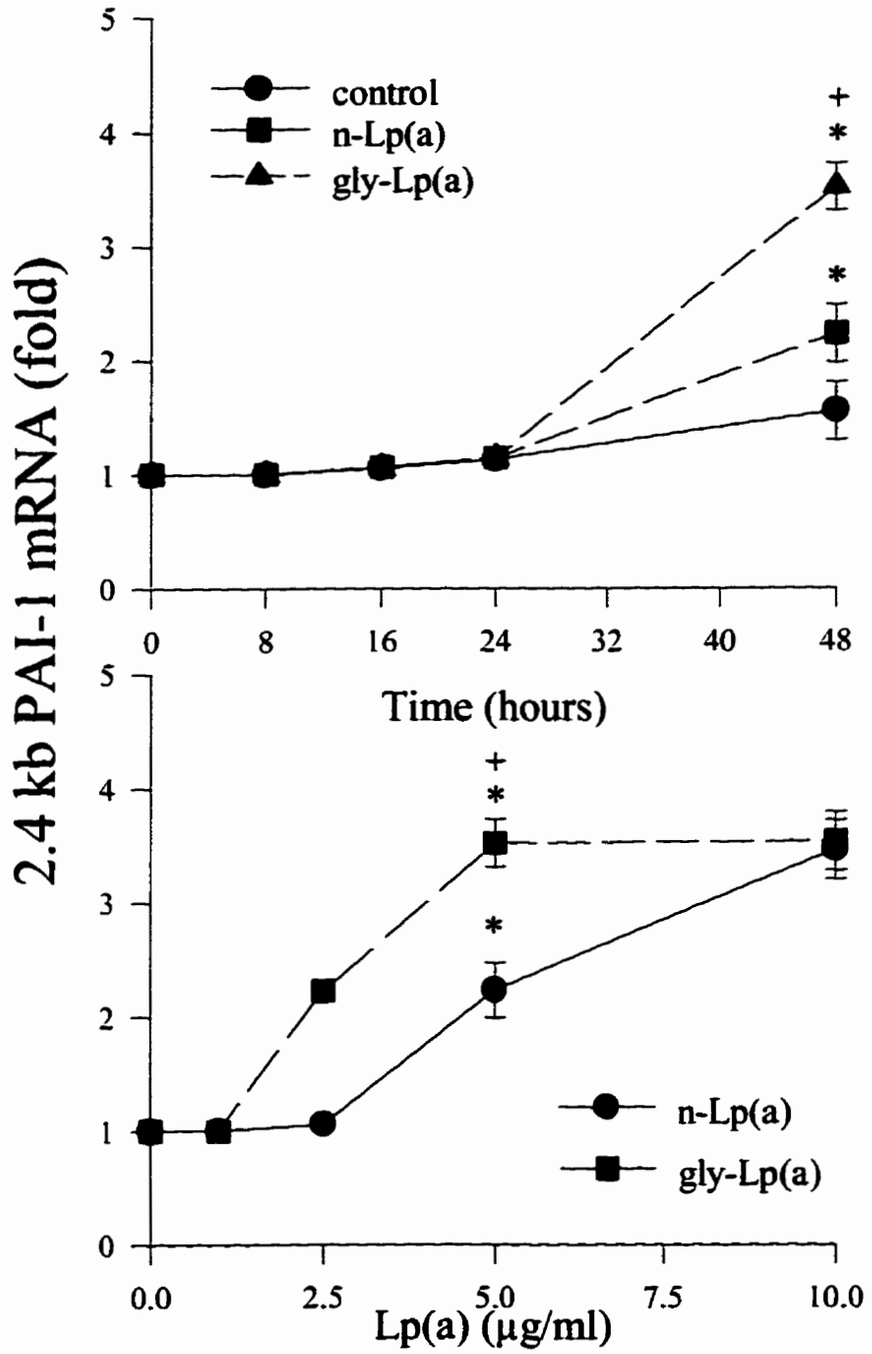


Figure 16. Time- and dose-dependence of glycated Lp(a) on PAI-1 mRNA levels in HUVEC.

Upper panel: cells were incubated without (control) or with the addition of 5 μg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 8 to 48 h. Lower panel: cells were incubated with the addition of 1, 2.5, 5 or 10 μg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 48 h. Total RNA (20 μg/lane) was subjected to Northern blotting analysis. Values are expressed as fold increases in 2.4 kb PAI-1 mRNA compared to control. The levels of PAI-1 mRNA were quantified by using scanning densitometry and corrected with β-actin mRNA levels in the same lane. *$p<0.01$ compared to control cultures; $^{+}p<0.01$ compared to native Lp(a).

5.3.3 Effect of glycated Lp(a) on t-PA mRNA and synthesis in EC.

The levels of t-PA mRNA in HUVEC treated with native or glycated Lp(a) (5 μg/ml) for 24 h were not significantly altered after adjustment with β-actin mRNA levels in the same lane (Figure 18).

The effect of glycated Lp(a) on *de novo* synthesis of t-PA was examined in HUVEC metabolically labelled with radioactive methionine and cystine. Treatment with 5 μg/ml of native Lp(a) for ≥ 16 h moderately reduced the levels of *de novo* synthesized t-PA accumulating in the media. t-PA synthesis in HUVEC treated with glycated Lp(a) was evidently reduced compared to that in cells treated with an equal amount of native Lp(a) (Figure 19). The levels of t-PA synthesis in cells treated with 5 μg/ml of glycated Lp(a) for 8-48 h were lower than those in cells treated with native Lp(a) for matching periods (Table 20).

Table 20. Time dependency of glycated Lp(a) on t-PA synthesis in HUVEC.

Time (hours)	t-PA synthesis (% of control)	
	native Lp(a)	glycated Lp(a)
0 (control)	100	100
8	101.91	88.82
16	93.64	79.42
24	85.21	78.12
48	86.82	76.57

Confluent HUVEC were treated with 5 μg/ml of native Lp(a), or glycated Lp(a) in methionine- and cystine-free DMEM medium containing 100 μCi/ml of Tran ^{35}S-label methionine (200 Ci/mM, 38 TBq/mM, 85% methionine and 15% cystine) for 8-48 h. The procedure for cell radiolabelling and immunoprecipitation of t-PA is the same as previously described in Methods. The levels of *de novo* synthesized t-PA were quantified by using scanning densitometry. Values are generated from the results of one experiment and presented as percentage of control.

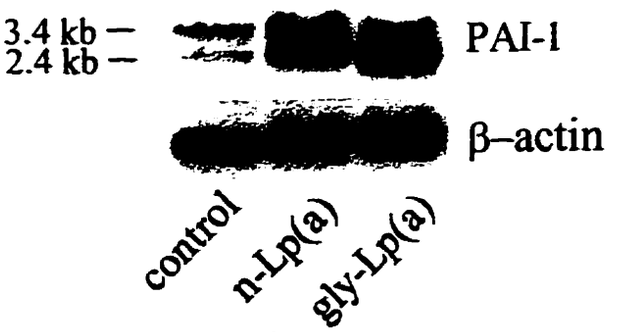


Figure 17. Northern blotting analysis of PAI-1 mRNA in HUVEC.
Cells were treated with 5 μg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 48 h. Total RNA was extracted and analyzed for PAI-1 mRNA by Northern blotting as described in Methods. Rehybridization of the blot with a ß-actin probe verified the presence of comparable amounts of total RNA in each lane.

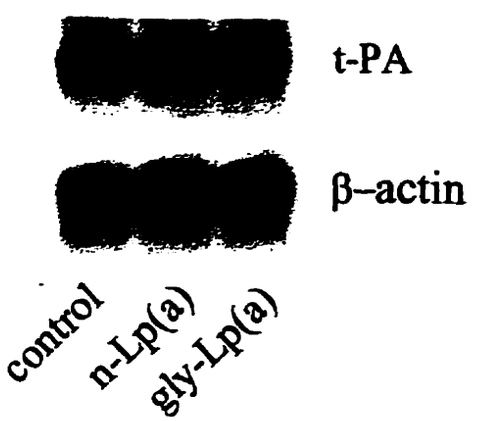


Figure 18. Northern blotting analysis of t-PA mRNA in cultured HUVEC.
Cells were treated with 5 μg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 24 h. Total RNA (20 μg/lane) extracted from the cells was analyzed by a 1% agarose-formaldehyde gel electrophoresis following by Northern blotting analysis with a human t-PA cDNA probe as described in Methods. The levels of ß-actin mRNA were estimated on stripped blots.

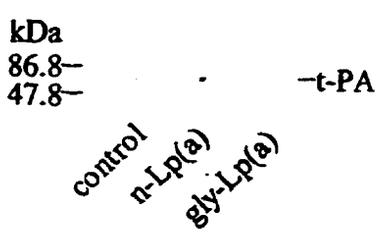


Figure 19. Synthesis of t-PA in HUVEC treated with glycated Lp(a).
Cells were metabolically labelled with 100 μCi/ml of ^{35}S-methionine in methionine- and cystine-free medium without (control) or with the addition of 5 μg/ml of native Lp(a) [n-Lp(a)] or glycated Lp(a) [gly-Lp(a)] for 24 h. Radioactive t-PA in the conditioned media (m) was immunoprecipited by goat anti-human t-PA IgG and was analyzed by a 12% SDS-PAGE. Molecular weight standards were analyzed in parallel lanes. Radioactive t-PA on dried gel was visualized by autoradiography.

5.4 Effect of aminoguanidine-treated glycated lipoproteins on PAI-1 and t-PA production in EC

Aminoguanidine is an inhibitor of the formation of AGEs. Addition of ≥ 25 mM aminoguanidine during the glycation of LDL effectively prevented the glycation of lysine in LDL (Table 21 and Figure 20). Increased PAI-1 and decreased t-PA secretion from HUVEC induced by glycated lipoproteins were normalized by treatment with 25 mM aminoguanidine during the glycation of lipoproteins (Table 22-25 and Figure 21,22). The decrease of t-PA synthesis in HUVEC induced by glycated LDL or Lp(a) also was normalized by aminoguanidine which was added during the lipoprotein glycation (Table 26, 27 and Figure 23).

Table 21. Effect of aminoguanidine on the formation of glucitollysine in glycated LDL.

Aminoguanidine (mM)	Ratio of glucitollysine/lysine residue (area)
0	1.24
5	1.19
10	1.20
25	0.20
50	0.19
100	0.21
200	0.20

LDL (2 mg/ml) was incubated with 200 mM glucose in the presence of 0 to 200 mM aminoguanidine at 37°C for 7 days. Samples were analyzed by HPLC as described in Methods. The ratio of peak area of glucitollysine to lysine was used to estimate the degree of incorporation of glucose into LDL. Values are generated from the results of one experiment.

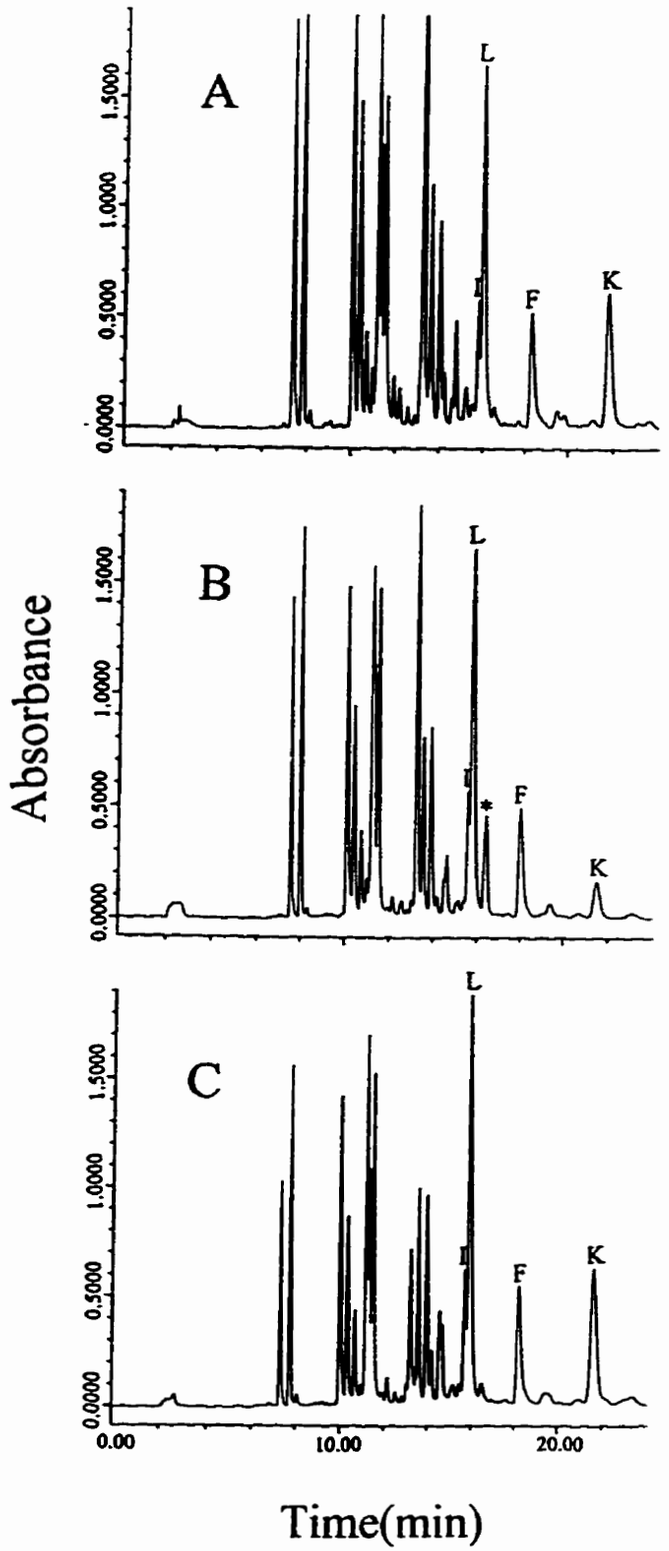


Figure 20. Amino acid profile of aminoguanidine-treated glycated LDL.

A: native LDL; B: glycated LDL, LDL was modified by 200 mM glucose; C: AG-glycated LDL, LDL was modified by 200 mM glucose in the presence of 25 mM aminoguanidine. Hydrolyzed LDL were derivertized with PITC and analyzed by a reverse phase HPLC with a solvent system of 100-70% of 70 mM sodium acetate and 0-30% of acetonitrile. I: isoleucine; L: leucine; *: putative glucitollysine; F: phenylalanine; K: lysine.

Table 22. Effect of aminoguanidine-treated glycated LDL on PAI-1 secretion from HUVEC.

Time (hours)	PAI-1 antigen (μg/mg protein)			
	control	native LDL	glycated LDL	AG-glycated LDL
24	2.33 ± 0.28	2.60 ± 0.15*	3.10 ± 0.11*	2.61 ± 0.27*$^{+}$
48	4.91 ± 0.4	5.56 ± 0.39*	6.36 ± 0.44*	5.56 ± 0.36*$^{+}$

Confluent HUVEC were incubated in heparin-free medium without (control) or with the addition of 100 μg/ml of native or glycated LDL and aminoguanidine-treated glycated LDL (AG-glycated LDL) for 24 to 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *$p<0.001$ compared to control cultures. $^{+}p<0.001$ compared to glycated LDL.

Table 23. Effect of aminoguanidine-treated glycated LDL on t-PA secretion from HUVEC.

Time (hours)	t-PA antigen (μg/mg protein)			
	control	native LDL	glycated LDL	AG-glycated LDL
24	1.14 ± 0.05	0.75 ± 0.04*	0.62 ± 0.06*	0.82 ± 0.06*$^{+}$
48	2.38 ± 0.29	2.07 ± 0.23*	1.58 ± 0.17*	2.09 ± 0.12*$^{+}$

Confluent HUVEC were incubated in heparin-free medium without (control) or with the addition of 100 μg/ml of native LDL, glycated LDL or aminoguanidine-treated glycated LDL (AG-glycated LDL) for 24 to 48 h. Conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *$p<0.001$ compared to control cultures; $^{+}p<0.001$ compared to glycated LDL.

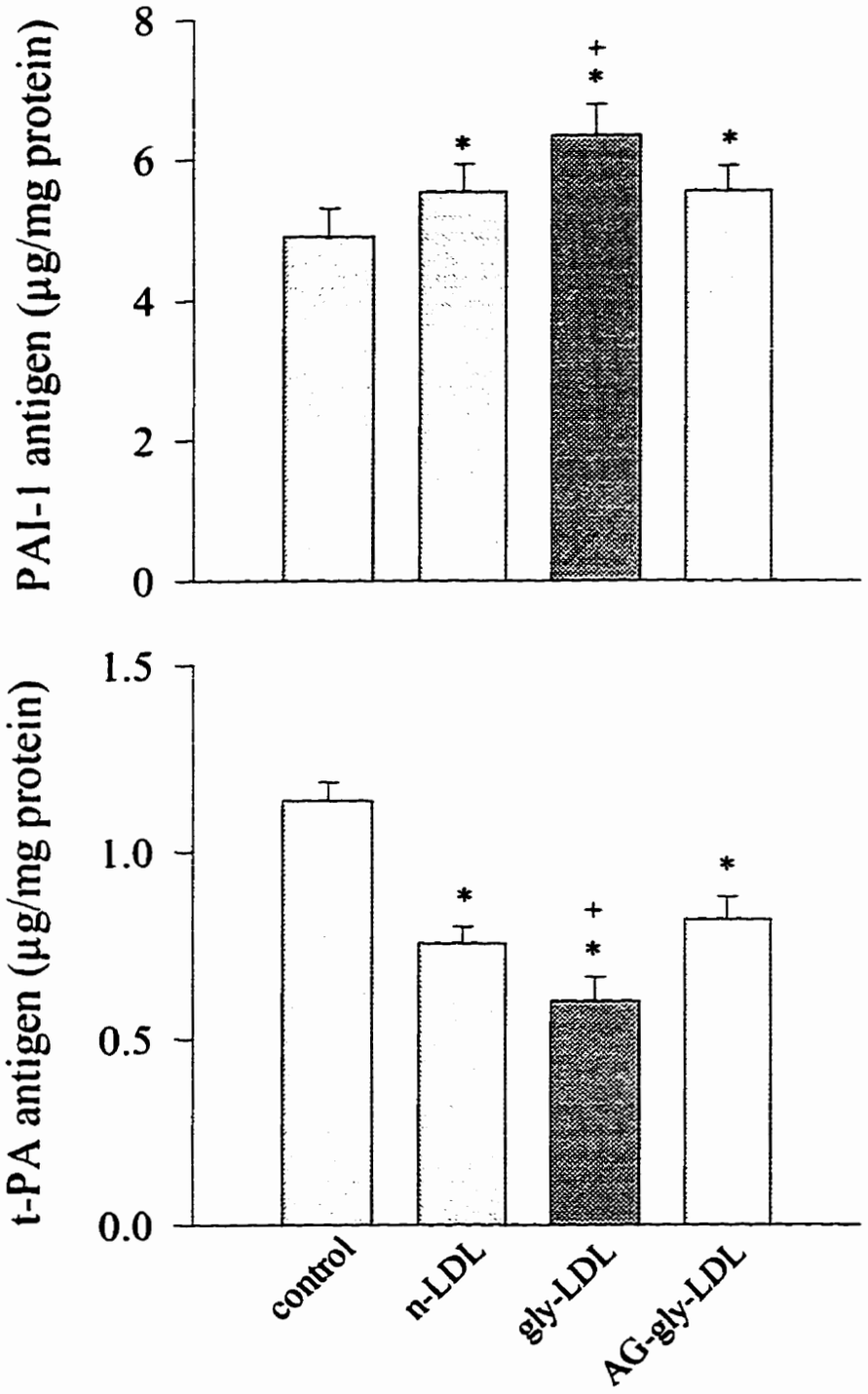


Figure 21. Effect of aminoguanidine-treated glycated LDL on PAI-1 and t-PA secretion from HUVEC.

Confluent HUVEC were incubated without (control) or with the addition of 100 µg/ml of native LDL (n-LDL), glycated LDL (gly-LDL) or aminoguanidine-treated glycated LDL (AG-gly-LDL) for 48 h (PAI-1, upper panel) or 24 h (t-PA, lower panel). The conditioned media were collected for measuring PAI-1 or t-PA antigen with ELISA. Values are presented as mean ± SD (n=4). *$p<0.001$ compared to control cultures; +$p<0.001$ compared to glycated LDL.

Table 24. Effect of aminoguanidine-treated glycated Lp(a) on PAI-1 secretion from HUVEC.

Lipoproteins	PAI-1 antigen (μg/mg cell protein)
control	4.29 ± 0.11
native Lp(a)	5.59 ± 0.31*
glycated Lp(a)	6.44 ± 0.26*$^{+}$
AG-glycated Lp(a)	5.70 ± 0.15*$^{\#}$

Confluent HUVEC were treated without (control) or with the addition of 5 μg/ml of native Lp(a) [n-Lp(a)], glycated Lp(a) [gly-Lp(a)] or aminoguanidine-treated glycated Lp(a) [AG-gly-Lp(a)] for 48 h. The conditioned media were collected for measuring PAI-1 antigen with ELISA. Values were presented as mean ± SD (n=4). *$p<0.001$ compared to control cultures; $^{\#}p<0.001$ compared to native Lp(a); $^{+}p<0.001$ compared to glycated Lp(a).

Table 25. Effect of aminoguanidine-treated glycated Lp(a) on t-PA secretion from HUVEC.

Lipoproteins	t-PA antigen (μg/mg cell protein)
control	1.45 ± 0.15
native Lp(a)	1.07 ± 0.17*
glycated Lp(a)	0.83 ± 0.05*$^{+}$
AG-glycated Lp(a)	1.10 ± 0.17*$^{\#}$

Confluent HUVEC were treated without (control) or with the addition of 5 μg/ml of native Lp(a) [(n-Lp(a)], glycated Lp(a) [gly-Lp(a)] or aminoguanidine treated glycated Lp(a) [AG-gly-Lp(a)] for 24 h. The conditioned media were collected for measuring t-PA antigen with ELISA. Values are presented as mean ± SD (n=4). *$p<0.001$ compared to control cultures; $^{\#}p<0.001$ compared to native Lp(a); $^{+}p<0.001$ compared to glycated Lp(a).

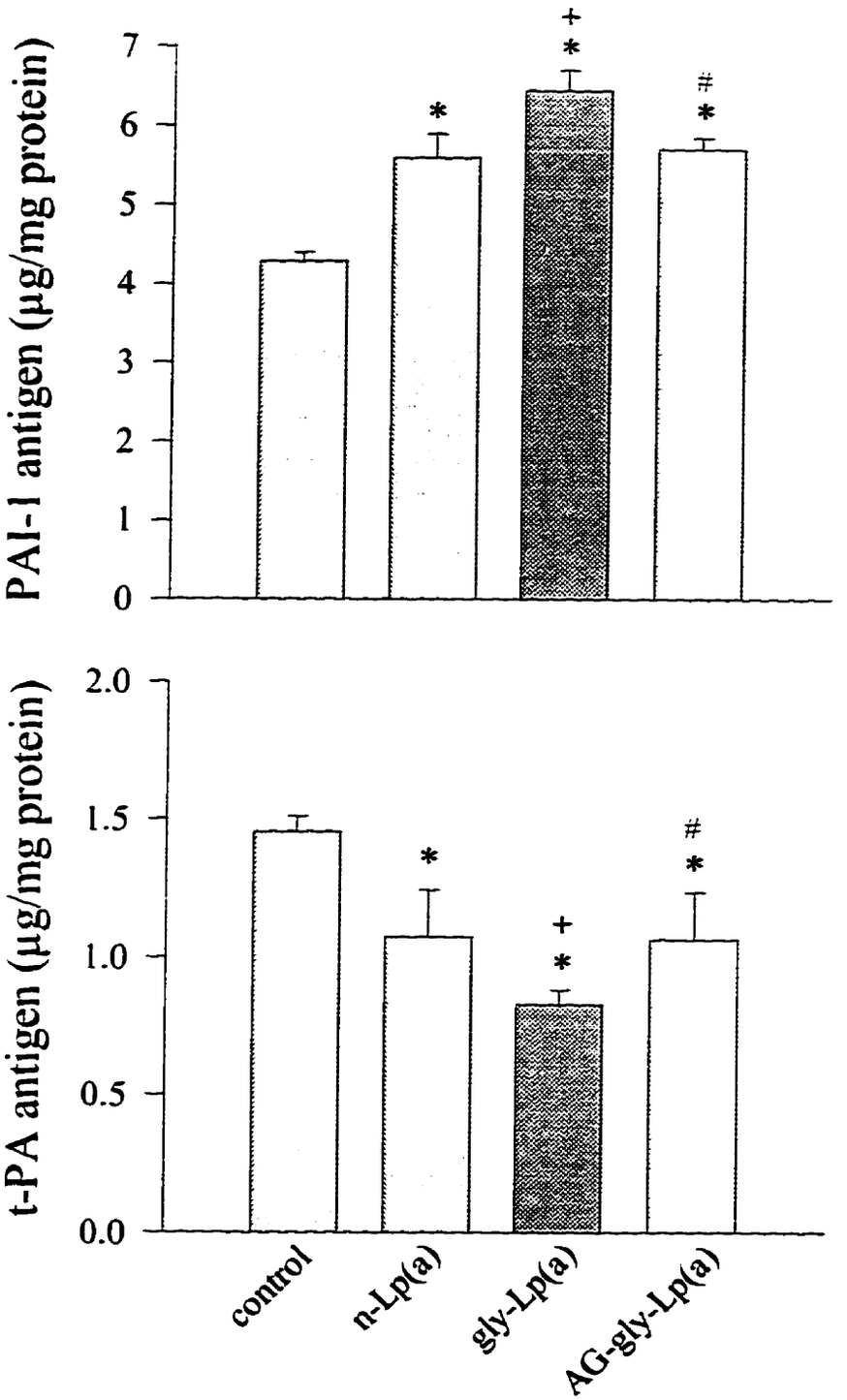


Figure 22. Effects of aminoguanidine-treated glycated Lp(a) on PAI-1 and t-PA secretion from HUVEC.

Confluent HUVEC were treated without (control) or with the addition of 5 µg/ml of native Lp(a) [n-Lp(a)], glycated Lp(a) [gly-Lp(a)] or aminoguanidine-treated glycated Lp(a) [AG-gly-Lp(a)] for 48 h (PAI-1) or 24 h (t-PA). The conditioned media were collected for measuring PAI-1 or t-PA antigen with ELISA. Values are presented as mean ± SD (n=4). *p<0.001 compared to control cultures; +p<0.001 compared to native Lp(a); #p<0.001 compared to glycated Lp(a).

Table 26. Effect of aminoguanidine-treated glycated LDL on t-PA synthesis in HUVEC.

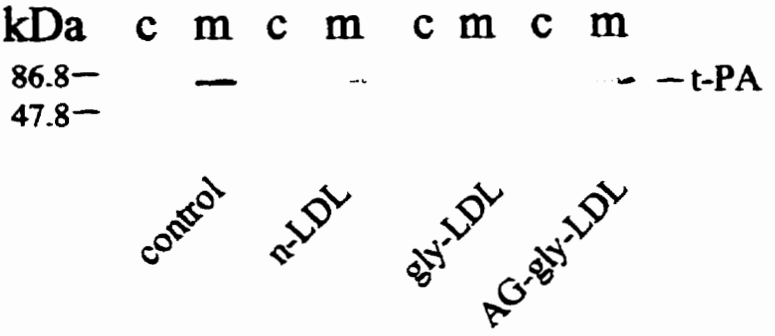
Time (hours)	t-PA synthesis (% of control)		
	n-LDL	gly-LDL	AG-gly-LDL
0 (control)	100	100	100
8	91.47	83.45	93.75
16	79.64	66.25	81.97
24	86.80	65.08	84.91
48	86.52	71.74	90.88

Confluent HUVEC were incubated with 100 μg/ml of native LDL (n-LDL), glycated LDL (gly-LDL) or AG-glycated LDL (AG-gly-LDL) in methionine-free medium containing 100 μCi/ml of Tran ^{35}S-label methionine (200 Ci/mM, 38 TBq/mM, 85% methionine and 15% cystine) for 8-48 h. The procedure for immunoprecipitation of t-PA is the same as previously described in Methods. The levels of *de novo* synthesized t-PA were quantified by using scanning densitometry. Values are generated from the results of one experiment and presented as percentage of control.

Table 27. Effect of aminoguanidine-treated glycated Lp(a) on t-PA synthesis in HUVEC.

Time (h)	t-PA synthesis (% of control)		
	native Lp(a)	glycated Lp(a)	AG-gly-Lp(a)
0 (control)	100	100	100
8	101.91	88.82	97.85
16	93.64	79.42	95.14
24	85.21	78.12	90.52
48	86.82	76.57	84.67

Confluent HUVEC were treated with 5 μg/ml of native Lp(a), glycated Lp(a) or aminoguanidine-treated glycated Lp(a) (AG-gly-LDL) in methionine- and cystine-free DMEM medium containing 100 μCi/ml of Tran ^{35}S-label methionine (200 Ci/mM, 38 TBq/mM, 85% methionine and 15% cystine) for 8-48 h. The procedure for cell radiolabelling and immunoprecipitation of t-PA is the same as previously described in text. The levels of *de novo* synthesized t-PA were quantified by using scanning densitometry. Values are generated from the results of one experiment and presented as percentage of control.



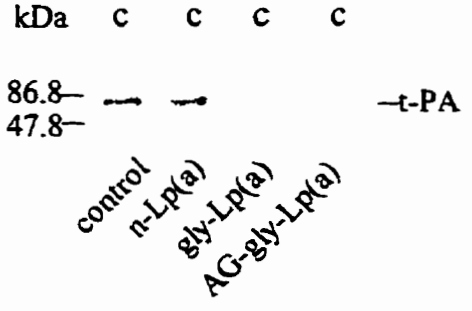


Figure 23. Synthesis of t-PA in HUVEC treated with AG-glycated LDL and AG-glycated-Lp(a).

Cells were metabolically labelled with 100 μCi/ml of ^{35}S-methionine in methionine- and cystine-free medium without (control) or with the addition of 100 μg/ml of native LDL (n-LDL), glycated LDL (gly-LDL) or aminoguanidine-treated glycated LDL (AG-gly-LDL); or with the addition of 5 μg/ml of native Lp(a) [n-Lp(a)], glycated Lp(a) [gly-Lp(a)] or aminoguanidine treated glycated Lp(a) [AG-gly-Lp(a)] for 24 h (bottom). Radioactive t-PA in the conditioned media (m) and cell associate pool (c) was immunoprecipited by goat anti-human t-PA IgG and was analyzed by a 12% SDS-PAGE. Molecular weight standards were analyzed in parallel lanes. Radioactive t-PA on dried gel was visualized by autoradiography.

5.5 Effect of glycated BSA on PAI-1 and t-PA secretion from EC

The level of PAI-1 antigen in the medium of HUVEC was significantly increased by treatment with 100 μg/ml of glycated LDL for 48 h compared to that in cells treated with an equal amount of native LDL ($p< 0.001$) as shown above. The secretion of t-PA from HUVEC was significantly reduced by treatment with 100 μg/ml of glycated LDL for 24 h compared to that from control and native LDL-treated cells ($p<0.001$). The equimolar concentration of glycated BSA modified by the identical procedure did not significantly alter the levels of PAI-1 and t-PA secreted from HUVEC (Table 28, 29 and Figure 24.)

Table 28. Effect of glycated BSA on PAI-1 secretion from HUVEC.

Lipoproteins	PAI-1 antigen (μg/mg protein)
control	4.12 ± 0.38
n-LDL	5.06 ± 0.35*
gly-LDL	6.12 ± 0.42*$^{+}$
n-BSA	4.13 ± 0.28
gly-BSA	4.13 ±0.43

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 100 μg/ml of native LDL, glycated LDL or equimolar concentration of native BSA or glycated BSA for 48 h. The conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native LDL.

Table 29. Effect of glycated BSA on t-PA secretion from HUVEC.

Lipoproteins	t-PA antigen (μg/mg protein)
control	1.61 ± 0.21
n-LDL	1.18 ± 0.10*
gly-LDL	0.97 ± 0.14*$^{+}$
n-BSA	1.67 ± 0.17
gly-BSA	1.57 ±0.15

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 100 μg/ml of native LDL, glycated LDL or equimolar protein concentration of native BSA or glycated BSA for 24 h and the conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native LDL.

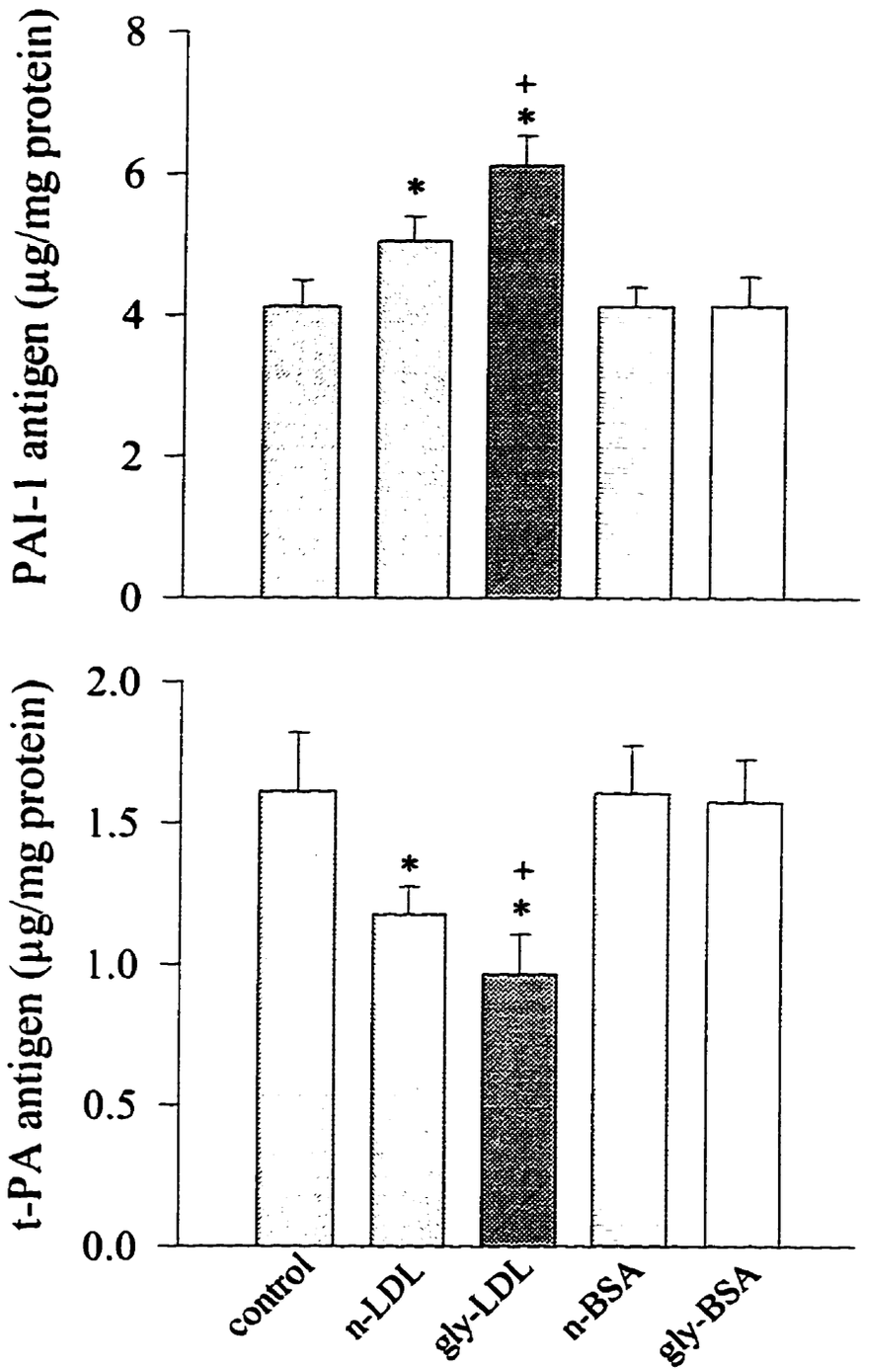


Figure 24. Effect of glycated BSA on the secretion of PAI-1 and t-PA from HUVEC.
Cells were incubated with 100 µg/ml of native LDL (n-LDL), glycated LDL (gly-LDL), native BSA (n-BSA), glycated BSA (gly-BSA) for 48 h (PAI-1, upper panel) or for 24 h (t-PA, lower panel). The levels of PAI-1 and t-PA antigen in conditioned media were determined by ELISA. Values are presented as mean ± SD (n=4). *$p<0.001$ compared to control cultures; +$p<0.001$ compared to native LDL.

5.6 Effects of oxidized lipoproteins on the secretion of PAI-1 and t-PA from HUVEC

The levels of PAI-1 antigen in the medium of HUVEC treated with 100 μg/ml of oxidized LDL or 5 μg/ml of oxidized Lp(a) for 48 h were significantly increased compared to those in the medium of cells treated with an equal amount of native LDL or Lp(a) ($p<0.001$, Table 30 and Figure 25, upper panel). The levels t-PA antigen in the medium of HUVEC treated with 100 μg/ml of oxidized LDL or 5 μg/ml of oxidized Lp(a) for 24 h were significantly ($p<0.001$) reduced compared to those in cells treated with an equal amount of native LDL or Lp(a) (Table 31 and Figure 25, lower panel). TBARS levels in the oxidized LDL and Lp(a) were 43.3 ± 5.7 nM MDA/mg protein (n=4) and 45.6 ± 6.3 nM MDA/mg protein (n=4), respectively. The levels of TBARS in native or glycated lipoproteins were undetectable.

Table 30. Effects oxidized LDL on PAI-1 secretion from HUVEC.

Lipoproteins	PAI-1 antigen (μg/mg protein)
control	4.12 ± 0.18
n-LDL	5.06 ± 0.15*
gly-LDL	6.12 ± 0.22*$^{+}$
ox-LDL	6.47 ± 0.28*$^{+}$

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 100 μg/ml of native LDL (n-LDL), glycated LDL (gly-LDL) or oxidized LDL (ox-LDL) for 48 h. The conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native LDL.

Table 31. Effects of oxidative LDL on t-PA secretion from HUVEC.

Lipoproteins	t-PA antigen (μg/mg protein)
control	2.39 ± 0.11
n-LDL	2.02 ± 0.11*
gly-LDL	1.64 ± 0.08*$^{+}$
ox-LDL	1.30 ± 0.07*$^{+\#}$

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 100 μg/ml of native LDL (n-LDL), glycated LDL(gly-LDL) or oxidized LDL (ox-LDL) 24 h. The conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native LDL; $^{\#}$p<0.001 compared to glycated LDL.

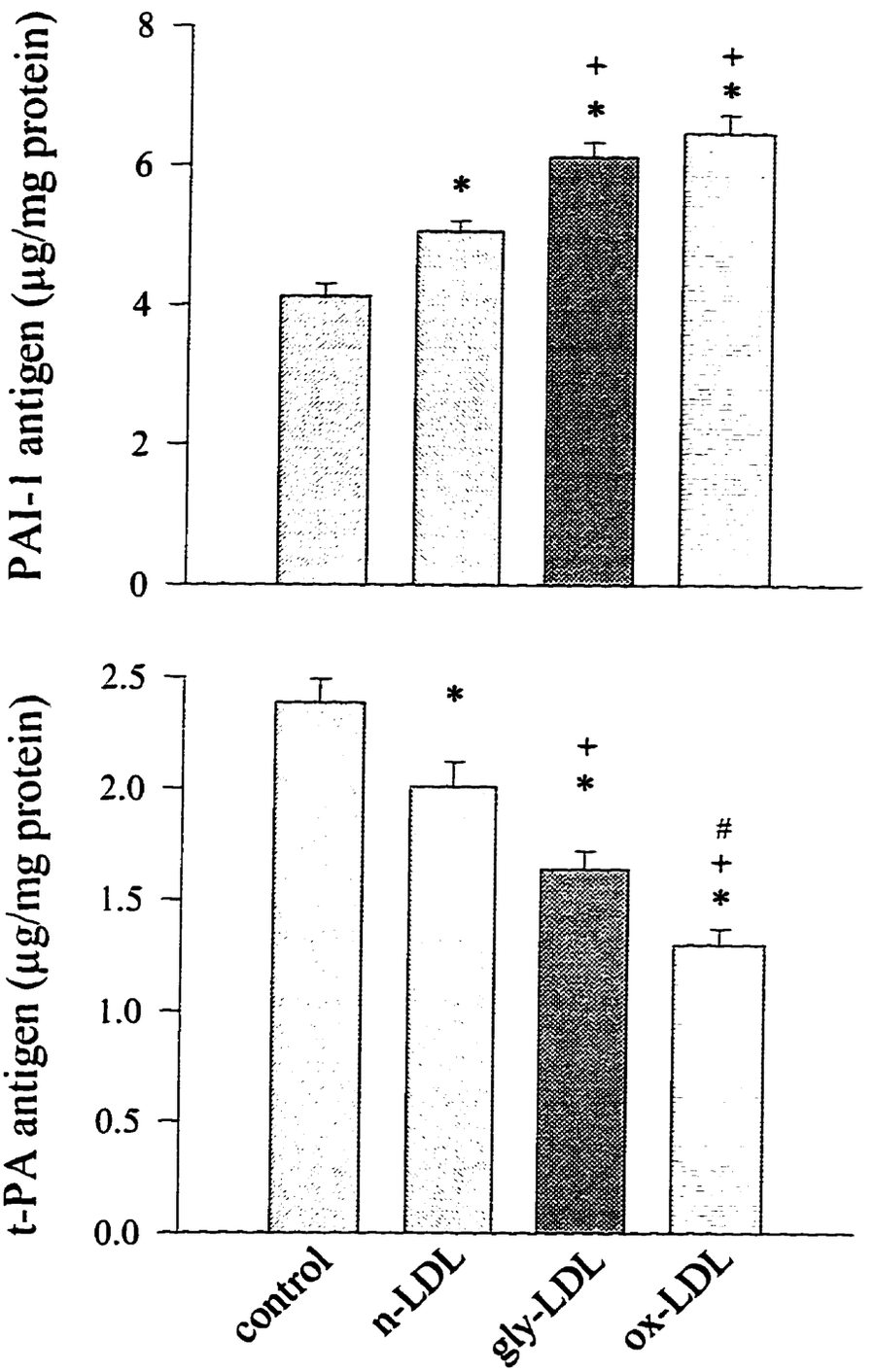


Figure 25. Effects of oxidized LDL on PAI-1 and t-PA secretion from HUVEC.
Confluent HUVEC were incubated without addition (control), with the addition of 100 µg/ml of native LDL (n-LDL), glycated LDL (gly-LDL) or oxidized LDL (ox-LDL) for 48 h (PAI-1, upper) or 24 h (t-PA, bottom). The conditioned media were collected for measuring PAI-1 or t-PA antigen with ELISA. Values are presented as mean ± SD (n=4). *p<0.001 compared to control cultures; $^{+}$p<0.001 compared to native LDL; $^{\#}$p<0.001 compared to glycated LDL.

Table 32. Effects of oxidized Lp(a) on PAI-1 secretion from HUVEC.

Lipoproteins	PAI-1 antigen (μg/mg protein)
control	3.78 ± 0.39
n-Lp(a)	5.07 ± 0.18*
gly-Lp(a)	6.14 ± 0.35*$^{+}$
ox-L(a)	6.03 ± 0.48*$^{+}$

Confluent HUVEC were treated in heparin-free medium without addition or with 5 μg/ml of native Lp(a), [n-Lp(a)], glycated Lp(a) [gly-Lp(a)], or oxidized Lp(a) [ox-Lp(a)] for 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA. Values are expressed as mean ± SD (n=4). *$p<0.001$ compared to control cultures. $^{+}p<0.001$ compared to native Lp(a).

Table 33. Effects of oxidative Lp(a) on t-PA secretion from HUVEC.

Lipoproteins	t-PA antigen (μg/mg protein)
control	1.33 ± 0.12
n-Lp(a)	1.06 ± 0.13*
gly-Lp(a)	0.78 ± 0.06*$^{+}$
ox-Lp(a)	0.54 ± 0.03*$^{+\#}$

Confluent HUVEC were incubated in heparin-free medium without or with the addition of 5 μg/ml of native [n-Lp(a)], glycated Lp(a) [gly-Lp(a)] or oxidized Lp(a) [ox-Lp(a)] for 24 h and the conditioned media were collected for measuring t-PA antigen with ELISA. Values are expressed as mean ± SD (n=4). *$p<0.001$ compared to control cultures. $^{+}p<0.001$ compared to n-Lp(a). $^{\#}p<0.001$ compared to gly-Lp(a).

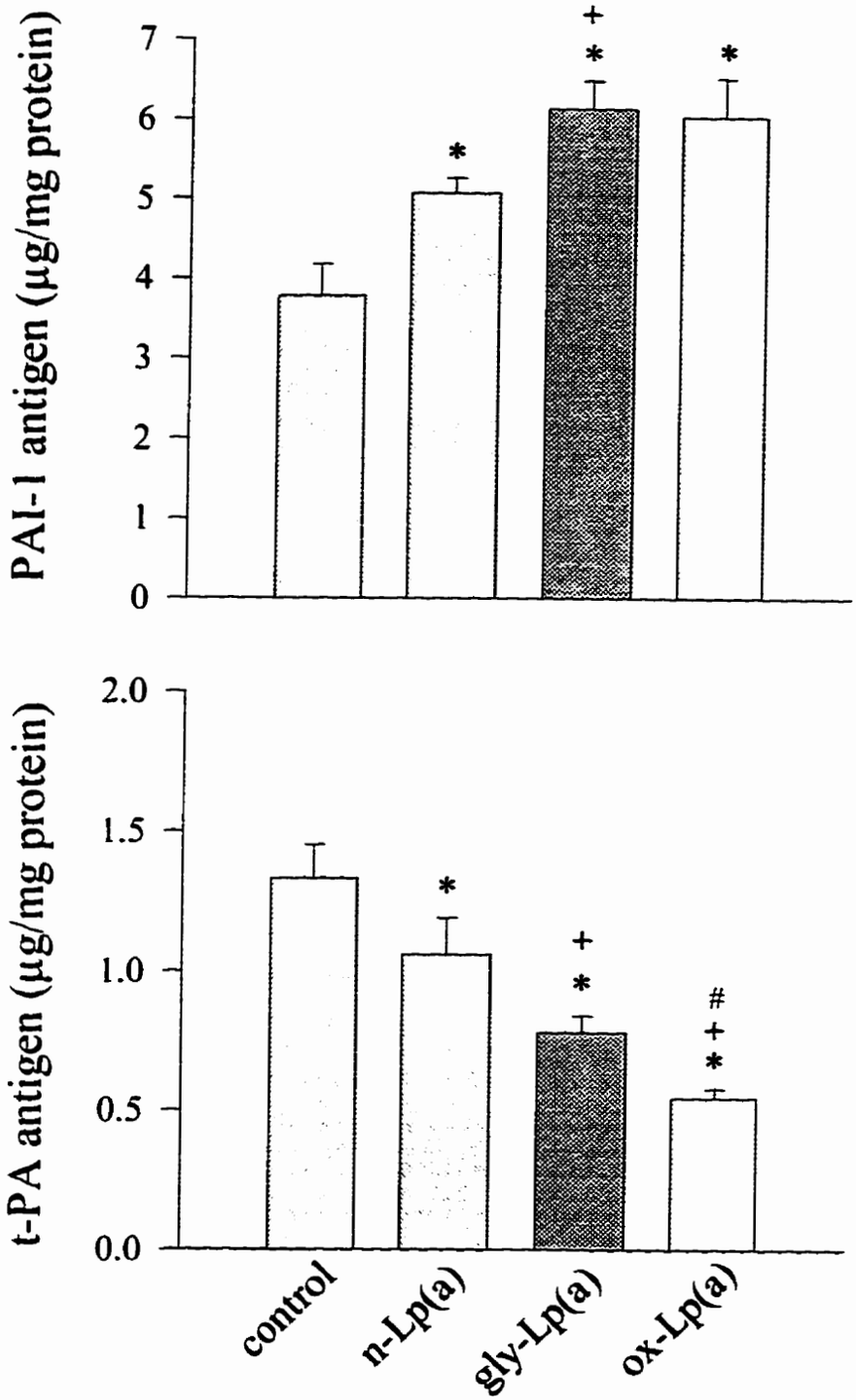


Figure 26. Effects of oxidized Lp(a) on PAI-1 and t-PA secretion from HUVEC.

HUVEC were treated without or with the addition of 5 µg/ml of native Lp(a) [n-Lp(a)], glycated Lp(a) [gly-Lp(a)] or oxidized Lp(a) [ox-Lp(a)] for 24 or 48 h. Conditioned media were collected for measuring PAI-1 antigen with ELISA after 48 h stimulation and conditioned media were collected for measuring t-PA antigen with ELISA after 24 h stimulation. Values are expressed in mean ± SD (n=4). *p<0.001 compared to control cultures. +p<0.001 compared to n-Lp(a). #p<0.001 compared to gly-Lp(a).

5.7 Cytotoxicity of native and glycated lipoproteins

To ensure that the decline in t-PA secretion was not caused by the loss of cell viability, ^{3}H-leucine incorporation assay was performed in cultures treated with or without the addition of native and glycated LDL or Lp(a) for 8-48 h. No significant decrease in ^{3}H-leucine incorporation into cells treated with native or glycated lipoproteins compared to control cultures.

5.8 TBARS in conditioned media of HUVEC

No detectable TBARS was found in the conditioned media of cultured HUVEC treated without (control) or with native and glycated lipoproteins for 8-48 h. In the conditioned media of HUVEC treated with oxidized LDL and Lp(a), the values of TBARS were 3.6 ± 0.4 nM MDA/mg protein (n=3) and 2.4 ± 0.3 nM MDA/mg protein (n=3) respectively.

6. DISCUSSION

The present study indicates that the exposure of HUVEC to native or glycated LDL and Lp(a) increases PAI-1 and decreases t-PA production in vascular EC. Glycation profoundly enhances the effects of native LDL and Lp(a) on the generation of these fibrinolytic regulators. Increased PAI-1 secretion from HUVEC treated with native or modified LDL and Lp(a) was associated with the increase in 2.4 kb PAI-1 mRNA levels.

The results of present study demonstrate that the levels of t-PA mRNA are not affected by native or glycated LDL or Lp(a). The levels of t-PA antigen in extracellular media and the *de novo* synthesis of t-PA were significantly reduced by treatments with glycated LDL or Lp(a) compared to those induced by native LDL or Lp(a). Since no

detectable amount of t-PA was found in the cell-associated pool, the amount of radioactive t-PA in the media represents the *de novo* synthesized t-PA in EC. Our findings suggest that the reduction of t-PA secretion from HUVEC induced by glycated lipoproteins is mainly due to the decrease in t-PA synthesis during post-transcription process instead of transcription process.

Increased plasma levels of PAI-1 activity were significantly associated with vascular events [80]. A supposedly causal relation between the PAI-1 elevation and the risk of recurrent myocardial infarction (MI) was found in longitudinal studies of young [81] and elderly MI survivors [82]. Moreover, plasma PAI-1 levels were significantly higher in subjects with unstable angina at rest compared with those in stable angina subjects [82]. The association between high PAI-1 levels and ischemic events was strong in peripheral vascular disease (PVD) subjects. Increased fibrin turnover, impaired fibrinolytic activity and elevated PAI-1 levels were found in subjects with atherosclerotic disease and these were considered as risk factors for developing thrombotic events [80].

Accumulation of LDL in the vessel wall contributes to the development of atherosclerosis[10, 83]. The rapid clearance of LDL from plasma depends on a receptor-mediated endocytotic process involving the recognition of apoB-100, the major protein of the LDL particle, by a high-affinity receptor present on cell membranes. Goldstein and Brown [10] demonstrated that the internalization of LDL by the LDL pathway leads to lysosomal degradation of apoB-100 and the hydrolysis of the cholesterol esters of LDL. Chemical modifications of lysine groups on apoB-100 interfere with the ability of LDL to bind to the LDL receptor. Interaction of glucose with the free amino groups of amino

acids, such as epsilon amino groups of lysine, potentially inhibits the binding of apoB-100 to the LDL receptor. Witztum *et al.* [84] found that there was good correlation between the number of glucose residues incorporated with LDL and the degree of inhibition of high-affinity degradation. The prolonged residence time of glycated LDL might contribute to elevated plasma LDL levels. The findings of our study indicate that glycation of LDL attenuates vascular EC-derived fibrinolytic activity which may contribute to thrombogenesis and atherogenesis.

Our study demonstrates that native and glycated Lp(a) are stronger agents than native and glycated LDL in stimulating PAI-1 production and reducing t-PA generation from vascular EC. Circulating Lp(a) concentrations were increased and the levels of glycated Lp(a) were positively correlated with the levels of HbA_1c in diabetic subjects [62]. It is reasonable to consider that increased glycated Lp(a) may attenuate fibrinolytic activity in diabetic subjects, which leads to a thrombotic tendency.

In diabetic subjects, approximately 75% of mortality is due to cardiovascular diseases [3]. A decrease of blood fibrinolytic activity was found in both IDDM and NIDDM subjects. Reduced plasma fibrinolytic activity negatively correlated to PAI-1 levels, which indicated the importance of PAI-1 in the regulation of fibrinolysis [50].

The results of the present study partially support the previous report from Levin *et al* [85] on the effects of LDL and Lp(a) on the secretion of t-PA in cultured HUVEC. In contrast to that study, we have not found that native LDL or Lp(a) in similar doses inhibits t-PA mRNA levels. However, our findings indicate that LDL and Lp(a) reduce

the *de novo* synthesis of t-PA in HUVEC. Glycation further reduced the level of t-PA synthesis induced by LDL or Lp(a).

Extended exposure of LDL or Lp(a) to glucose increased the formation of AGEs on protein or lipid moiety of lipoprotein particles. Cell surface receptors that are specific for the recognition and degradation of AGE-modified proteins have been identified on circulating monocytes, lymphocytes, endothelial and renal mesangial cells [63]. Studies of human vascular segments showed that the expression of RAGE was usually at low levels in healthy individuals. In contrast, a prominent increase in the expression of RAGE in endothelial cells was found in individuals with a range of peripheral occlusive vascular diseases, with or without underlying diabetes [86]. The effects of glycated lipoproteins on PAI-1 and t-PA production may be regulated by the reaction of glycated lipoproteins with RAGE. The interaction of AGEs with the RAGE directly generates oxygen free radicals and these second messengers may result in a variety of activation changes in EC [65]. The results of the present study indicate that aminoguanidine not only inhibited the glycation of amino acid residues in glycated LDL, but also effectively prevented the glycated lipoprotein-induced overproduction of PAI-1 and the reduction in t-PA synthesis in HUVEC. Aminoguanidine effectively inhibits the formation of AGEs *in vitro* and *in vivo* [67, 87]. It has been suggested that the primary mechanism by which aminoguanidine inhibits the formation of AGEs is by reacting with Amadori-derived fragmentation products [88]. Previous studies demonstrated a significant correlation between the extent of glycation of LDL and the susceptibility of LDL to oxidation [89]. Aminoguanidine is also able to prevent oxidative modification of LDL [90, 91]. It can trap reactive

breakdown products of lipid peroxidation and prevent apoB modification in a manner similar to the prevention of AGEs formation. In present study, the levels of lipid peroxidation products in glycated lipoproteins were undetectable in terms of TBARS levels. The presence of EDTA in dialysis buffer may prevent the peroxidation of glycated lipoproteins. The inhibitory effects of aminoguanidine on glycated lipoprotein-induced generation of PAI-1 and t-PA in EC is likely due to its inhibition on AGEs formation instead of its antioxidation effect. However, it does not exclude the possibilities that a trivial amount of peroxidative products present in glycated lipoprotein solution which have not been detected by TBARS assay. More sensitive methods, such as the measurement of the loss of unsaturated fatty acids and the formation of conjugated-diene may help to detect low level of oxidative products in glycated lipoproteins.

Brown *et al.* [74] described that glucitollysine migrated between leucine and phenylalanine in glycated BSA on reverse phase HPLC. In the present study, an amino acid peak migrated to the similar position between leucine and phenylalanine in glycated BSA, LDL and Lp(a) but it was not found in the native counterparts. The appearance of this novel peak was associated with a decrease in lysine abundance in glycated BSA and glycated lipoproteins. An additional evidence to clarify this novel peak which is a glycated lysine product is the high radioactivity of this compound in glycated LDL incubated with ^{14}C glucose. The area of this novel peak in amino acid profile obtained from glycated LDL treated with aminoguanidine was reduced compared to that in glycated LDL without exposure to aminoguanidine. These findings support the hypothesis that this glycation-related amino acid is glucitollysine.

In the present study, glycation of BSA did not alter the secretion of either PAI-1 or t-PA, which suggests that the structure of lipoproteins may play a critical role in the regulation of PA-1 and t-PA production. It has been reported that incubation of LDL with glucose produced AGE moieties attaching to both lipid and apolipoprotein components [92]. Higher levels of both apolipoprotein- and lipid-linked AGEs in LDL specimens isolated from diabetic individuals were found compared to that from non-diabetic controls [92]. Determination of the molecular structure of LDL or Lp(a) associated with AGEs may help to clarify the molecular basis of the alteration in the generation of PAI-1 and t-PA induced by glycated lipoproteins in EC.

Both glycated LDL and Lp(a) increased the production of PAI-1 and reduced the secretion of t-PA in HUVEC compared to their native counterparts. The effect of Lp(a) or its glycated form on production of fibrinolytic regulators was stronger than native and glycated LDL based on protein concentration of lipoproteins. LDL and Lp(a) have similar lipid cores. Apo(a) is the unique structure in Lp(a). The kringle IV domains of apo(a) contain relative less lysine compared to apoB-100 [61]. Therefore, most glycation of lysines takes place in apoB moiety in Lp(a). It is possible that an unidentified structure(s) in apo(a) has/have greater stimulatory effect on the production of fibrinolytic regulators in EC. The effects on the production of fibrinolytic regulators induced by lipid components of glycated lipoproteins have not been addressed in this study.

It should be pointed out that the detectable effects on t-PA and PAI-1 production induced by glycated lipoproteins are evident in HUVEC treated with lipoproteins modified by $\geq$ 25 mM or 50 mM glucose. Such high levels of glucose are uncommon in clinical

presentation of the majority of diabetic subjects under conventional treatment. It implies that intense changes in EC-derived PAI-1 and t-PA production may occur in poorly controlled diabetic subjects but the alterations may be in a moderate extent in subjects with mild hyperglycemia. Besides, the glycation of lipoproteins may take place in a different way between *in vitro* and *in vivo*.

The results of the present study should help to explain the attenuation of fibrinolytic activity in diabetes. Although atherosclerosis is exclusively developed in arteries, the response of HUVEC to glycated lipoproteins on the generation of PAI-1 and t-PA is considered to be close to that of arterial EC. The reason is that the umbilical vein carries oxygenated and nutrient-bearing blood from the placenta to the fetus. HUVEC may behave functionally close to arterial EC than other venous EC [22].

7. CONCLUSION

Glycation increases the production of PAI-1 and attenuates the synthesis of t-PA induced by LDL and Lp(a) in vascular EC. The effects of glycated lipoproteins on the generation of PAI-1 and t-PA were effectively normalized by treatments with aminoguanidine during the glycation of lipoproteins. These findings suggest that the glycation of lipoproteins may contribute to attenuation of fibrinolytic activity and development of thrombotic vascular complications in diabetic subjects. Management of hyperglycemia and hyperbetalipoproteinemia or pharmacological intervention on AGEs formation by aminoguanidine or its analogues may improve fibrinolytic activity in diabetic subjects through the normalization of PAI-1 or t-PA production in vascular EC.

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