INFLUENCE OF ATHEROGENIC FACTORS ON PRODUCTION OF FIBRINOLYTIC REGULATORS IN CULTURED VASCULAR CELLS AND INVOLVED MECHANISMS

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

The generation of thrombin is increased at the sites of vascular injury. The production of plasminogen activator inhibitor-1 (PAI-1), the major physiological inhibitor for tissue-type and urokinase-type plasminogen activators, from cultured vascular smooth cells (SMC) is elicited by thrombin. The results of my study demonstrated that thrombin increases PAI-1 antigen, activity and mRNA levels in cultured baboon aortic smooth muscle cells (BASMC). Thrombin treatments elevated the levels of PAI-1 antigen in the conditioned medium of BASMC within 10 min of the treatment. Overexpression of PAI-1 gene was detected in the cultures exposed to thrombin for at least 1 h. The maximal induction of PAI-1 mRNA was found in cultures treated with 10 U/ml of thrombin for 4 h. The thrombin-induced early increase of PAI-1 antigen (up to 0.5 h of stimulation) was blocked by hirudin (a specific inhibitor of thrombin) and was not suppressed by cycloheximide (a protein synthesis inhibitor). The majority of metabolically labelled PAI-1 associated with BASMC was present in extracellular matrix. The level of extracellular matrix-associated PAI-1 was reduced 40 % by 0.5 h of thrombin treatment. My results suggest that thrombin not only increases PAI-1 transcription but also proteolytically cleaves PAI-1 from extracellular matrix of vascular SMC. PAI-1 released by thrombin from the extracellular matrix did not alter PAI activity in extracellular fluid but may reduce the storage of active PAI-1 in vessel wall.

My studies also investigated the signal transduction pathway involved in thrombin-

induced PAI-1 production in BASMC. Thrombin receptor activating peptide mimicked the effect of thrombin on PAI-1 production in BASMC. Thrombin-induced PAI-1 production was blocked by specific protein kinase C (PKC) inhibitors. Both basal and thrombin-induced PAI-1 production were suppressed by adenylate cyclase agonists or cAMP homologue, forskolin or 8-bromo-cAMP. Addition of 8-bromo-cGMP also inhibited thrombin-stimulated PAI-1 production. Pertussis toxin, a G-protein inhibitor, partially inhibited thrombin-induced PAI-1 production. Sodium fluoride, a G protein agonist, stimulated PAI-1 production. Genistein and tyrphostin 25, two tyrosine kinase inhibitors, blocked thrombin-induced PAI-1 production. PAI-1 generation in BASMC was also impeded by neomycin, an inhibitor for phospholipase C and D, and a selective phospholipase C inhibitor, U73122. My results indicate that pertussis toxin-sensitive G protein-coupled receptor, tyrosine kinase, phospholipase C and PKC possibly mediate thrombin-induced PAI-1 overproduction. Increased intracellular cAMP or cGMP levels negatively regulate the synthesis of PAI-1.

The pharmacological modulation of PAI-1 production was investigated by intervening in the involved signal transduction pathway. Hirulog-1 is a thrombin inhibitor based on hirudin that effectively prevents thrombosis in certain clinical situations associated with low incidence of bleeding complications. Hirulog-1 alone did not significantly alter PAI-1 production in BASMC. Treatment with 10-20 μg/ml Hirulog-1 completely inhibited thrombin-induced PAI-1 release from BASMC. Significant reduction of thrombin-induced PAI-1 release occured in cultures treated with Hirulog-1 for 1 h. The maximal inhibitory effect of Hirulog-1 was reached in cultures following 6-8 h of

treatment. The inhibitory effect of Hirulog-1 on PAI-1 production was also detected at mRNA level in BASMC. Nitroprusside, which can increase intracellular cGMP level, suppressed thrombin-induced PAI-1 production in a dose- and time-dependent manner. These results suggest that treatment with hirulog-1 and nitroprusside effectively inhibited thrombin-induced PAI-1 synthesis in cultured vascular SMC.

Increased plasma lipoprotein(a) [Lp(a)] has been considered a strong risk factor for atherosclerotic coronary artery disease. Oxidization of lipoproteins may promote the formation of atherosclerosis. The results of my studies demonstrated that Lp(a) elevated the mRNA levels of PAI-1 in cultured human umbilical vein endothelial cells (HUVEC). The maximum effect of Lp(a) on PAI-1 mRNA was found in HUVEC treated with 10-20 μg/ml of Lp(a) for 48 h. Lp(a)-free LDL in a comparable range of concentrations did not affect the levels of PAI-1 mRNA. Oxidative modification of Lp(a) by $CuSO_4$ increased Lp(a)-induced PAI-1 mRNA levels 1.8- to 2.5-fold in HUVEC. Oxidized LDL at equimolar concentration moderately increased PAI-1 production in HUVEC. Comparable increases of PAI-1 activities were detected in the conditioned media of HUVEC treated with oxidized Lp(a) in comparison with native Lp(a). The results of my study indicated that Lp(a) increased the production of PAI-1 in cultured vascular endothelial cells at mRNA levels. Oxidization enhanced the effect of Lp(a) and LDL on PAI-1 production in cultured vascular endothelial cells.

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LIST OF ABBREVIATIONS:

min	minute
h	hour
°C	degree centigrade
ml	millilitre
ng	nanogram
µg	microgram
mg	milligram
µM	micromolar
mM	millimolar
%	percent
kD	kilodalton
BSA	bovine serum albumin
CsCl	cesium chloride
ddH_2O	distilled deionized water
EDTA	ethylene-diamine-tetraacetic-acid
TE	Tris-EDTA
DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
mRNA	messenger RNA
OD	optical density

UV	ultraviolet
EC	endothelial cell
HUVEC	human umbilical vein endothelial cell
SMC	smooth muscle cell
BASMC	baboon aortic smooth muscle cell
PAI-1	plasminogen activator inhibitor-1
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
PBS	phosphate buffered saline
ELISA	enzyme-linked immunosorbent assay
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis.
Lp(a)	lipoprotein (a)
apo(a)	apolipoprotein (a)
apoB	apolipoprotein B
LDL	low density lipoprotein
VLDL	very low density lipoprotein
HDL	high density lipoprotein
CAD	coronary artery disease
MI	myocardial infarction
TRAP	thrombin receptor activating peptide

PKC	protein kinase C
PMA	phorbal myristate acetate
NP	nitroprusside
NO	nitric oxide
CL	cell layer
EM	extracellular matrix
TBARS	thiobarbituric acid reactive substances
NaF	sodium fluoride
PLC	phopholipase C
PC	phosphatidylcholine
LPC	lysophosphatidylcholine
$CuSO_4$	cupric sulfate
cAMP	cyclic adenosine monophophate
cGMP	cyclic guanosine monophophate
8-br-cAMP	8-bromo-cAMP
8-br-cGMP	8-bromo-cGMP
APMSF	amidinophenylmethanesulfonyl fluoride
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone

INTRODUCTION

Cardiovascular disease is the principle cause of death in Western populations. Atherosclerosis, the major underlying mechanism, has been recognized as a multifactorial process. Endothelial injury, thrombosis, smooth muscle cell proliferation and lipid deposition were considered as the critical underlying mechanisms of atherogenesis. Thrombosis may contribute to atherosclerotic plaque formation. Fibrinolysis is the principle defense system in the body against intravascular clots. The fibrinolytic system is mainly composed of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitors. Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor for tPA and uPA. An increase in the production of PAI-1 or the reduction of plasminogen activators may cause reduced formation of plasmin and attenuate fibrinolytic activity (Andreasen et al. 1990). Fibrinolytic activity is frequently reduced in patients with cardiovascular disease. Elevated PAI-1 activity has been described in coronary artery disease (CAD) patients (Yorimuitsu et al. 1993). Circulatory fibrinolytic activity is mainly regulated by tPA, uPA and PAI-1. Vascular endothelial cells (ECs) and smooth muscle cells (SMCs) are capable of producting those fibrinolytic regulators (Clowes et al. 1990). Thrombin is a plasma serine protease and a potent regulator of coagulation. Production of thrombin is magnified at sites where platelet aggregation and endothelial layer injury occur. SMCs are anatomically protected from blood components by non-thrombotic endothelium. When endothelium is

denuded, SMC may be exposed to blood components. Increased circulatory lipoprotein(a) [Lp(a)] has been considered a strong risk factor for CAD (Amstrong et al. 1986, Rhoads et al. 1988). Lp(a) particles have been immunohistochemically detected in ECs and SMCs of human atherosclerotic lesions (Yorimuitsu et al. 1993). The production of fibrinolytic regulators in vascular cells is regulated by several biological stimulators, including thrombin and Lp(a).

1. Fibrinolytic system

The fibrinolytic system is critical in maintaining normal haemostasis. Plasmin, the major product of the fibrinolytic system, dissolves fibrin-thrombus thereby restoring blood flow within obstructed vessels. Plasminogen, the precursor of plasmin, is abundant in the circulation and most body fluids and serves as a limitless supply of proteolytic capacity. Conversion of plasminogen to plasmin is primarily promoted by tPA and uPA. The activity of tPA and uPA is regulated by their major physiological inhibitor PAI-1. PAI-1 can form complexes with tPA or uPA and reduces the catalytic activity of tPA and uPA (Saksela and Rifkin 1988, Andreasen et al. 1990).

Inherited deficiencies of plasminogen activator activities were described in familial thrombotic disease (Johansson et al. 1978, Stead et al. 1983). Thrombosis plays a crucial role in the pathogenesis and development of CAD. Increased levels of PAI-1 activity with or without a decrease of tPA activity have been described in myocardial infarction (MI) survivors (Hamsten et al. 1985, Estelles et al. 1985), acute MI, unstable angina pectoris

(Huber et al. 1988, Munkvad et al. 1990), angiographically-verified CAD (Francis et al. 1988, Sakata et al. 1990) and restenosis after percutaneous transluminal coronary angioplasty (Huber et al. 1993). In eight large-scale randomized controlled trials, early death and severe complications in acute MI were significantly reduced by infusion of recombinant tPA (O'Rourke et al. 1988, Held et al. 1990). In patients who experienced re-infarction or tPA infusion resistance after acute MI or re-stenosis after percutaneous transluminal coronary angioplasty, increased PAI-1 or decreased tPA was detected (Huber et al. 1988, Barbash et al. 1989, Kristensen et al. 1985). Circulatory PAI-1 activity strongly correlates with the occurrence of ischemic thrombotic events in CAD. Epidemiological studies indicate that an increase of PAI-1 in plasma is a risk factor for CAD (Cortellaro et al. 1993). PAI-1 has been immunohistochemically demonstrated in EC, SMC and collagen fibres of atherosclerotic coronary artery (Yorimuitsu et al. 1993). This theory has been strengthened by the findings of the presence of PAI-1 in human coronary atherosclerotic lesions (Yorimuitsu et al. 1993) and increased levels of PAI-1 mRNA in thrombosed arteries compared to normal arteries (Arnman et al. 1994). Increased levels of PAI-1 protein and mRNA are detected in arteriosclerotic blood vessels (Schneiderman et al. 1992, Lupu et al. 1993). Available evidence strongly suggests that the imbalance between fibrinolytic regulators, especially the increased levels of PAI-1 in blood, may play a critical role in atherothrombotic vascular diseases.

2. Regulation of fibrinolytic mediators in vascular cells

Several studies have demonstrated that vascular EC and SMC produce tPA, uPA

and PAI-1 (Emeis and Kooistra 1986, Laug et al. 1989, Bell and Madri 1990, Clowes et al. 1990). Vascular endothelium behaves not only as a selective permeability barrier between blood and other tissues but also as an active secretory source of numerous biological mediators. Cultured EC synthesize tPA, uPA and PAI-1 (Kristensen et al. 1985, van Hinsbergh et al. 1988, Booyse et al. 1988). The generation of PAs or PAI-1 in EC is regulated by a variety of biological stimulants, including thrombin (Loskutoff 1979, Hanss and Collen 1987), heparin (Konkle and Ginsberg 1988), tumor growth factor (van Hinsberg et al. 1994), active phorbol ester (Grulich-Henn et al. 1990) interleukin-1 and endotoxin (Emeis and Kooistra 1986). Recent studies indicated that lipoproteins may be active agonists for PAI-1 synthesis in EC. Very low density lipoprotein (VLDL) from hypertriglyceridemic subjects increased PAI-1 antigen in EC cultures (Stiko-Rahm et al. 1990). Native and modified low density lipoprotein (LDL) elevated PAI-1 antigen level in EC cultures, and their effects were not inhibited by antibody against LDL receptor (Tremoli et al. 1993). Addition of Lp(a) induced an increase in mRNA, antigen and activity of PAI-1 in EC cultures (Etingin et al. 1991).

Vascular SMCs are capable of performing multiple functions. Two different phenotypes of SMC have been described in cell culture as well as in arterial wall (Chamley-Campbell et al. 1981.). Contractile phenotype of SMC contain abundant myofilaments and do not respond to mitogens. When SMCs are appropriately stimulated, they lose cytoplasmic myofilaments and become synthetical phenotype, which are able to synthesize numerous secretory proteins, including several fibrinolytic mediators: tPA,

uPA and PAI-1 (Laug et al. 1989, Au et al. 1991). Vascular SMCs may be exposed to circulatory components when injury to a vessel wall involves denudation of endothelium, which promotes the formation of thrombosis and atherogenesis at the injury sites. Thrombin and apoB-rich lipoproteins are common constituents of these lesions. Increased levels of tPA and uPA mRNA were detected in tissue extracts of rat carotids following balloon catheter-induced intima injury (Clowes et al. 1990). Heparin selectively inhibits tPA gene expression but not uPA or PAI-1 (Au et al. 1991). Thrombin increased mRNA as well as antigen levels of PAI-1 in bovine aortic SMC after incubating beyond 4 hours (Noda-Heiny et al. 1993).

3. Lp(a) and cardiovascular disease

Lp(a) is composed of an apoB 100 containing LDL particle and one on two apolipoprotein(a) [apo(a)] polypeptide. Concentrations of Lp(a) in human plasma vary a thousand-fold (from undetectable to >200 mg/dl). Numerous case-control studies demonstrate a strong correlation between plasma Lp(a) levels and premature cardiovascular disease (Breckenridge 1990). The effect of Lp(a) in atherosclerosis has been fortified by the findings that the development of atherosclerosis in transgenic mice express apo(a) following high fat diet (Hajjar et al. 1989) and the detection of apo(a) in human atherosclerotic lesions (Lawn et al. 1992).

Apo(a) has a high degree structural homology of plasminogen. Lp(a) competes with the binding of plasminogen to the endothelial cell surface and inhibits the activation

of plasminogen induced by tPA (Lawn et al. 1992). Since the decrease in plasminogen binding on EC surface impairs plasminogen activity, Lp(a) has been considered as a potential link between atherosclerosis and thrombosis (Scott 1989). The structure of apo(a) also resembles hepatocyte growth factor, prothrombin, tPA, uPA and several coagulation factors, which implies that Lp(a) may interfere with other atherosclerosis-related processes, such as SMC proliferation, fibrinolysis and coagulation. Numerous studies suggest that oxidative modification enhances atherogenicity of LDL (Latron et al. 1991). The uptake of Lp(a) and apo(a) by monocytes was increased by the oxidative modification of Lp(a) (Naruszewicz et al. 1994, Naruszewicz 1995). Available evidence implies that Lp(a) and its oxidative form may play a multi-functional role in the pathogenesis of atherothrombosis.

Vascular endothelium not only serves as a selectively permeable barrier between blood and other tissues, but also as an active secretory source of numerous biological mediators. Cultured vascular ECs synthesize tPA, uPA and PAI-1 (Kristensen et al. 1985, Booyse et al. 1988). The production of fibrinolytic regulators by ECs is regulated by a variety of circulatory activators, including several kinds of plasma lipoproteins. VLDL from hypertriglyceridemic subjects increases PAI-1 in the conditioned medium of EC (Stiko-Rahm et al. 1990). LDL modified by ultraviolet radiation or $CuSO_4$ induced greater release of PAI-1 from EC than native LDL (Latron et al. 1991, Kugiyama et al. 1993, Tremoli et al. 1993). Lp(a), LDL and high density lipoprotein (HDL) decreased tPA release from EC but none of them affected the level of PAI-1 in the conditioned medium

after a 16 h incubation (Levin et al.1994). Etingin et al. (1991) found that Lp(a) increased PAI-1 mRNA, antigen and activity levels but not tPA mRNA in cultured EC following ≥24 h of treatments. Addition of lysophosphatidylcholine (LPC), a peroxidation product of phosphatidylcholine (PC) and a component of oxidized LDL, promotes the release of PAI-1 from EC (Kugiyama et al. 1993). Lp(a) particles have been immunohistochemically detected in endothelial and SMC of human atherosclerotic lesions (Hajjar et al. 1989).

4. Central regulatory role of thrombin in haemostasis

Thrombin is the key enzyme that regulates haemostasis and catalyses several steps in blood coagulation: the conversion of fibrinogen to clottable fibrin and the activation of factor XIII, which stabilizes fibrin clot (Fenton 1988a, Berliner 1992). Thrombin is a highly specific serine protease that also has important nonenzymatic (receptor-mediated) activities. Thrombin is generated from prothrombin by the action of factor Xa and cofactor Va within the cell surface-bound prothrombinase complex. The generation of thrombin takes place on phospholipid-rich cell surfaces via a series of proteolytic reactions. The surface of activated platelets provides an optimal locus for several critical coagulation reactions and accelerates the process and thrombin generation. Thrombin activates factor V and VIII and binds to an endothelial cell surface protein, thrombomodulin, resulting in more efficient activation of protein C. Activated protein C inactivates factors Va and VIIIa by limited proteolysis, resulting in a reduction in thrombin formation. The binding of thrombomodulin to thrombin competitively inhibits thrombin's ability to cleave fibrinogen and also blocks the platelet activation reaction.

Several protease inhibitors are involved in controlling the activity of thrombin in blood. The most important one is antithrombin III (Rosenberg 1987), whose affinity for thrombin is enhanced by heparin, the classical anticoagulant (Hirsh 1991). Thrombin is a potent agonist for platelet secretion and aggregation, which further accelerates the formation of thrombus. Heart attacks and strokes are usually caused by the formation of occlusive thrombi in coronary arteries at the sites of atherosclerotic stenosis and plaque rupture (Chesebro et al. 1987, Heras et al. 1989). At the sites of vascular injury, thrombin is the primary mediator of platelet recruitment and activation (Kelly et al. 1991).

5. Thrombin and vascular cell-derived fibrinolytic regulators

Thrombin is a plasma serine proteinase and a crucial factor in coagulation, it is also a potent agonist for the production of fibrinolytic regulators in vascular cells (Levin et al. 1984, Gelehrter and Szycer-Laszuk 1986, Dichek and Quertermous 1989). Addition of physiological concentrations of thrombin dose-dependently elevate PAI-1 production in cultured vascular endothelial cells (EC) (Gelehrter and Szycer-Laszuk 1986, Dichek and Quertermous 1989). Thrombin-induced PAI-1 production may be secondary to interleukin-1 in EC (Heaton et al. 1992). Thrombin forms a complex with PAI-1 in the extracelluar matrix of EC, which causes a mutual neutralization of thrombin and PAI-1 (Ehrlich et al. 1991). Thrombin also increases the generation of tPA from EC (Levin et al. 1984). PAI-1 may complex with tPA and neutralize the activity of tPA. Thrombin increased uPA mRNA content in porcine aortic EC (Sun et al. 1994), whereas, no detectable uPA was found in primary cultures of human EC (Philips et al. 1984, Levin

et al. 1984). Production of thrombin is magnified where platelet aggregation and endothelial injury occur. A slight increase in tPA release was found in the conditioned medium of human vascular SMC treated with thrombin (Wojta et al. 1993). Noda-Heiny et al. (1993) reported that thrombin increases the activity, antigen and mRNA levels of PAI-1 in cultured bovine aortic SMC. These findings suggest that thrombin may cause an imbalance among fibrinolytic regulators derived from vascular cells.

6. Transcellular signalling in vascular cells activated by thrombin

Signal transduction pathway mediating thrombin-induced production of PAI-1 has been examined in endothelial (Francis et al. 1989, Grulich-Henn and Muller-Berghaus 1990), glomerular mesangial (Villamediana et al. 1990) and epithelial cells (He et al. 1992). Receptors on the cell surface are targets for ligands. Ligand binding initiates an intracellular signalling process, which further induces a series of biochemical events and functional changes in the cells. The thrombin receptor has been cloned from hamster and human cells (Rasmussen et al. 1991, Vu et al. 1991). The deduced sequence determined from cloned thrombin receptor cDNA shows that the receptor has an unique long extracelluar extension, which contains the cleavage site sensitive to thrombin. The remaining shortened extracelluar portion after cleavage contains a newly exposed NH_2 terminus that binds to the thrombin receptor, and functions as a "tethered ligand" to activate the receptor. The thrombin receptor has seven hydrophobic segments spanning lipid bilayer and its intracellular extension is coupled with G protein (Rasmussen et al. 1991, Vu et al. 1991). Synthetic peptides corresponding to the newly exposed NH_2

terminus are named as thrombin receptor activating peptides (TRAP). They elicit cellular responses similar to native thrombin (Huang et al. 1991, Seiler et al. 1995). Addition of TRAP activates protein kinase C (PKC) and increases Ca^{2+} in vascular EC (Lum et al 1993). Thrombin and TRAP activate protein tyrosine phosphorylation in BC_3H1 muscle cells (Seiler et al. 1995). TRAP induced rapid morphological changes in endothelial cells, with marked increase in the release of prostacyclin, endothelin, platelet activating factor, tPA and PAI-1 (Maruyama et al. 1994). γ-Thrombin, a degradation product of thrombin, retains some enzymatic activity of α-thrombin but does not bind to the thrombin receptor (Gordon and Carney 1986).

Like many mitogens and other polypeptides, thrombin may activate phospholipase C thus causes the generating of IP_3 and diacylglycerol (DAG) (Jaffe et al. 1987). IP_3 mobilizes Ca^{2+} from endoplasmic reticulum, whereas DAG stimulates protein kinase C (PKC) (Nishizuka 1984). PAI-1 production in thrombin-treated endothelial cells was inhibited by PKC inhibitors (Grulich-Henn et al. 1990) and cAMP agonists (Francis et al. 1989). Genistein, a tyrosine kinase inhibitor, suppressed PAI-1 gene expression in tumor necrosis factor- and thrombin-stimulated endothelial cells (van Hinsbergh et al. 1994). Thrombin also stimulated Na^+/H^+ exchange in vascular SMC (Huang and Ives 1987, Berk et al. 1990). The metabolic pathway for the thrombin-induced fibrinolytic regulators in SMC has not been well defined.

7. Thrombin inhibitors and anti-coagulants

Plasma antithrombin III inhibits thrombin acting by forming complex with thrombin. In the absence of heparin, these reactions are very slow. Heparin increases the effect of antithrombin III by several thousand-fold and it is the most commonly used anticoagulant. High molecular weight heparin has higher affinity to antithrombin III. Low molecular weight heparin inhibits activated factor X and has less antithrombin activity. Heparin frequently causes bleeding and thrombocytopenia (Hull and Pineo 1992). Another limitation in the application of heparin is that clot-bound thrombin is protected from the effect of heparin (Weitz et al. 1990).

Hirudin is a highly specific and the most potent thrombin inhibitor derived from the medicinal leech (Johnson et al. 1989). Hirudin forms exceedingly high-affinity noncovalent complexes with thrombin, which potentially cause complete thrombin consumption. Hirulogs are a group of novel synthetic small peptides based on the structure of hirudin. They are designed to inhibit both catalytic center and fibrinogen-recognition exosite of α-thrombin using the model of hirudin (Maraganore et al. 1990). Hirulog-1 is one of the most potent hirulogs and effectively inhibits thrombin-induced activation of clotting factors (Ofosu et al. 1992). It is a 20 amino acid synthetic peptide consisting of a part which binds to the active site cleft and a hirudin-like C-terminal region that binds to the anion-binding exosite of thrombin linked by four glycines (Maraganore et al. 1990). Compared to heparin, hirudin and hirulogs have the following theoretic advantanges: 1) they inhibit clot-bound thrombin, which heparin can not. 2) they do not require anti-thrombin III for cofactor as heparin does and 3) they are not

inhibited by the products of activated platelets as heparin is (Johnson 1994). Hirulog-1 is weaker than hirudin on tight-binding inhibition on thrombin (Fenton 1995) but more potent than hirudin on anticoagulation activity (Maraganore et al. 1990). Recent phase III clinical studies have shown hirulog-1 to be equivalent or more effective than high doses of heparin in the treatment of unstable angina associated with a significant lower rate of bleeding complications (Lidon et al. 1993). Hirulog-1 treatment also benefits to the management of acute myocardial infarction (Lidon et al. 1993) and the prevention of thrombosis during coronary angiography (Topol et al. 1993). The effectiveness of hirulog-1 in thrombolysis and fewer bleeding complications suggests Hirulog-1 is likely to be a component of thrombolytic therapy for selected patients. The mechanism of of acting of hirulog-1 on the prevention of thrombosis has not been completely understood. The influence of hirulogs on fibrinolytic activity, another important factor of thrombogenesis, has not been documented.

HYPOTHESIS:

The atherogenic effects of certain risk factors of cardiovascular disease such as thrombin and Lp(a) may result in part from their influence on the production of fibrinolytic regulators in vascular cells. Thrombin may stimulate the generation of PAI-1 from vascular SMC via membrane receptor and multiple signal transduction pathways. Thrombin-induced PAI-1 production in vascular cells may be modulated by mediators through the regulation of receptor-signal transduction pathway in vascular cells. The overproduction of PAI-1 in vascular cells induced by thrombin stimulation may be

pharmacologically modulated by intervening signal transduction pathway. Hirulog-1 will be a suitable candidate based on its effect on thrombin inhibition and the prevention of thrombotic complications. Lp(a) may regulate the production of fibrinolytic mediators in vascular EC. Oxidative modification may enhance the effect of Lp(a) on the production of fibrinolytic regulators. The studies on thrombin- and oxidized Lp(a)-induced PAI-1 production in cultured vascular cells may help to understand the atherothrombogenicity of thrombin and Lp(a). The generated information may help to rationally design pharmacotherapy to potentiate fibrinolysis and prevent thrombosis in hypercoagulation and hyperlipoprotein(a) states.

MATERIALS AND METHODS

Cell Culture

Baboon aortic SMCs (BASMCs) were originally characterized and kindly provided by Dr. A.W. Clowes (University of Washington, Seattle, WA). These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Burlington, Ont) containing 10% fetal bovine serum (FBS), penicillin (50 U/L), streptomycin (50 mg/L), 10 g/L MEM vitamins and 10 g/L MEM non-essential amino acids, in a humidified incubator with 95% air/5% CO_2 at 37°C and subcultured every 7 to 10 days (Au et al. 1991). Human umbilical vein ECs (HUVECs) were obtained by collagenase digestion and verified by non-overlaping cobblestone culture morphology and the presence of factor VIII antigen. HUVECs were cultured in M-199 medium supplemented with 10% fetal bovine serum, 30 μg/ml of endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO), 100 μg/ml of heparin, 0.1 mM non-essential amino acids and 4 mM L-glutamine until confluence in a humidified incubator under 95% air/5% CO_2 at 37°C. Cells were grown in 150 mm dishes for mRNA evaluation and in 12-well plates for measuring PAI-1 antigen and activity. Endotoxin levels in medium were monitored by Limulus lysate assay.

Stimulation of cells

Confluent BASMCs were rinsed with serum-free medium before additions. Stimulants or inhibitors were diluted in medium containing 1% FBS and incubated with BASMC at 37°C under 95% air/5% CO_2. The presence of 1% FBS in medium did not affect thrombin-induced PAI-1 production compared to serum-free medium. Bovine thrombin (Sigma Chemical Co., St Louis MO) was freshly dissolved in phosphate buffered saline (PBS). For preparation of hirudin-inactivated thrombin, thrombin was pre-incubated with hirudin (Sigma Chemical Co.) in a ratio of 1:2 (thrombin:hirudin) by units of activity, on ice for 20 minutes. Thrombin activity was completely inhibited by hirudin treatment, as estimated by measuring thrombin-time using normal human plasma. For the experiments using phorbal myristate acetate (PMA) and nitroprusside, the additions of these reagents were made in dim light. For the experiments using specific inhibitors for signal transduction pathways, cells were pre-treated with the inhibitor as follows: 30 min pre-treatment for calphostin C, genistein, U73122, U73343 or neomycin, and 5 h pre-treatment for pertussis toxin. At the end of the pretreatment, the media were removed and incubations were continued with fresh media containing identical concentrations of the inhibitors with or without thrombin. Confluent endothelial monolayers were stimulated in heparin-free medium with and without addition of studied lipoproteins. The effect of Lp(a) on PAI-1 production was not significantly affected by the presence of endothelial cell growth supplement or hirudin, a specific thrombin inhibitor. No visible morphological impairment was observed under light microscope in the cells treated with the inhibitors or stimulants.

Enzyme-linked immunosorbent assay (ELISA) of PAI-1 antigen

At the end of incubation, conditioned medium was harvested and frozen at -20°C until analysis. PAI-1 antigen levels in conditioned media were determined using IMUBIND PAI-1 ELISA kit with a monoclonal antibody against human PAI-1 (American Diagnostica Inc., Greenwich, CT) following the manufacturer's instructions. Absorbance at 490 nm was read on a THERMOmax micro-test plate spectrophotometer (Molecular Devices, Menlo Park, CA).

Metabolic labelling and immunoprecepitation of PAI-1

Confluent cells in 60 mm dishes were labelled for 16 h with Tran^{35}S-label (200 μCi/ml, 38 TBq/ml, >70% methionine and <15% cystine, ICN Radiochemicals, Irvine CA) in 1 ml methionine- and cystine-free DMEM (ICN Radiochemicals) supplemented with 2 mM glutamine and 5% dialysed FBS. Labelled cells were chased with the medium with or without the addition of 10 U/ml of thrombin for 0.5 and 5 h. Cell monolayer was lysed in 0.5 ml PBS/well containing 0.5% Triton X-100, and extracelluar matrix was then harvested into 0.5 ml PBS/well containing 0.1% sodium dodecyl sulphate (SDS) as previously described (Mimuro et al. 1987). The lysates of cellular fractions were centrifuged at 4°C for 2 min at 12,000xg. The resulting supernatant was then subjected to immunoprecipitation as previously described (Cockell et al. 1995). Briefly, samples were pre-cleared 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 conjugate (Sigma Chemical Co.) with incubation for 30 min. The supernatants were incubated with

monoclonal antibody against human PAI-1 (American Diagnostica) at 25°C for 2 h. Immunoprecipitates recovered from the beads were suspended in 125 mM Tris buffer (pH 6.8) containing 20% glycerol and 4.6% SDS, boiled for 5 min under non-reducing conditions and analyzed in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Dried gels were subjected to autoradiography.

Northern analysis of PAI-1 gene expression

Total cellular RNA was extracted from cells at the end of incubations, by the guanidine isothiocyanate-cesium chloride method (Chirgwin et al. 1979). Northern analysis was performed as previously described (Southern 1975). Plasmid containing cDNA for human PAI-1 (Wun et al. 1987) was labelled with ^{32}P-dCTP (>111 TBq/mmole, ICN Radiochemicals, Irvine, CA) by a random primer labelling kit (BRL, Burlington, Ont.). Hybridization and autoradiography were performed as previously described (Shen et al. 1989). Expression of β-actin gene measured on rehybridized blot was used as an internal control for the quantity of total mRNA loaded on each lane. The levels of PAI-1 and β-actin mRNA were quantified from autoradiographs by Ultrascan XL laser scanning densitometry (LKB, Sweden).

Thrombin preparations

Human α- and γ-thrombin, and TRAP (SFLLRN) were kindly provided by Drs. J.W. Fenton II and J.M. Maraganore (Fenton et al. 1977, Bing et al. 1977, Beecher et al. 1994, Maraganore et al. 1990). Catalytically inactivated thrombin was obtained by

incubation of thrombin with 0.6 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, Calbiochem, La Jolla, CA) at 37°C for 0.5 h. The activity of thrombin was assessed by measuring thrombin time with fresh human plasma.

Isolation of lipoproteins

Density<1.21 plasma fractions were separated from fresh human plasma by ultracentrifugation in the presence of 1 mM EDTA. After dialysis, plasma lipoprotein fractions were applied to lysine-Sepharose chromatography (Snyder et al. 1992). The unbound fraction of elution was used to prepare Lp(a)-free LDL by ultracentrifugation (density 1.019-1.063). Lp(a) bound to the affinity column was eluted by 20 mM 6-amino hexanoic acid in 0.1 M phosphate buffer (pH 7.4), 1 mM benzamidine and 0.01% EDTA. Concentrations of Lp(a) in eluted fractions were estimated by Macro Lp(a) enzyme immunoassay kit (Terumo, Elkon, MD). No detectable Lp(a) was found in LDL isolated from lipoproteins eluted from affinity column. Lp(a) size isoforms were determined by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described (Shen et al. 1995). Lp(a) and LDL isolated from the plasma of 5 donors, in the apo(a) phenotypes of S3, S4/S2, B, S2 or S1/S4, were applied in the present study. All lipoproteins were stored in sealed tubes filled with nitrogen at 4°C in dark to prevent oxidization. Protein levels of Lp(a) or LDL were measured by a modified Lowry method (George et al. 1987). Molar concentrations of Lp(a) were determined from protein levels and molecular weights of apo(a).

Oxidative modification of lipoproteins

Aliquots of Lp(a) or LDL were modified by 5 μM $CuSO_4$ following a dialysis against an EDTA-free buffer (Liu et al. 1994). Lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) as previously described (Ohkawa et al. 1979) and tetramethoxypropane was used as a standard. Undetectable or very low TBARS levels were found in native LDL or Lp(a). Oxidative modification raised TBARS levels in both LDL and Lp(a) more than 20-times.

Enzymatic bioassay for PAI activity

Conditioned medium and extracelluar matrix of cell were incubated with 0.4 U/ml of human tPA (American Diagnostica Inc.) at 37°C for 30 min, then with 0.6 mM S-2390 (Val-Phe-Lys-ponitroaniline, Kabi Co., Vitrum AB, Sweden), 50 μg/ml of soluble fibrin (Kabi Co.) in 50 mM Tris buffer (pH 8.8) with 0.01% Triton X-100 at 37°C for 2 h (Chmielewska and Wiman 1986). Absorbancy at 405 nm was read with a micro-test spectrophotometer for evaluation of activity of tPA. One unit of PAI-1 activity was defined as the amount that inhibits 1 unit of tPA activity under these conditions.

Protein concentration measurements

Protein concentrations of lipoproteins were analyzed by a modified Lowry method (George et al. 1987) and expressed in μg protein/ml of lipoprotein preparation. For measuring the amount of protein in cultured cells, the cells were first washed with PBS three times, then solubilized in 0.1 N NaOH. Protein concentrations in the mixtures were

Statistical analysis

Student's t test for paired and unpaired data was used where appropriate. For multiple groups, analysis of variance was performed and followed by Duncan's test to detect individual differences. P value less than 0.05 was considered to be statistically significant.

RESULTS

1. EFFECT OF THROMBIN ON RELEASE AND PRODUCTION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) FROM CULTURED VASCULAR SMOOTH MUSCLE CELLS

1.1 Effects of thrombin on PAI-1 release:

Significant increases in PAI-1 antigen level ($p<0.05$ or 0.01) were detected in the conditioned media of BASMC treated with 10 U/ml of human α-thrombin from 0.5 to 24 h compared to time-matched control cells (without exposure to thrombin) (Fig.1 A). In separate experiments, the effect of thrombin incubation (10 U/ml) for 5 min to 1 h on PAI-1 release from BASMC was examined. Significant elevations of PAI-1 antigen in the conditioned media of BASMC were detected in the cultures incubated with thrombin ≥10 min ($p<0.01$, Fig.1 B).

1.2 Time-dependence of thrombin treatment on PAI-1 gene expression:

PAI-1 mRNA in HUVEC is present in two distinguishable species with apparent sizes of 3.4 kb and 2.4 kb. In BASMC, the 2.4 kb band of PAI-1 mRNA is barely detectable, and the majority of PAI-1 mRNA revealed by a human PAI-1 cDNA probe is in the 3.4 kb band (Fig. 2). The overexpression of PAI-1 gene was first seen in the cultures receiving over 1 h of thrombin stimulation, and reached a peak after 4 h (Fig. 3). No detectable increase of PAI-1 gene expression was found in the cells treated with thrombin for 0.5 h. The levels of PAI-1 gene expression declined in the cultures with 8 h stimulation. No apparent increase was found in PAI-1 gene expression in a time-

matched control cultures (without an addition of thrombin).

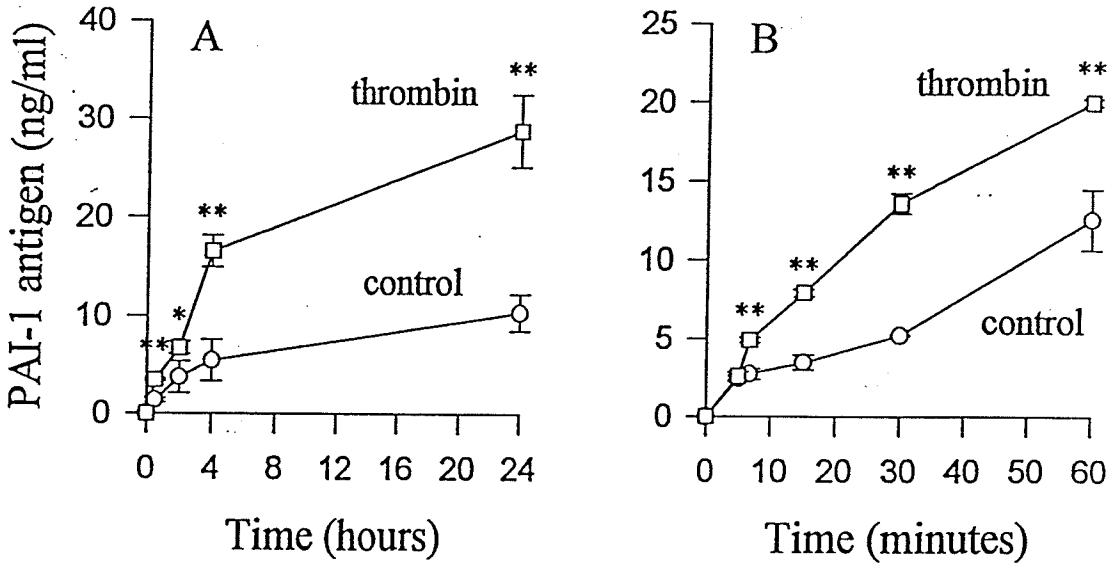


Fig.1. Time course of thrombin-stimulated accumulation of PAI-1 in conditioned medium of BASMC. Confluent cells were incubated in the medium containing 1% FBS with or without the addition of 10 U/ml of thrombin for 0.5 to 24 h (A) or 5 to 60 min (B) in separate experiments. PAI-1 antigen levels in conditioned media were analyzed by ELISA with anti-human PAI-1 monoclonal antibody. Values are expressed as mean±SD from triplicate cultures. Circle: control; square: 10 U/ml of thrombin. *,** : $p<0.05$ or 0.01 vs control.

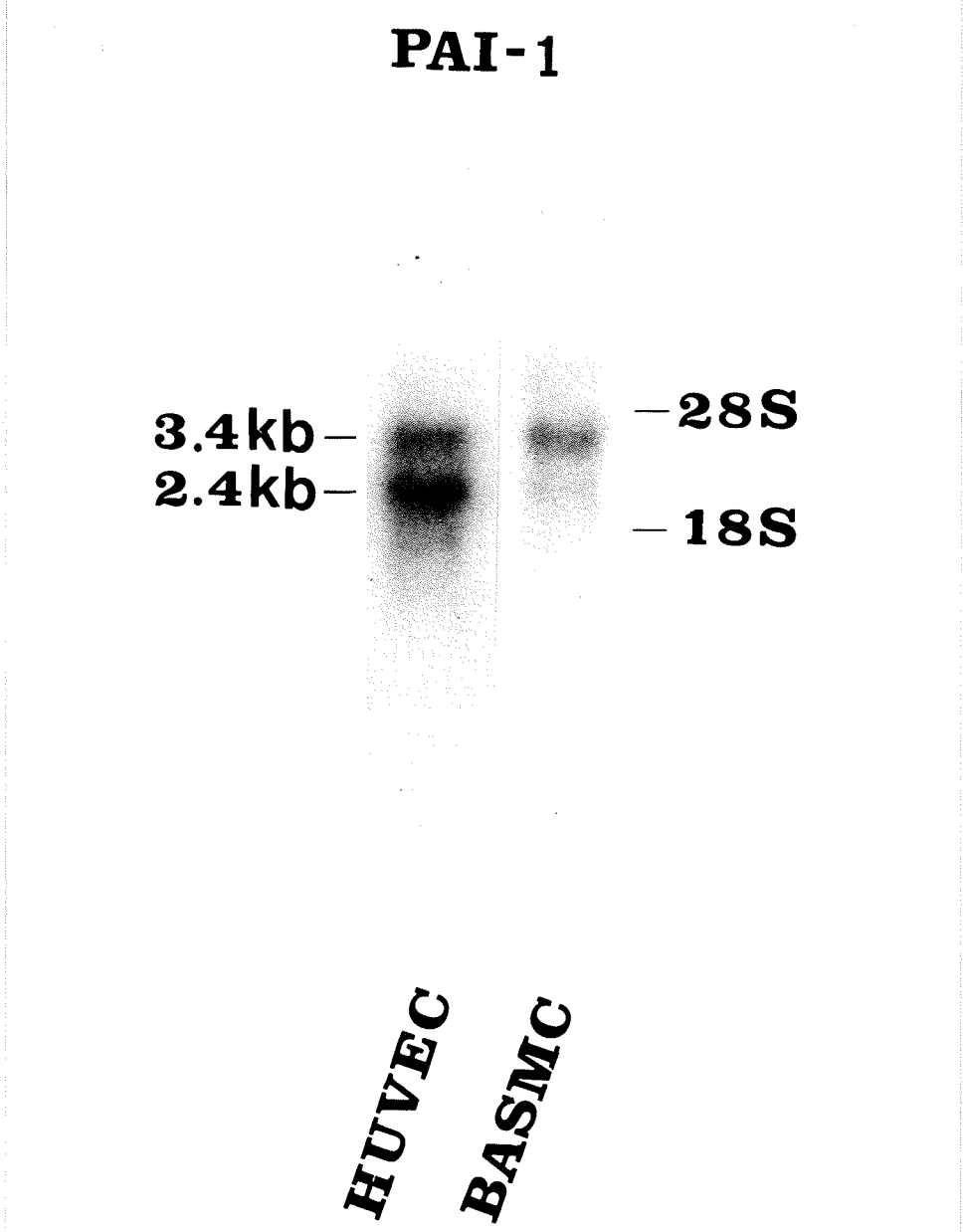


Fig. 2. PAI-1 mRNA species in baboon aortic smooth muscle cell (BASMC). Total RNA (20 μg/lane) isolated from human umbilical vein endothelial cells (HUVEC) and BASMC was separated in 10 g/L agarose-formaldehyde gel and blotted to nitrocellulose membrane. The membrane was hybridized with ^{32}P-dCTP labeled human PAI-1 cDNA. Specific gene expression was visualized by autoradiography. Location of 28S and 18S ribosomal RNAs were determined by ethidium bromide staining. The sizes of two species of PAI-1 mRNA were 3.4 kb and 2.4 kb as calibrated by lambda Hind III fragments.

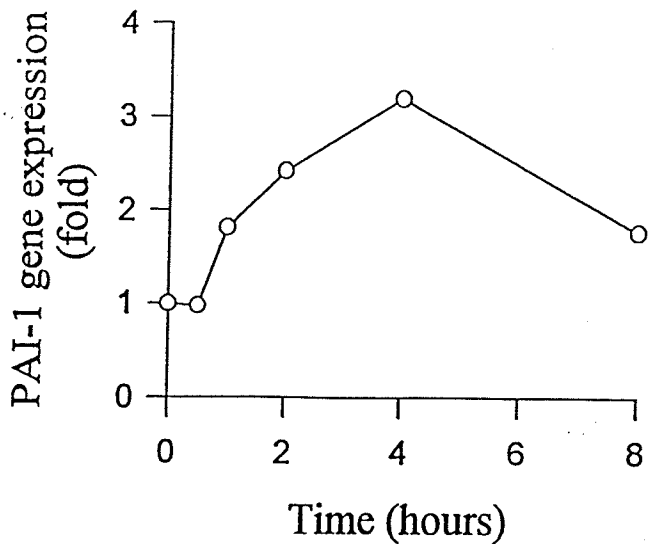


Fig.3. Time-course of thrombin on PAI-1 gene expression in BASMC. Cells were incubated with 10 U/ml of thrombin for 0.5-8 h. Total RNA (20 μg/lane) was subjected to Northern analysis. The specific expression of 3.4 kb species of PAI-1 gene was quantified by densitometry. Values are expressed as fold-increase over time-matched control after adjustment for expression of β-actin gene on rehybridized blot. Each point represents the average of two determinations.

1.3 Dose-dependence of thrombin on PAI-1 production

α-Thrombin dose-dependently increased the levels of PAI-1 antigen in the conditioned medium of cultured BASMC. Significant increase in PAI-1 antigen was found in the cultures treated with 1 U/ml of thrombin for 4 h ($p<0.01$). The effect of thrombin on PAI-1 release reached a plateau when thrombin concentrations were above 10 U/ml. Dose-response of thrombin-induced PAI-1 production in BASMC was also examined. The effect of thrombin on PAI-1 mRNA levels reached a plateau in BASMC treated with over 10 U/ml of thrombin. Increase in the 3.4 kb PAI-1 mRNA level by over half of the maximal effect was detected in BASMC incubated with 1 U/ml of thrombin (Fig.4).

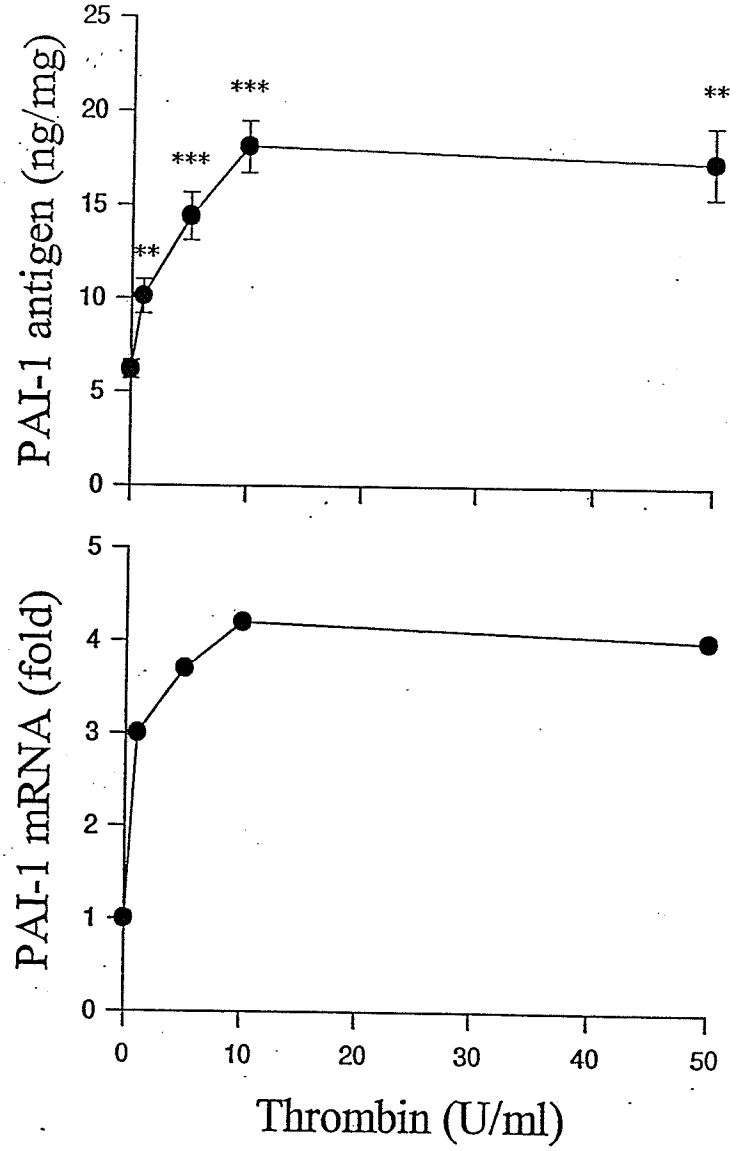


Fig. 4. Dose-response of thrombin-induced production of plasminogen activator inhibitor-1 (PAI-1) in baboon aortic smooth muscle cells (BASMC). Confluent BASMC were treated with 1-50 U/ml of thrombin for 4 h. Upper: Conditioned media were collected for PAI-1 antigen measurement using ELISA. The cells were harvested for cellular protein determination (mean ± SD, n=4). PAI-1 antigen levels were expressed in ng/mg of protein. **,***: $p<0.01$ or <0.001 versus cultures without addition. Bottom: Total RNA isolated from BASMC was separated in 10 g/L agarose-formaldehyde gel and blotted to nitrocellulose membrane. Membranes were hybridized with ^{32}P-dCTP labeled human PAI-1 cDNA then rehybridized with β-actin. PAI-1 mRNA levels were expressed in fold increase compared to no addition controls after adjustment with β-actin mRNA levels (average of two experiments).

1.4 Effect of protein synthesis inhibitor:

To determine whether protein synthesis was required for thrombin-induced early increase of PAI-1 antigen (≤0.5 h) in the conditioned medium, BASMC were treated with 10 μg/ml of cycloheximide along with 10 U/ml of thrombin for 0.5 h. The level of PAI-1 antigen in the cultures treated with cycloheximide plus thrombin did not differ from those stimulated by thrombin alone (17.1 ± 1.7 versus 20.2 ± 2.2 ng/ml, mean ± SD, $p>0.1$, $n=3$).

1.5 Influences of growth cycle and serum supplementation:

In order to answer the question whether the components of FBS affect the release of PAI-1 from BASMC, the cells were treated with serum-free medium or medium containing 1% FBS with or without the addition of 10 U/ml of thrombin for 0.5 h. Absolute levels of PAI-1 in the conditioned medium of the cultures incubated with 1% FBS were 25% higher than cultures exposed to serum-free medium, with or without the addition of thrombin. The relative increase of PAI-1 antigen in the conditioned media of BASMC receiving 0.5 h of thrombin treatment were essentially the same between the cells maintained in serum-free (1.98-fold, $p<0.001$) or 1% FBS medium (2.0-fold, $p<0.001$) (Table 1). A similar pattern of thrombin-induced PAI-1 increase was observed in quiescent cells compared to growing cells (Fig.1 A), and the levels of PAI-1 antigen in the conditioned media of quiescent cells were relatively lower than in growing cultures under both control and thrombin-stimulated conditions. These findings indicate that the thrombin-induced early increase of PAI-1 antigen in BASMC is likely independent of the growth stages of the SMC and the components of FBS.

Table 1. Effect of serum on thrombin-induced early increase of PAI-1 antigen in conditioned medium of BASMC

Treatment	PAI-1 antigen (ng/ml)	
	serum-free	1% FBS
Control	7.08 ± 0.51	9.40 ± 0.53
Thrombin	14.01 ± 0.96^{+}	19.10 ± 0.70^{+}

Confluent cells were incubated in serum-free or 1% FBS medium with or without the addition of 10 U/ml of human α-thrombin for 0.5 h.

Values are expressed as mean ± SD (n=3). $^{+}$: $p<0.001$ compared to control cultures.

1.6 Effect of thrombin on PAI-1 in extracelluar matrix:

The majority of metabolically labelled PAI-1 was detected in the extracelluar matrix of BASMC. The apparent molecular weight of the PAI-1 in extracelluar matrix is around 46 kDa (Fig.5). No labelled PAI-1 was detected in Triton X-100-soluble cell layer. In cultures chased by thrombin for 0.5 h, The radioactivity in extracelluar matrix containing PAI-1 was reduced 38% compared to the control cultures. The radioactivity in extracelluar matrix containing PAI-1 of control cultures chased by medium without thrombin addition for 5 h was 48% less than the control cultures receiving 0.5 h of chase by the same medium. In BASMCs chased by 10 U/ml of thrombin for 5 h, no detectable PAI-1 radioactivity was remained in the extracelluar matrix.

The results of this section were published in Thromb. Res. (Cockell et al. 1995).

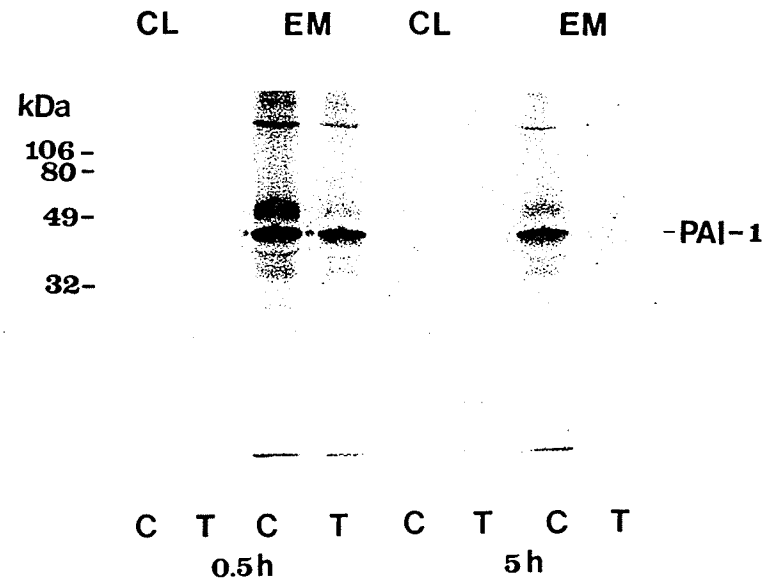


Fig.5. Effect of thrombin on metabolically labelled PAI-1 in cell-associated fractions of BASMC. Confluent BASMC were labelled with 200 μCi/ml of Tran^{35}S label in methionine- and cystine-free DMEM for 16 h followed by incubation with and without bovine thrombin (10 U/ml) for 0.5 and 5 h. Cell layer (CL) was lysed in PBS containing 0.5% Triton X-100; extracelluar matrix (EM) was harvested in PBS containing 0.1% SDS. Immunoprecipitated PAI-1 was analyzed by 10%SDS-PAGE and visualized by autoradiography. C : control; T : thrombin.

2. EFFECTS OF NATIVE AND OXIDIZED LP(a) ON THE PRODUCTION OF PAI-1 IN VASCULAR ENDOTHELIAL CELLS.

2.1 Effects of lipoproteins on PAI-1 antigen and activity levels in conditioned media of HUVEC.

The levels of PAI-1 antigen and activity were determined in the post-culture medium of HUVEC treated with medium alone (control), 10 nM LDL, oxidized LDL, Lp(a) or oxidized Lp(a) for 48 h (Fig.6.). Native LDL at 10 nM concentration did not affect the levels of PAI-1 antigen or activity released from EC. Oxidized LDL at the same concentration moderately but significantly increased the levels of PAI-1 antigen by 35% and its activity by 39% in post-culture medium compared to native LDL. The levels of PAI-1 antigen and activity in HUVEC treated with 10 nM Lp(a) were 67% and 63% higher than that treated with native LDL ($p<0.01$ or 0.001). Oxidized Lp(a) (10 nM) profoundly elevated PAI-1 antigen levels ($p<0.001$), which was 74% higher than that in oxidized LDL-treated cells and 40% higher than that in native Lp(a)-treated cells. The levels of PAI-1 activity generated from oxidized Lp(a)-treated EC were 46% higher than oxidized LDL-treated cells and 21% higher than native Lp(a)-treated cells ($p<0.001$).

2.2 Effects of native and oxidized LDL and Lp(a) on cell-associated PAI-1 antigen and activity.

HUVEC were incubated with medium without addition (control) or containing 10 nM LDL, oxidized LDL, Lp(a) or oxidized Lp(a) for 48 h. Neither the addition of native nor oxidized LDL affected cell-associated PAI-1 antigen and activity in HUVEC. Native and

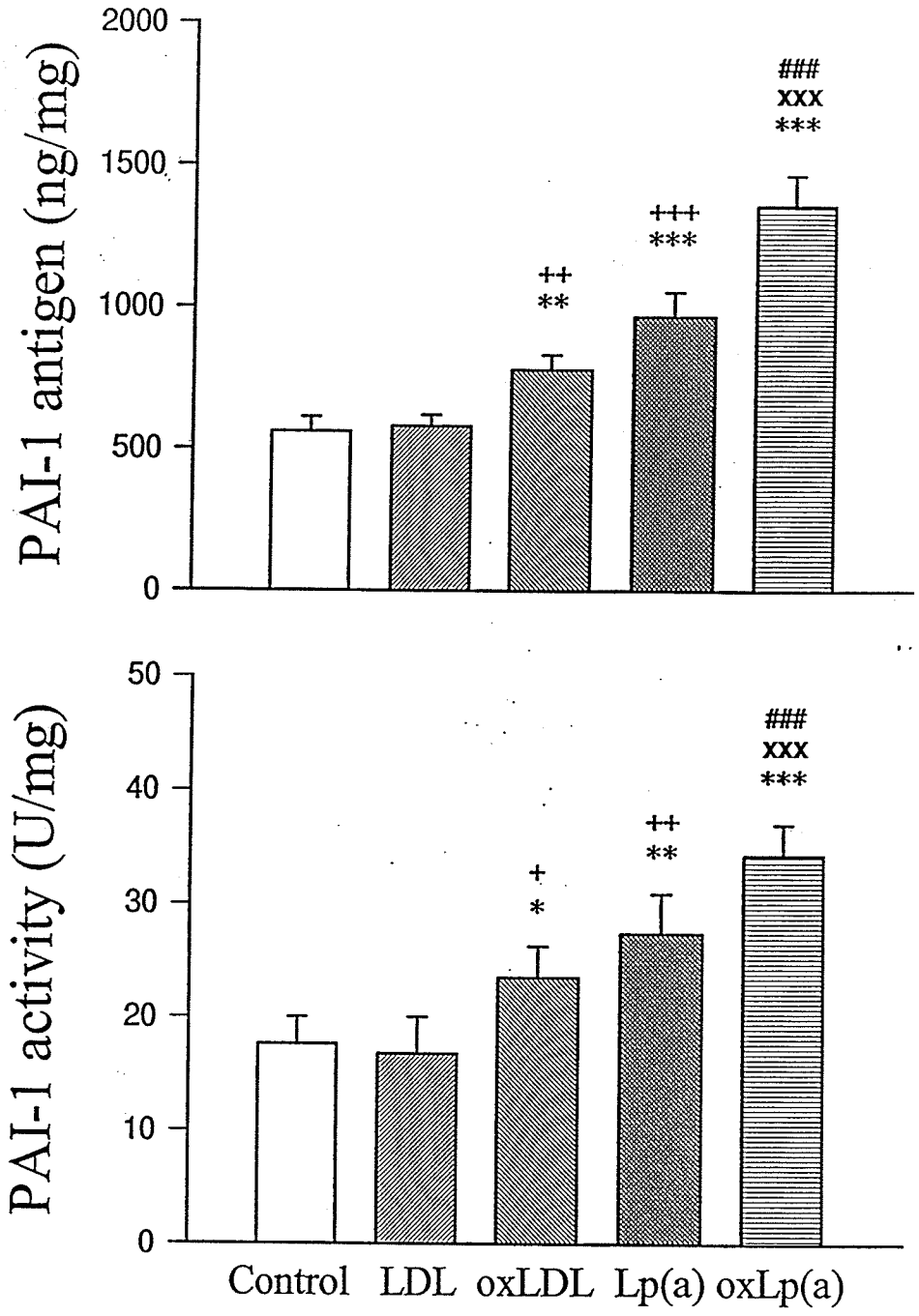


Fig.6. Comparison of PAI-1 antigen and activity levels in the conditioned media of HUVEC treated with various lipoproteins. HUVEC were incubated with control medium (Control) or medium containing 10 nM native Lp(a)-free LDL (LDL), oxidized Lp(a)-free LDL (oxLDL), native Lp(a) (Lp(a)) or oxidized Lp(a) (oxLp(a)) for 48 h. Total PAI-1 antigen was analyzed by ELISA with monoclonal antibody against human PAI-1 (top). PAI activity was estimated by a bioassay using chromogenic substrate S-2390 (bottom). Values are expressed in mean ± SD (n=4). *,**,***: $p<0.05$, <0.01 or <0.001 versus control; +,++,+++: $p<0.05$, <0.01 or <0.001 versus LDL; xxx: $p<0.001$ versus oxLDL; ###: $p<0.001$ versus Lp(a).

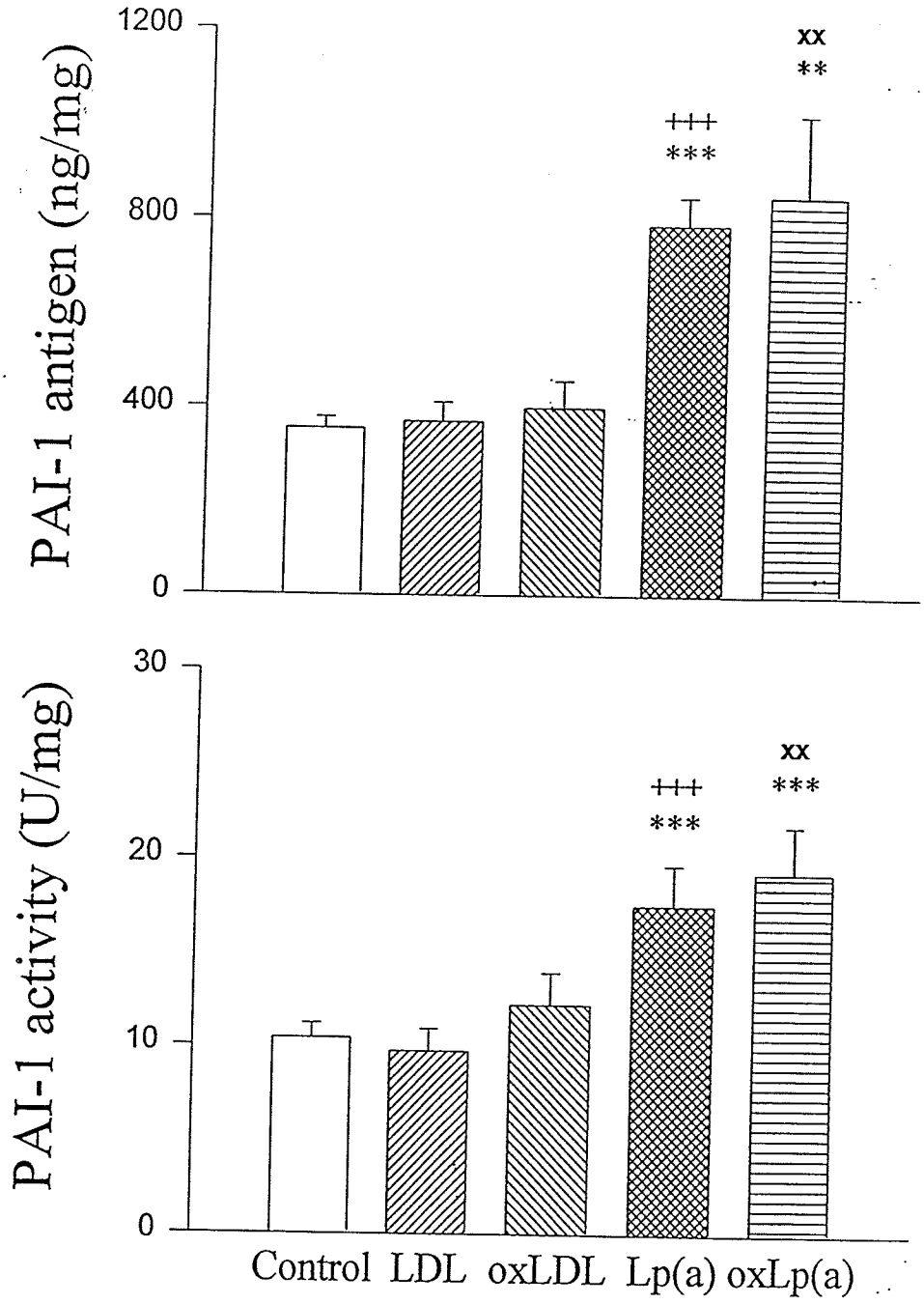


Fig.7. Comparison of the levels of PAI-1 antigen and activity in a cell-associated pool of HUVEC treated with various lipoproteins. Cells were treated with control medium (Control) or medium containing 10 nM native Lp(a)-free LDL (LDL), oxidized Lp(a)-free LDL (oxLDL), native Lp(a) (Lp(a)) or oxidized Lp(a) (ox-Lp(a)) for 48 h. Cells were lysed in PBS containing 0.1% SDS. The procedures for measuring PAI-1 antigen (top) and activity (bottom) are the same as described in the legend of Fig.4. Values are expressed in mean ± SD (n=4). **,***: $p<0.01$ or <0.001 versus control; +++: $p<0.001$ versus LDL; xx: $p<0.01$ versus oxLDL.

oxidized Lp(a) at the same concentration significantly increased cell-associated PAI-1 antigen (by 122%-140%) and activity (by 68%-85%) compared to control cultures, but no significant difference was found between cells treated with native and oxidized Lp(a) (Fig.7).

2.3 Effects of lipoproteins on PAI-1 accumulation in cell-associated pool.

To verify the finding on the effect of Lp(a) and its oxidized form on PAI-1 accumulation in cell-associated pool, HUVEC were metabolically labelled with ^{35}S-methionine. The subsequent treatment with 10 nM native Lp(a) increased PAI-1 in cell-associated pool by 2.1-fold compared to control cultures. Cell-associated PAI-1 in oxidized Lp(a) (10 nM) treated EC was 2.3-fold higher than control cultures. The apparent molecular mass of cell-associated PAI-1 was around 46 kDa. This implies that the cell-associated PAI-1 in EC was intact PAI-1. Native and oxidized LDL did not affect the levels of cell-associated PAI-1 (Fig.8.).

2.4 Dose-dependence of native and oxidized Lp(a) on PAI-1 mRNA in HUVEC

In unstimulated condition, 3.4 kb PAI-1 mRNA in HUVEC is more abundant than the 2.4 kb species. Native Lp(a) dose-dependently increased the levels of 2.4 kb PAI-1 mRNA but not that of 3.4 kb PAI-1 mRNA in HUVEC following a 48 h of incubation. The levels of both 3.4 kb and 2.4 kb PAI-1 mRNA were increased in HUVEC treated with oxidized Lp(a). The maximal effects of native and oxidized Lp(a) achieved in the cells treated with above 10-20 nM oxidized Lp(a) (Fig.9).

2.5 Time-dependence of native and oxidized Lp(a) on PAI-1 mRNA level in HUVEC

In the cultures treated with 10 nM native or oxidized Lp(a) for 12 h, no increase in PAI-1 mRNA was detected. Twenty-four hours of treatment with 10 nM native Lp(a) induced

a mild increase of 2.4 kb PAI-1 mRNA without a detectable change in 3.4 kb PAI-1 mRNA. A prominent increase in 3.4 kb PAI-1 mRNA associated with an elevation of 2.4 kb PAI-1 mRNA was found in HUVEC treated with 10 nM oxidized Lp(a) for 24 h. PAI-1 mRNA levels were further increased in HUVEC treated with native and oxidized Lp(a) for 48 h (Fig.10). Following a 72 h of treatment of native or oxidized Lp(a), considerable amounts of cells detached from culture dishes.

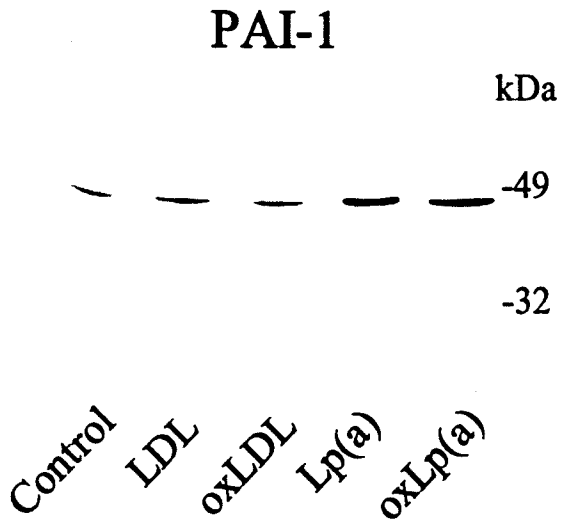


Fig.8. Cell-associated PAI-1 in HUVEC treated with native and oxidized LDL or Lp(a). HUVEC were labelled with ^{35}S-methionine for 20 h in methionine-free medium then incubated for 48 h with medium without addition (Control) or medium containing 10 nM native LDL (LDL), oxidized LDL (oxLDL), native Lp(a) (Lp(a)) or oxidized Lp(a) (oxLp(a)). At the end of the incubation, medium was removed and cells were rinsed with PBS. Cells were harvested with PBS containing 0.1% SDS. PAI-1 in cell lysates was immunoprecipitated with monoclonal antibody against PAI-1 then analyzed on 4.5-18% SDS-PAGE. Radioactivity was visualized by autoradiography.

2.6 Effects of lipoproteins on PAI-1 mRNA in HUVEC.

Native LDL at 10 nM did not obviously alter the levels of PAI-1 mRNA in HUVEC. Oxidized LDL treatment at the same concentration moderately but significantly increased both 3.4 kb and 2.4 kb PAI-1 mRNA levels compared to native Lp(a)-free LDL ($p<0.01$). The mean value of the 2.4 kb PAI-1 mRNA in 10 nM native Lp(a) treated HUVEC was 2.8-fold compared to control ($p<0.01$) without an increase in the 3.4 kb species. In oxidized Lp(a)-treated HUVEC, the levels of 3.4 kb PAI-1 mRNA was over 3-fold compared to control and native Lp(a)-treated EC ($p<0.01$). The levels of 2.4 kb PAI-1 mRNA in EC treated with oxidized Lp(a) were increased 3-fold compared to control cultures ($p<0.01$) but no significant difference was found in comparison to native Lp(a)-treated cells (Fig.11 A and B).

The results of this section were described in a paper accepted by Atherosclerosis and in press (Ren et al. 1996a).

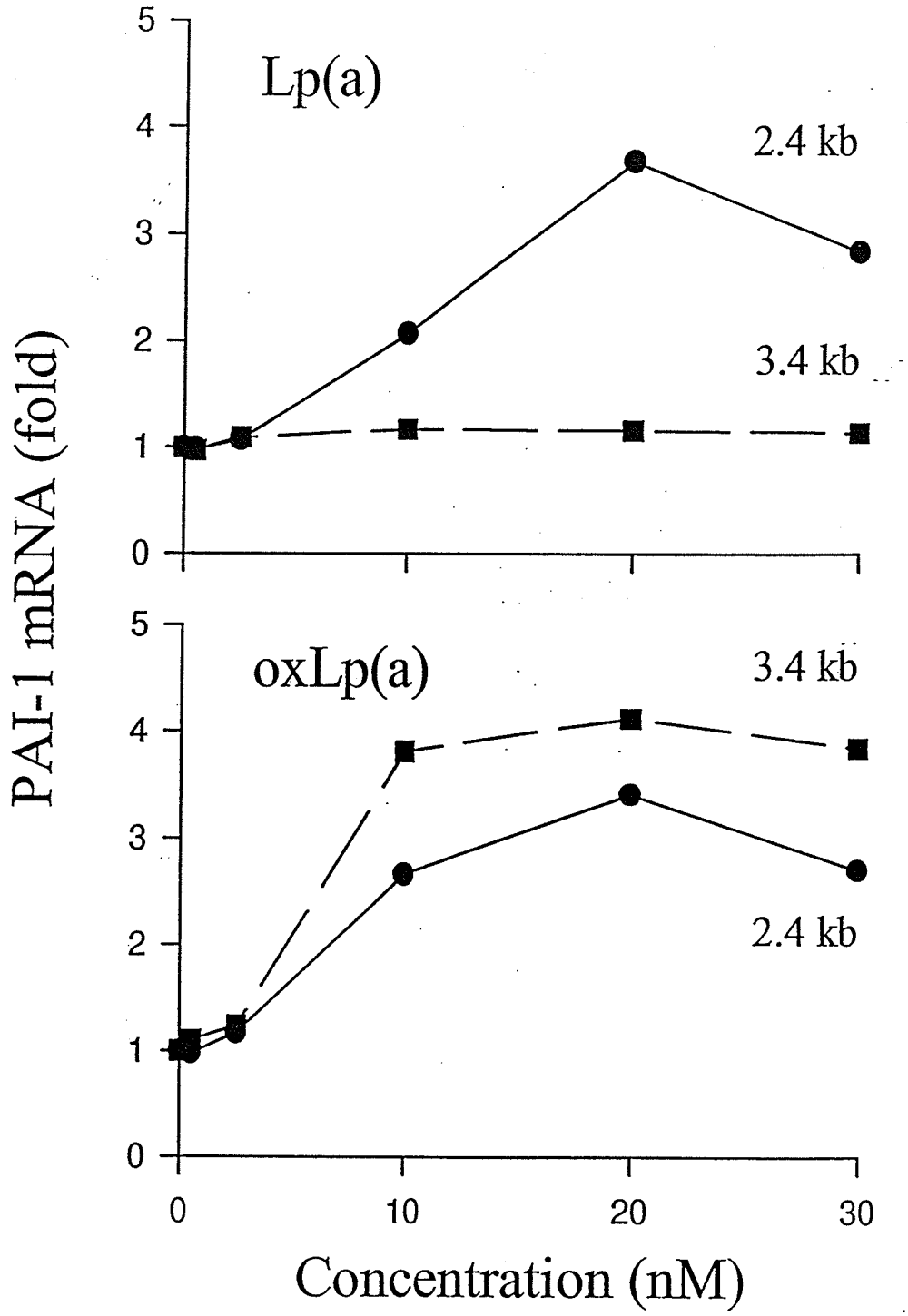


Fig.9. Dose-dependence of native and oxidized Lp(a) on PAI-1 mRNA levels in HUVEC. HUVEC were incubated with 0.5-30 nM Lp(a) for 48 h. Fold increases in 3.4 kb and 2.4 kb PAI-1 mRNA induced by Lp(a) were quantified by densitometer and corrected with β-actin mRNA levels. Values presented in the figure are averages from two separate experiments. Top: native Lp(a); bottom: oxidized Lp(a).

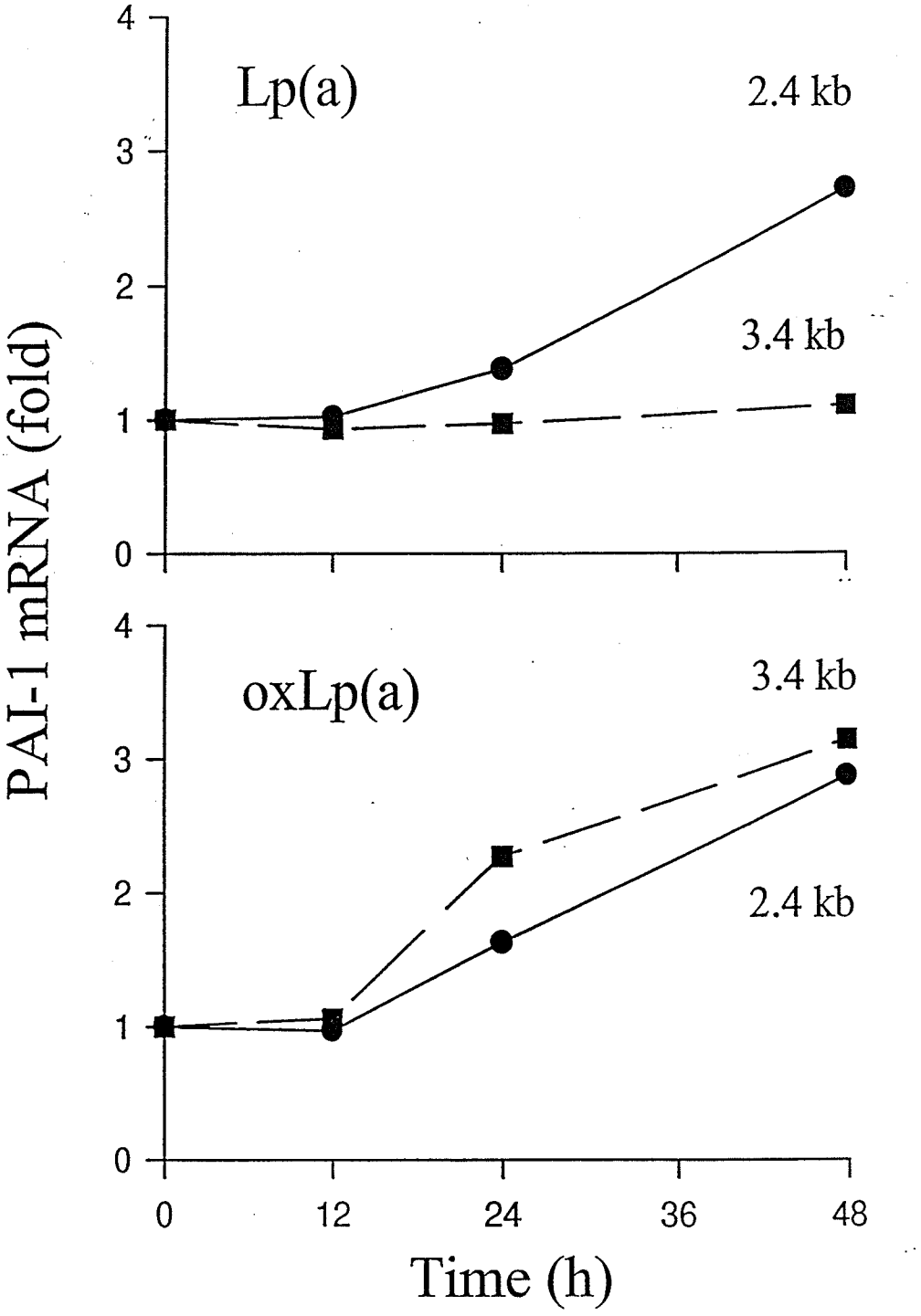
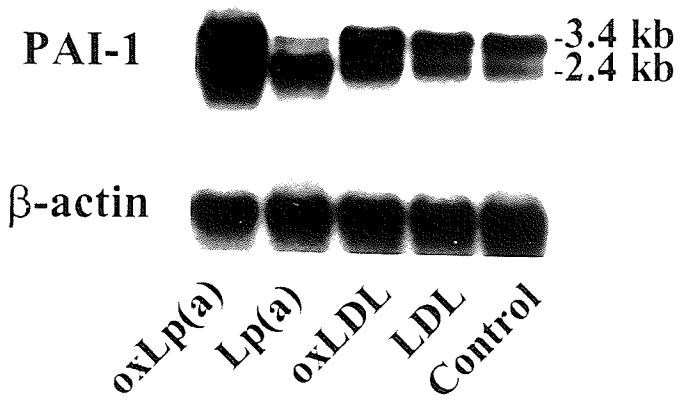
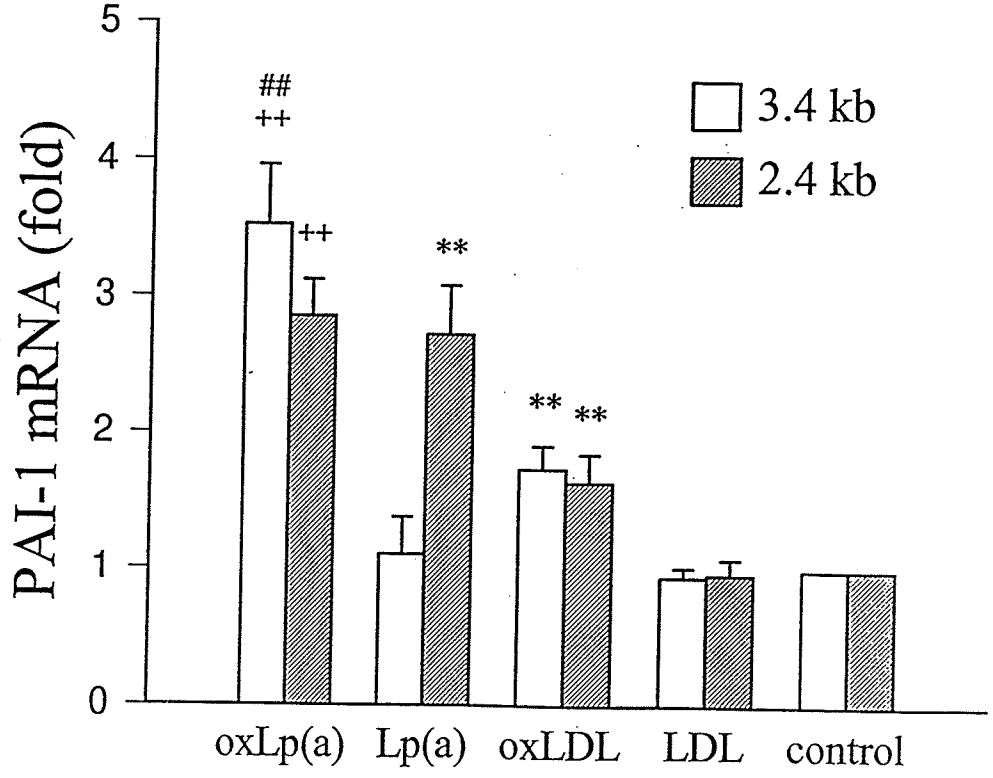


Fig.10. Time-dependence of native and oxidized Lp(a) on PAI-1 mRNA level in HUVEC. HUVEC were treated by 10 nM native Lp(a) for 12-48 h. Values presented in the figure are averages from two separate experiments. Top: native Lp(a); bottom: oxidized Lp(a)

Fig.11. PAI-1 mRNA in HUVEC treated with native and oxidized Lp(a) or LDL. HUVEC were treated with medium without addition (control) or containing 10 nM native Lp(a)-free LDL (LDL), oxidized Lp(a)-free LDL (oxLDL), native Lp(a) (Lp(a)) or oxidized Lp(a) (oxLp(a)) for 48 h. Upper: Northern blot of PAI-1 mRNA: total RNA (20 μg/lane) on 1% agarose-formamide gel was transferred to nitrocellulose membrane then hybridized with ^{32}P-dCTP labelled human PAI-1 cDNA. The sizes of mRNA species was determined by lamda HindIII standards by using ethidium bromide-staining. Bottom: Fold increase in PAI-1 mRNA levels: fold-increase in 3.4 and 2.4 kb PAI-1 mRNA levels in HUVEC stimulated as described above were quantified by using densitometry and corrected with β-actin mRNA levels. Values are expressed as mean ± SE, n=5. **: $p<0.01$ versus LDL; ++: $p<0.01$ versus oxLDL; ##: $p<0.01$ versus Lp(a).


PAI-1
-3.4 kb
-2.4 kb
β-actin
oxLp(a)
Lp(a)
oxLDL
LDL
Control


PAI-1 mRNA (fold)
5
4
3
2
1
0
##
++
++
**
** **
3.4 kb
2.4 kb
oxLp(a)
Lp(a)
oxLDL
LDL
control

3. INVOLVEMENT OF SIGNAL TRANSDUCTION PATHWAY IN THROMBIN-INDUCED PAI-1 PRODUCTION IN VASCULAR SMC.

3.1 Involvement of thrombin receptor :

The role of thrombin receptor in thrombin-induced PAI-1 production in BASMC was investigated. Treatment of BASMC with thrombin receptor activating peptide (SFLLRN) at 11 μM effectively elevated PAI-1 mRNA levels from BASMC (Fig.12). α-Thrombin at the levels of 10 U/ml or 0.11 μM (3 hour treatment) induced a substantial increase in PAI-1 mRNA compared to the cultures without addition (control). γ-Thrombin at an equimolar concentration (0.11 μM) did not increase PAI-1 mRNA level. Moderate increase in PAI-1 mRNA levels was detected in BASMC treated with 5-10 times higher concentrations of γ-thrombin.

3.2 Requirement of enzymatic activity:

Requirement of enzymatic activity for thrombin-induced PAI-1 synthesis was evaluated by using enzymatically inactivated thrombin. Pre-treatment of thrombin with hirudin, a specific thrombin inhibitor, completely blocked the effect of thrombin-induced PAI-1 mRNA in BASMC. The effect of thrombin on PAI-1 mRNA was eliminated by pre-treatment of thrombin with protease inhibitors, amidinophenylmethanesulfonyl fluoride (APMSF) or D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Fig.13)

3.3 Involvement of G protein:

Thrombin-induced PAI-1 antigen was reduced by 85% in BASMC pre-treated with 100 ng/ml of pertussis toxin for 5 h. The levels of PAI-1 mRNA in BASMC stimulated

with 10 U/ml of thrombin were reduced by 63% following pre-treatment with pertussis toxin. Matching doses of pertussis toxin alone did not affect PAI-1 antigen or mRNA levels in BASMC (Fig.14a). Sodium fluoride (NaF), a G-protein agonist, greatly elevated PAI-1 antigen levels (Fig.14b). Combination of the results suggest that a G protein is involved in thrombin-induced PAI-1 synthesis in BASMC.

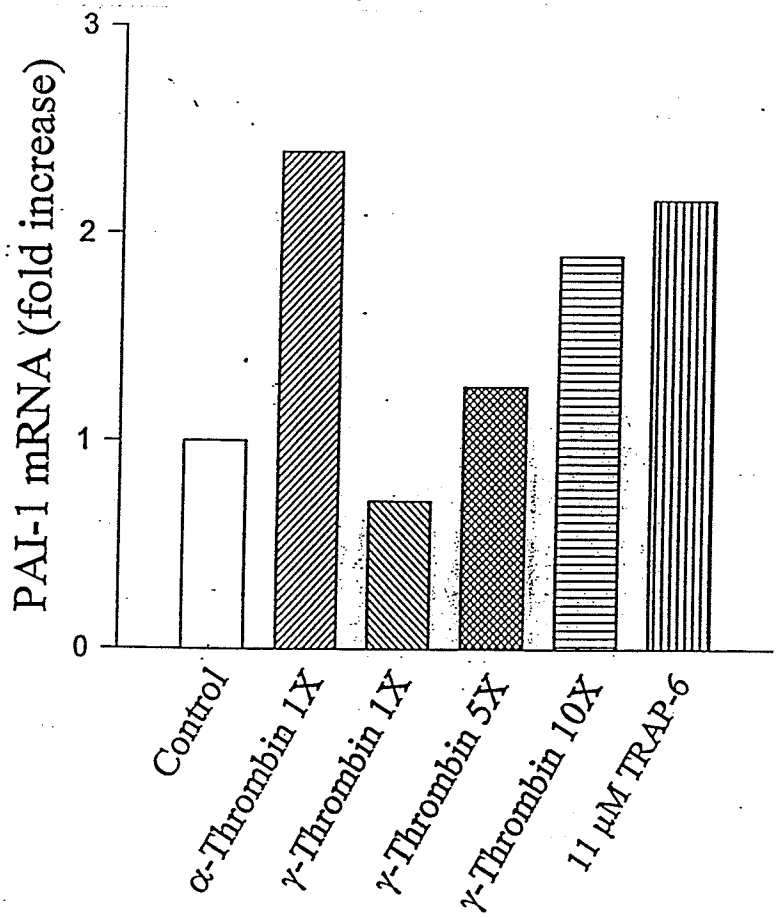


Fig.12. Comparison of α-thrombin, γ-thrombin and thrombin receptor activating peptide (TRAP) on PAI-1 mRNA in BASMC. Confluent BASMC were incubated with the medium without addition (control), with 0.11 μM α-thrombin (1X), 0.11 μM (1X), 0.55 μM (5X), 1.1 μM γ-thrombin (10X) or 11 μM TRAP for 3 h. Northern analysis was performed as described in the legend of Fig.4. Upper panel: PAI-1 mRNA; bottom: β-actin.

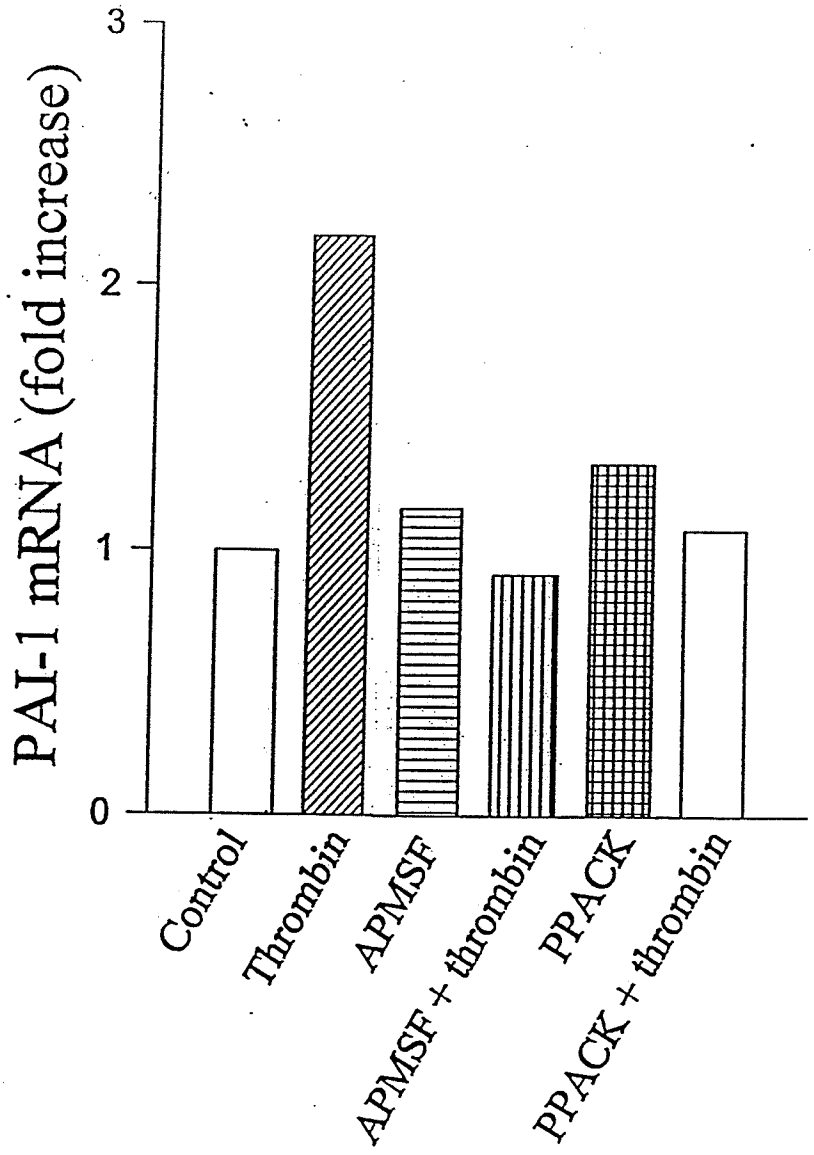


Fig.13. Effect of non-specific protease inhibitors on the thrombin-induced PAI-1 mRNA in BASMC. Thrombin was pretreated by hirudin, amidinophenylmethanesulfonyl fluoride (APMSF) or D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) as described in the methods. BASMC were incubated with medium without addition (control), 10 U/ml of thrombin, 10 U/ml thrombin pre-treated with hirudin, APMSF or PPACK, or the medium contain equivalent amounts of the inhibitors for 3 h. Northern analysis was performed as described in the legend of Fig.4. Upper panel: PAI-1 mRNA; bottom: β-actin.

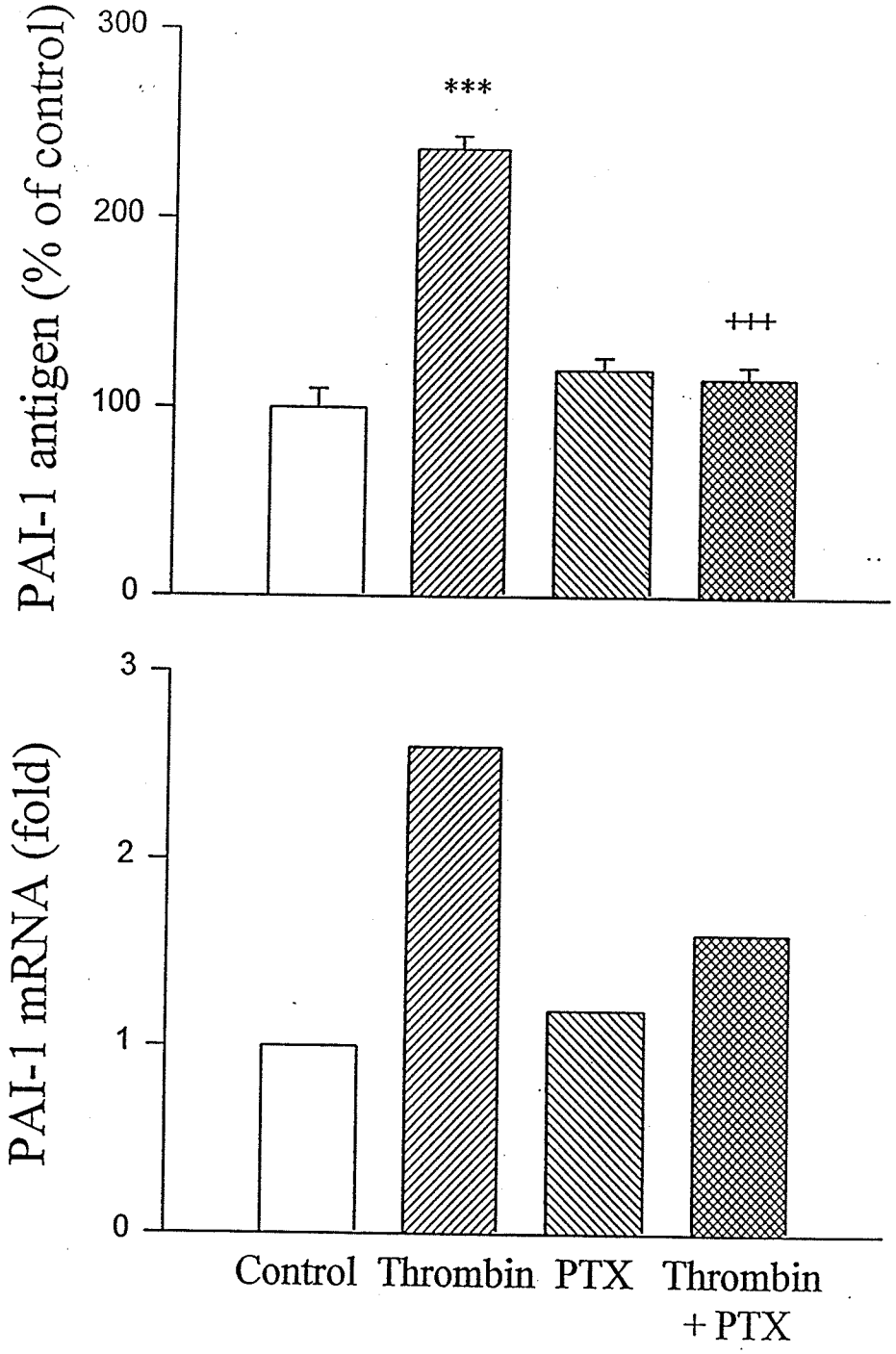


Fig.14a. Effect of pertussis toxin (PTX) on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were pre-treated with medium without addition (control), 10 U/ml of thrombin for 3 h, 100 ng/ml of PTX, 100 ng/ml of PTX plus 10 U/ml of thrombin. The procedures for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. ***: $p<0.001$ versus controls; +++: $p<0.001$ versus thrombin treated cells.

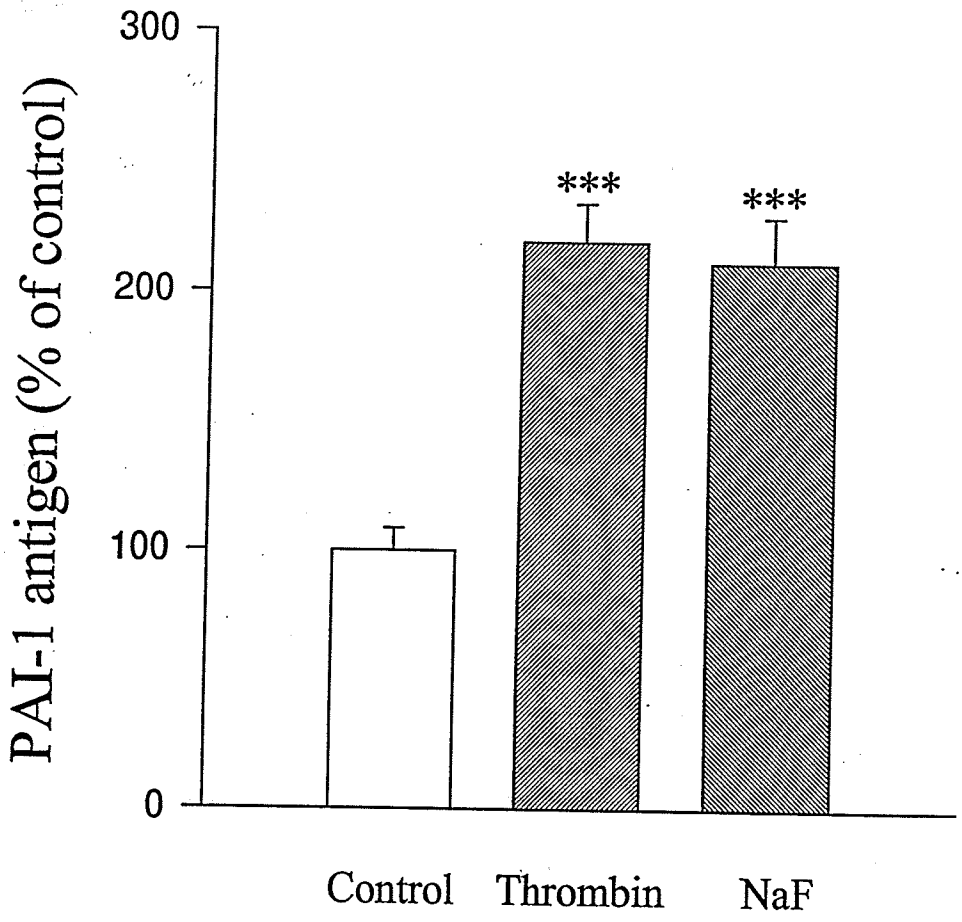


Fig.14b. Effect of sodium fluoride (NaF) on PAI-1 production in BASMC.

Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin or 10 μM NaF for 3 h. The procedures for analyses of PAI-1 antigen (n=4, mean ± SD) were the same as described in the legend of Fig.1. ***: $p<0.001$ versus controls.

3.4 Possible involvement of tyrosine kinase:

Thrombin-stimulated PAI-1 release from BASMC was completely inhibited by 10

μg/ml of genistein, a potent inhibitor of tyrosine kinase ($p<0.001$). Genistein at the same concentration moderately reduced the release of PAI-1. Tyrphostin 25 (25 μM), a specific inhibitor for tyrosine kinase significantly reduced thrombin-induced increase of PAI-1 antigen. Its structural homologue, tyrphostin 1 (25 μM), had no effect on thrombin-induced PAI-1 production (Fig.15). These results suggest that tyrosine kinase may be involved in thrombin-induced PAI-1 production in BASMC.

3.5 Involvement of phospholipase C (PLC):

Neomycin (5 mM), an inhibitor for PLC and PLD, significantly reduced thrombin-induced increase of PAI-1 antigen in the conditioned medium of BASMC but not the basal levels of PAI-1 release. Treatment with neomycin completely inhibited thrombin-induced increase of PAI-1 mRNA levels in BASMC. Neomycin alone did not affect basal levels of PAI-1 mRNA (Fig.16a). U73122 (10 μM), a specific inhibitor for PLC, significantly reduced thrombin-induced increase of PAI-1 antigen, but not the basal level of PAI-1 release. Its structural homologue, U73343 (10 μM), had no effect on thrombin-induced PAI-1 production (Fig.16b). These results implied that PLC may also be involved in thrombin-induced PAI-1 production.

3.6 Involvement of protein kinase C (PKC):

Phorbol myristate acetate (PMA), a potent PKC agonist, at 10 nM (3 h treatment) greatly elevated PAI-1 mRNA levels (Fig. 17, first lane from right). Calphostin C, a specific PKC inhibitor, at 1 μM concentration, inhibited thrombin-induced PAI-1 antigen and mRNA levels in BASMC. Calphostin C alone suppressed basal levels of PAI-1 antigen and mRNA (Fig.18). These results suggest that PKC is required for PAI-1 production in BASMC at basal and thrombin stimulated conditions.

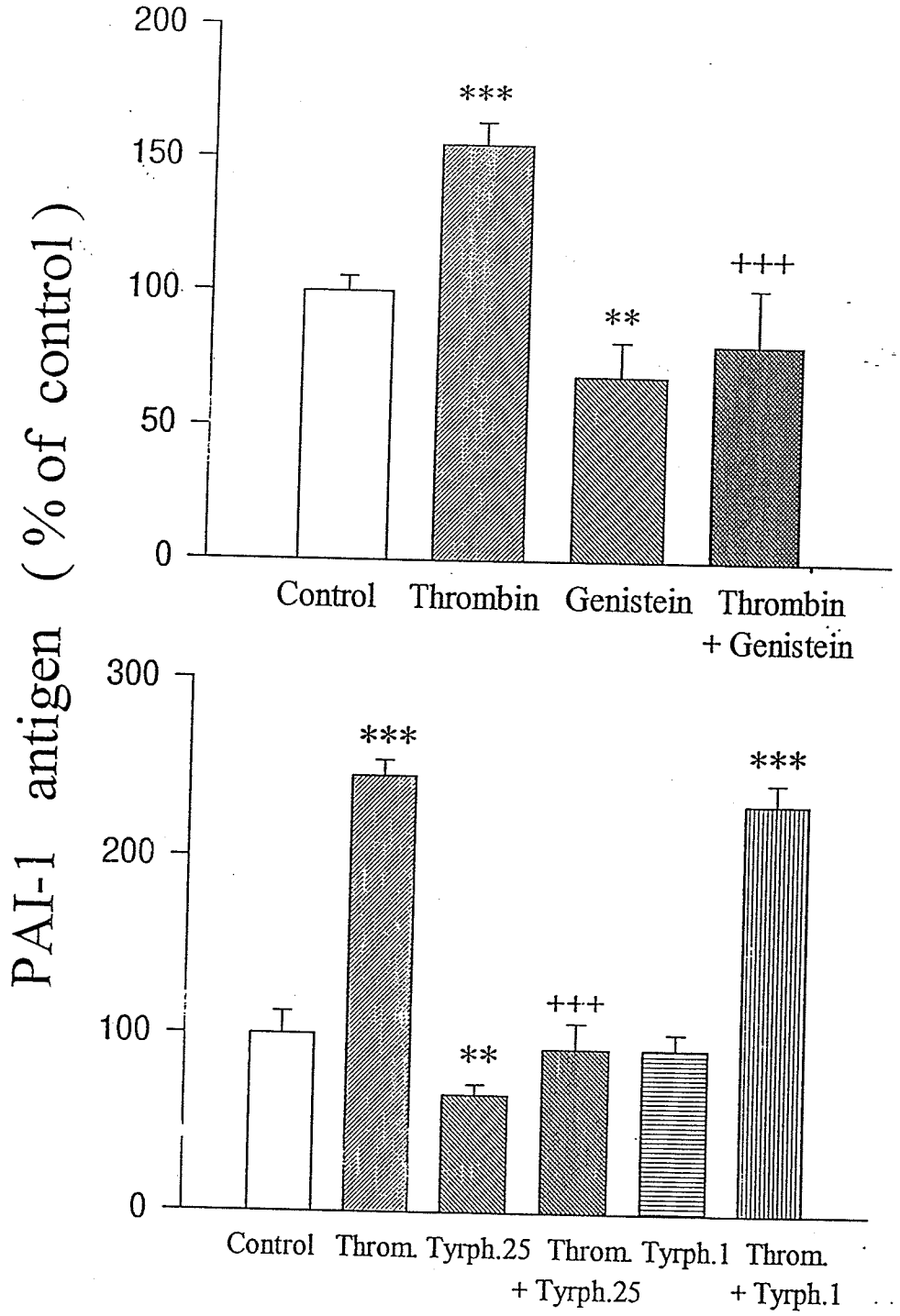


Fig.15. Effect of genistein on thrombin-induced PAI-1 production from BASMC. Upper: Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 10 μg/ml of genistein or 10 U/ml of thrombin plus 10 μg/ml of genistein for 3 h. Bottom: BASMC were treated with control medium, 10 U/ml of thrombin, 25 μM tyrphostin 25, 10 U/ml of thrombin plus 25 μM tyrphostin 25, 25 μM tyrphostin 1 or 10 U/ml of thrombin plus 25 μM tyrphostin 1 for 3 h. The procedures for analyses of PAI-1 antigen (n=4, mean ± SD) were the same as described in the legend of Fig.1. **,***: $p<0.01$ or 0.001 versus controls; +++: $p<0.001$ versus thrombin treated cells.

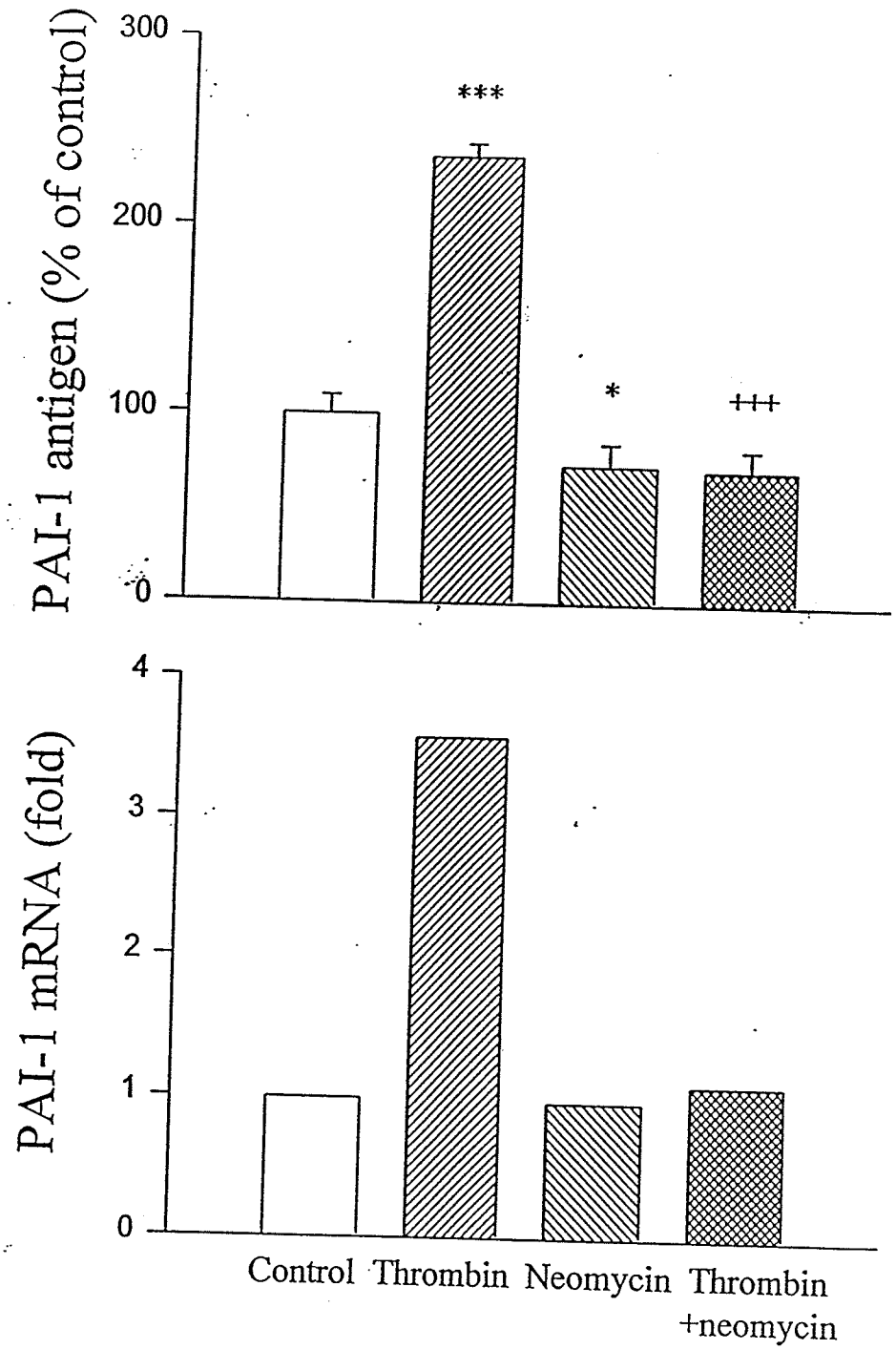


Fig.16a. Effect of neomycin on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin for 3 h, 5 mM neomycin or 10 U/ml of thrombin plus 5 mM neomycin. The procedures for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. *,***: $p<0.05$ or <0.001 versus controls; +++: $p<0.001$ versus thrombin treated cells.

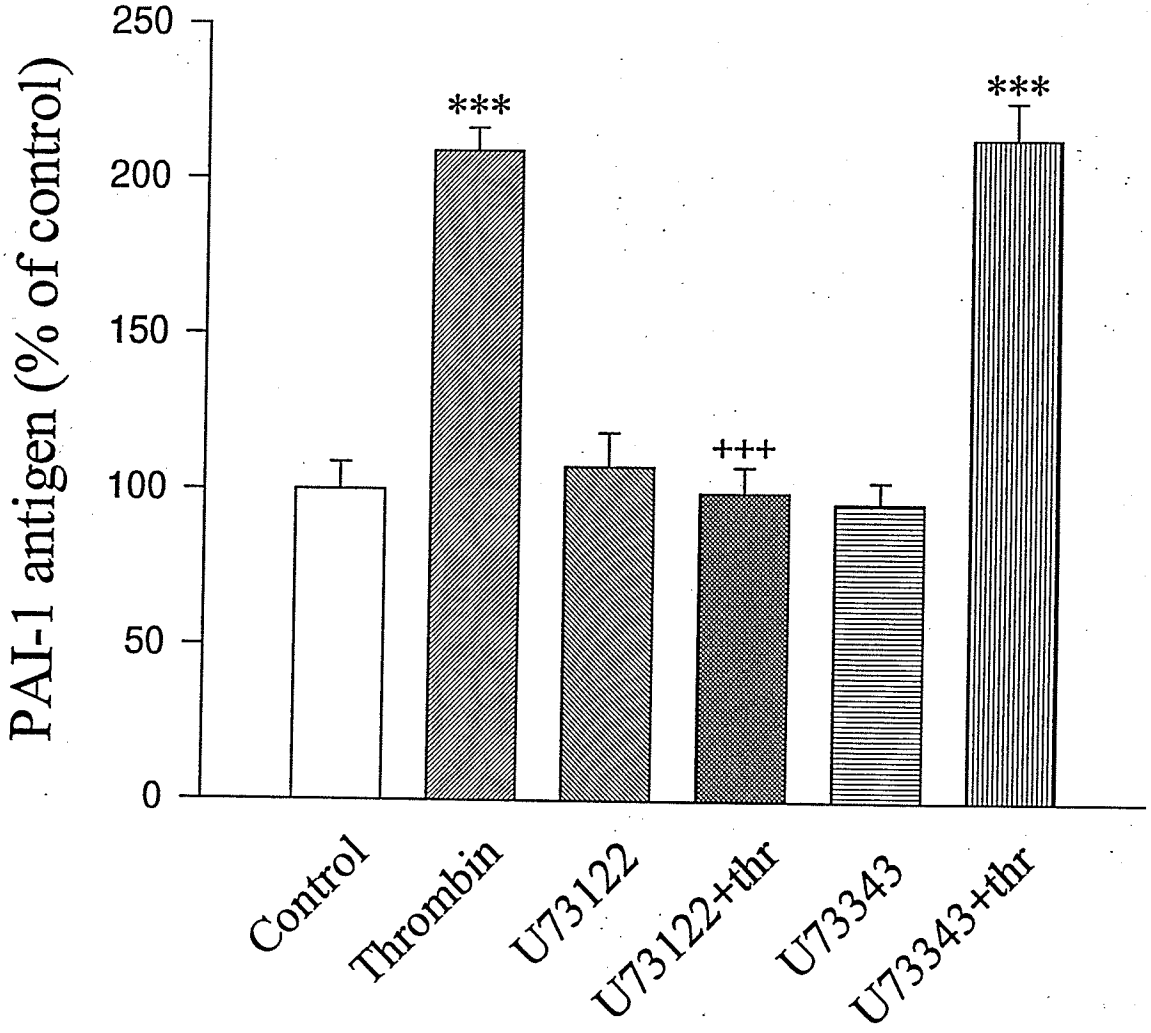


Fig.16b Effect of U73122 and U73343 on thrombin-induced PAI-1 production in BASMC. BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 10 μM U73122, 10 U/ml of thrombin plus 10 μM U73122 (U73122+thr), 10 μM U73343 or 10 U/ml of thrombin plus 10 μM U73343 (U73343+thr). The procedures for analyses of PAI-1 antigen (n=4, mean ±SD) were the same as described in the legend of Fig.1. ***: $p<0.001$ versus controls; +++: $p<0.001$ versus thrombin treated cells.

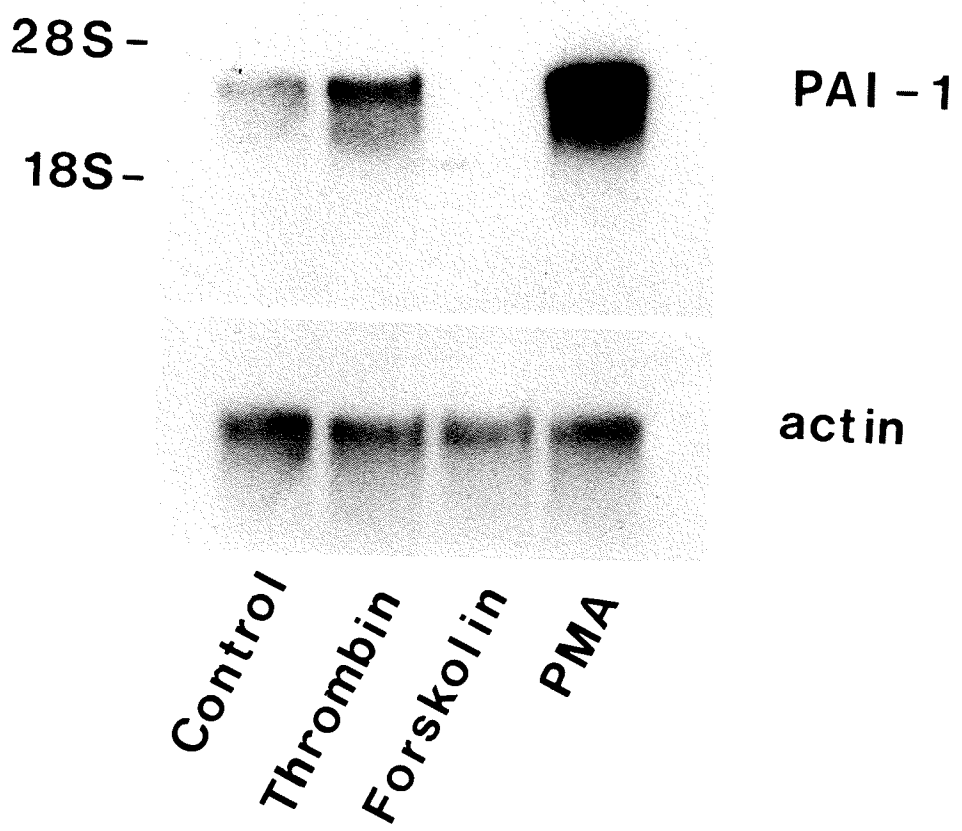


Fig.17. Phorbol myristate acetate (PMA) and forskolin on PAI-1 mRNA in BASMC. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 100 μM forskolin or 10 nM PMA for 3 h. Northern analysis was performed as described in the legend of Fig.2. Upper panel: PAI-1 mRNA; bottom: β-actin.

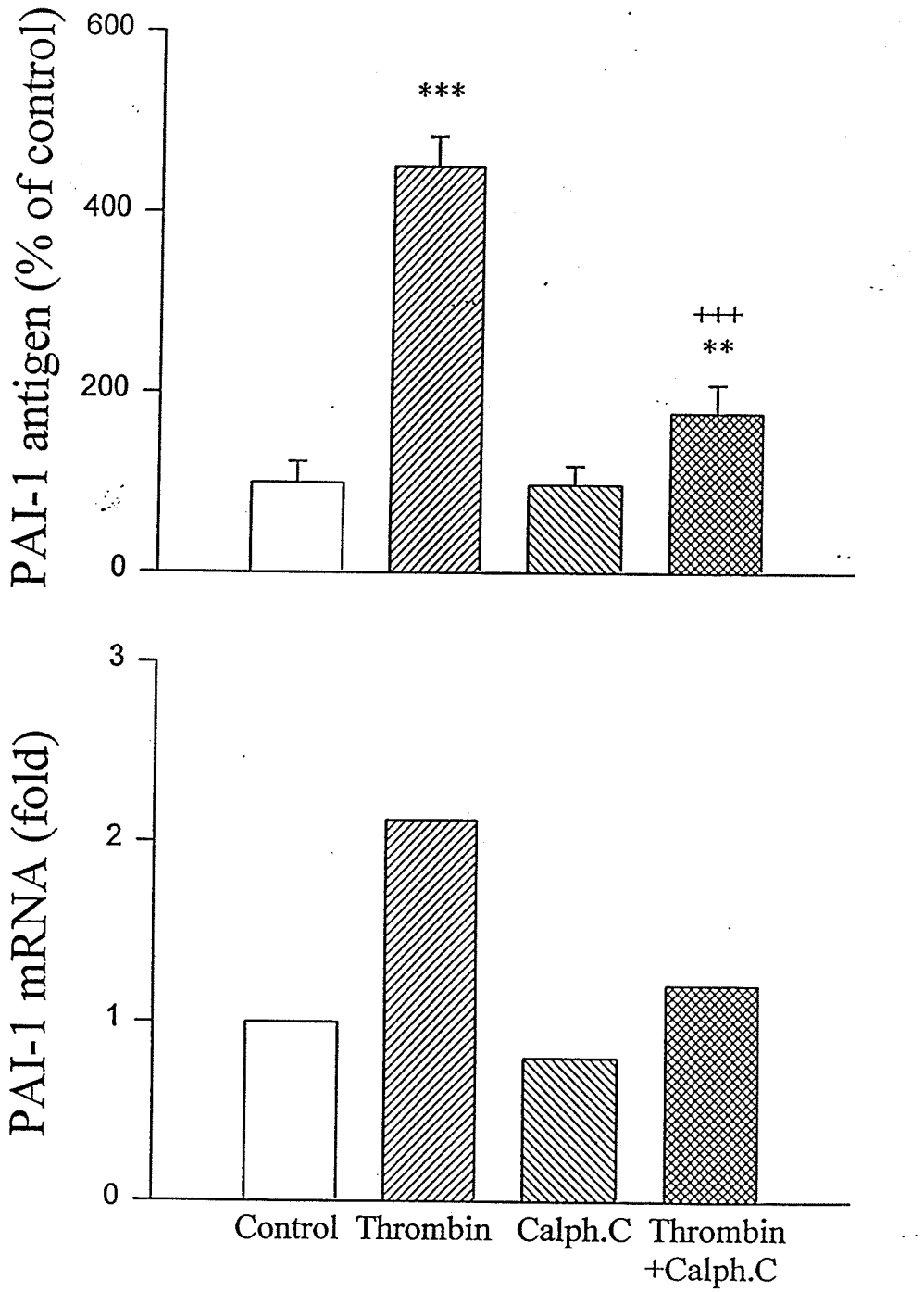


Fig. 18. Effect of calphostin C on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were incubated with medium without addition (control), 10 U/ml of thrombin, 1 μM calphostin C (Calph.C) or 10 U/ml of thrombin plus 1 μM calphostin C (cells pre-treated with calphostin C for 30 min) for 3 h. The procedures for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. **,***: $p<0.01$ or 0.001 versus controls; +++: $p<0.001$ versus thrombin treated cells.

3.7 Involvement of cAMP-dependent pathway:

Forskolin (100 μM), an adenylate cyclase agonist, reduced basal level of PAI-1 mRNA in BASMC to undetectable level (Fig. 12, second lane from right). 8-Bromo-cAMP (100 μM), a cAMP structural analogue, was applied to verify the effect of elevated cAMP on thrombin-induced PAI-1 production. Addition of 8-bromo-cAMP alone reduced 45% of basal PAI-1 mRNA. The basal level of PAI-1 antigen was slightly but not significantly reduced by 8-bromo-cAMP. Thrombin-induced PAI-1 release and the increase of PAI-1 mRNA were effectively inhibited by the treatment with 8-bromo-cAMP (Fig. 19). These results suggest that increased intracellular cAMP level may negatively regulate basal and thrombin-induced PAI-1 production.

3.8 Involvement of cGMP-dependent pathway:

8-bromo-cGMP alone has no effect on PAI-1 antigen level. Zaprinast, a cGMP-dependent phosphodiesterase inhibitor, reduces the metabolism of cGMP and maintains the levels of cGMP in cells. Addition of 8-bromo-cGMP with zaprinast significantly inhibited thrombin-induced PAI-1 production (Fig.20). Addition of 8-bromo-cGMP effectively reduced basal and thrombin-induced PAI-1 mRNA (Fig.21). These results suggest that the maintaince of cGMP level in cells is required for a significant increase in the generation of PAI-1 from BASMC. Continuous increase in intracellular cGMP levels may reduce PAI-1 production in BASMC.

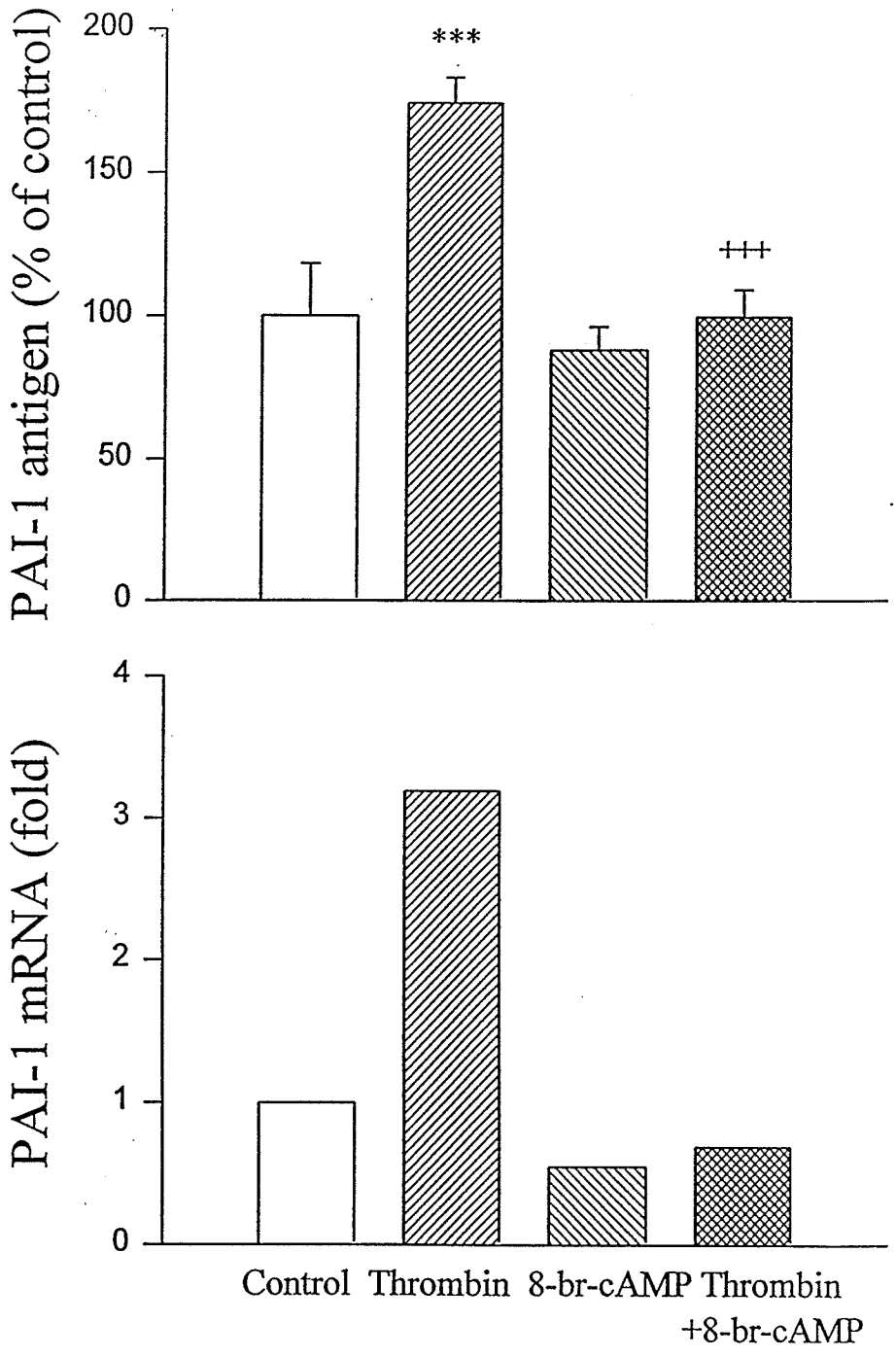


Fig.19. Effect of 8-bromo-cAMP on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 100 μM 8-bromo-cAMP (8-br-cAMP), 10 U/ml of thrombin plus 100 μM 8-br-cAMP for 3 h. The procedure for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. ***: $p<0.001$ versus controls; +++: $p<0.001$ versus thrombin-treated cells.

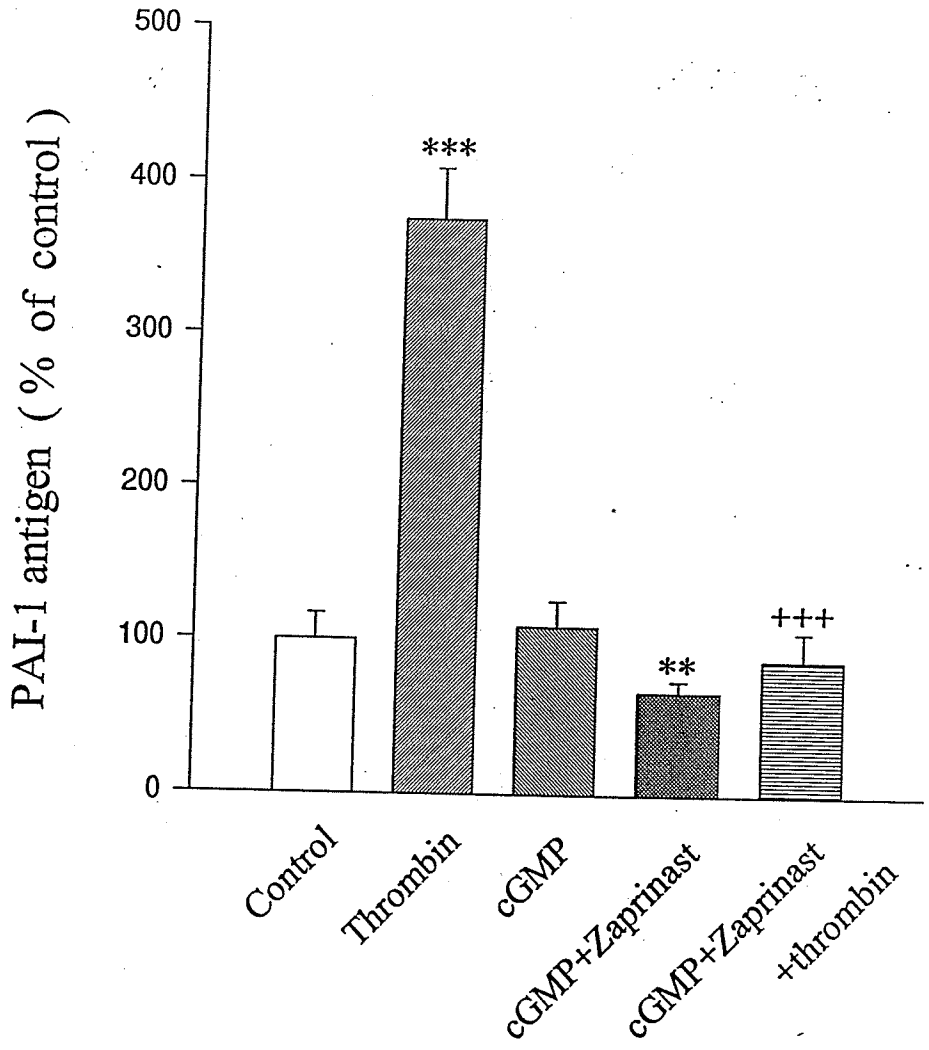


Fig.20. Effect of 8-bromo-cGMP on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 100 μM 8-bromo-cGMP (cGMP), 10 U/ml of thrombin plus 10 μM Zaprinast, 10 U/ml of thrombin plus 100 μM 8-br-cGMP and 10 μM Zaprinast for 3 h. The procedures for analyses of PAI-1 antigen (n=4, mean ± SD) were the same as described in the legend of Fig.1. **,***: $p<0.01$ or <0.001 versus controls; +++: $p<0.001$ versus thrombin treated cells.

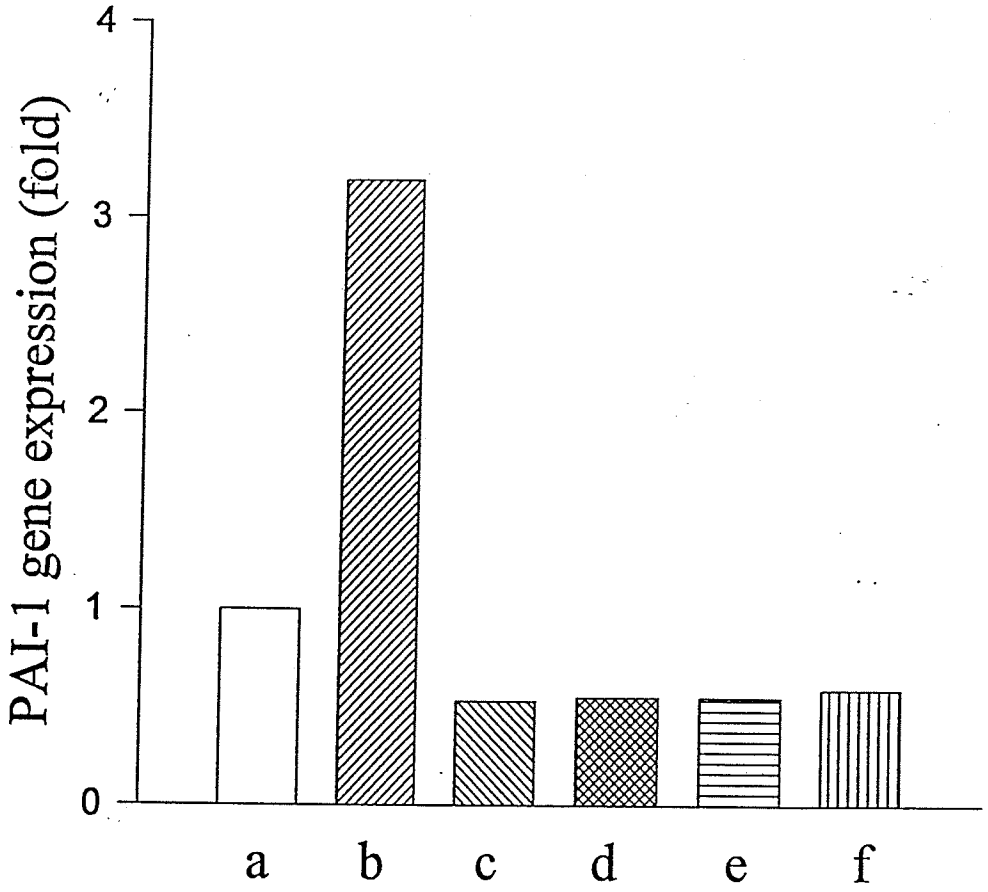


Fig.21. Effect of forskolin and 8-bromo-cGMP on thrombin-induced PAI-1 mRNA in BASMC. Confluent BASMC were incubated with the medium without addition, with 10 U/ml of thrombin, 100 μM forskolin plus 10 U/ml of thrombin, 100 μM 8-bromo-cGMP or 100 μM 8-bromo-cGMP plus 10 U/ml of thrombin for 3 h. The procedures for Northern blotting and quantitative analysis of PAI-1 mRNA were the same as described in the Fig.4.

3.9 Effect of interleukin 1α (IL-1α): Treatment with 10 U/ml of IL-1α achieved the maximal release of PAI-1 antigen from BASMC. The levels of PAI-1 antigen in the conditioned medium of BASMC treated with a combination of thrombin and IL-1α at their maximal stimulation conditions were significantly higher than that treated with thrombin or IL-1α alone (Table 2). This suggests that thrombin induces additional production of PAI-1 beyond the effect of IL-1α in BASMC.

Table 2. Effect of thrombin and IL-1α on PAI-1 generation from BASMC

Additions	PAI-1 antigen (ng/mg protein)
No addition	12.1 ± 2.9
Thrombin (5 U/ml)	39.7 ± 3.2 ***
Thrombin (10 U/ml)	52.3 ± 3.8 ***
IL-1α (5 U/ml)	28.0 ± 4.0 **
IL-1α (10 U/ml)	42.4 ± 4.7 ***
Thrombin (5 U/ml)+IL-1α (5 U/ml)	51.3 ± 3.7 ++,###
Thrombin (10 U/ml)+IL-1α (10 U/ml)	65.7 ± 7.0 +, xx

Baboon aortic SMC were treated with indicated amounts of thrombin or interleukin 1α (IL-1α) or medium without addition for 3 h. The levels of PAI-1 antigen in the conditioned medium were measured by ELISA. Values are expressed in mean ± SD (n=4). *, **, ***: $p<0.05$, <0.01 or <0.001 versus no addition control. ++: $p<0.01$ or <0.001 versus 5 U/ml of thrombin. ###: $p<0.001$ versus 5 U/ml of IL-1α. +: $p<0.05$ versus 5 U/ml of thrombin. xx: $p<0.01$ versus 10 U/ml of IL-1α.

3.10 Involvement of signal transduction pathways in thrombin-induced PAI-1 production in growth quiescent BASMC.

Effect of stimulators and inhibitors of signal transduction systems were examined in growth quiescent BASMC (Table 3). Similar effects were obsered as those in growing cells (see previous sections).

Table 3. Effect of stimulators and inhibitors on PAI-1 production in quiescent BASMC

Additions	PAI-1 antigen (ng/mg protein)
No addition	11.2 ± 1.0
Thrombin (10 U/ml)	24.7 ± 1.7 ***
TRAP (11 μM)	20.8 ± 1.3 ***
PMA (10 nM)	31.6 ± 2.2 ***
NaF (1 mM)	21.5 ± 1.4 ***
Calph.C (1 μM)	9.4 ± 0.6
Thr.(10 U/ml) + Calph.C (1 μM)	10.6 ± 0.8 +++
Genistein (10 μg/ml)	9.4 ± 0.7
Thr.(10 U/ml) + Gen.(10 μg/ml)	9.7 ± 0.7 +++
PTX (100 ng/ml)	9.8 ± 0.8
Thr.(10 U/ml) + PTX (100 ng/ml)	14.1 ± 1.1 +++
Neomycin (5 mM)	10.3 ± 1.0
Thr.(10 U/ml) + Neomycin (5 mM)	16.3 ± 1.1 +++
Forskolin (100 μM)	7.1 ± 0.4 *
Thr.(10 U/ml) + Forskolin (100 μM)	13.9 ± 1.3 +++
8-Br-cAMP (100 μM)	9.9 ± 0.9
Thr.(10 U/ml) + 8-Br-cAMP (100 μM)	11.8 ± 1.0 +++

Quiescent BASMC were obtained by feeding cells with medium without FBS for 48 hours. Then the cells were treated with medium containing 1% FBS with the additions of indicated amounts of thrombin, stimulators or inhibitors with or without thrombin for 3 h. The levels of PAI-1 antigen in the conditioned medium were measured by ELISA. Values are expressed in mean ± SD (n=4). *,***: $p<0.05$, 0.001 versus control. +++: $p<0.001$ versus 10 U/ml of thrombin. Calph.C: calphostin C. Thr.: thrombin. Gen.: genistein.

The results in this section were submitted to Journal of Vascular Research (paper in revision, Ren et al. 1996b).

4. PHARMACOLOGICAL MODULATION ON THROMBIN-INDUCED PAI-1 PRODUCTION IN BASMC

4.1 Effect of nitrovasodilator on thrombin-induced PAI-1 production:

Sodium nitroprusside (10 μM, 3 h treatment), which can increase intracellular cGMP level through nitric oxide (NO) pathway, significantly reduced thrombin-induced increase of antigen in the conditioned medium of BASMC but not the basal level of PAI-1 release. Treatment with nitroprusside completely inhibited thrombin-induced increase of PAI-1 mRNA levels in BASMC. Nitroprusside alone did not affect the basal level of PAI-1 mRNA. (Fig.22)

4.2 Dose response of nitroprusside on thrombin-induced PAI-1 production:

Treatments with nitroprusside (<5 μM) did not affect thrombin-induced PAI-1 release from BASMC. Nitroprusside at concentrations beyond 10 μM significantly inhibited thrombin-induced PAI-1 production ($p<0.001$). Nitroprusside (1-40 μM) alone did not alter PAI-1 antigen level. (Fig.23)

4.3 Time course of nitroprusside on thrombin-induced PAI-1 production:

Significant increases in PAI-1 antigen level ($p<0.01$) were detected in the conditioned media of BASMC treated with α-thrombin from 0.5 to 9 h compared to time-matched control cells. Up to 3 h, nitroprusside (10 μM) significantly inhibited thrombin-induced PAI-1 production ($p<0.01$) (Fig.24).

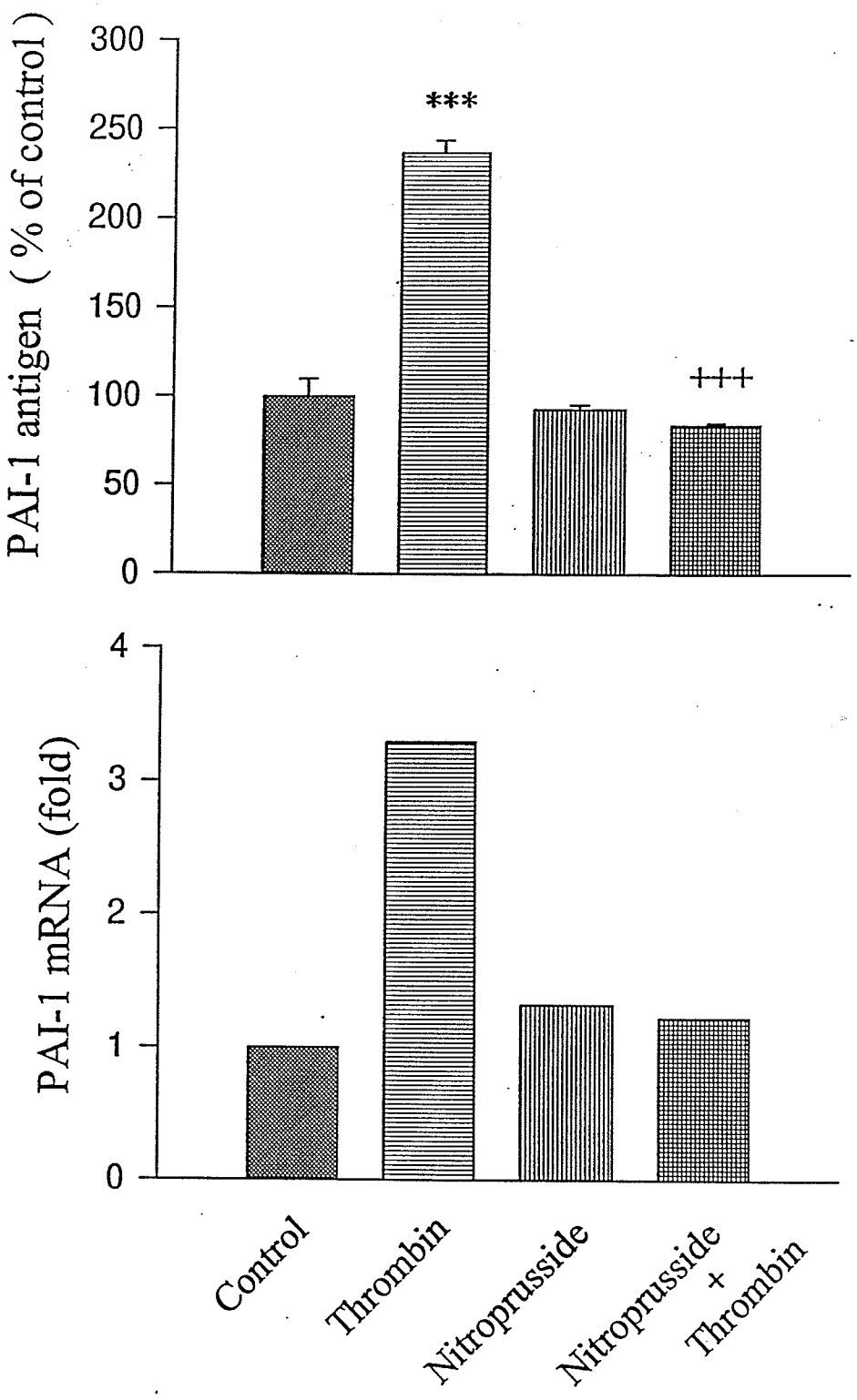


Fig.22. Effect of nitroprusside on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 10 μM of nitroprusside or 10 U/ml of thrombin plus 10 μM of nitroprusside for 3 h. The procedures for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. ***: $p<0.001$ versus controls; +++: $p<0.001$ versus thrombin treated cells.

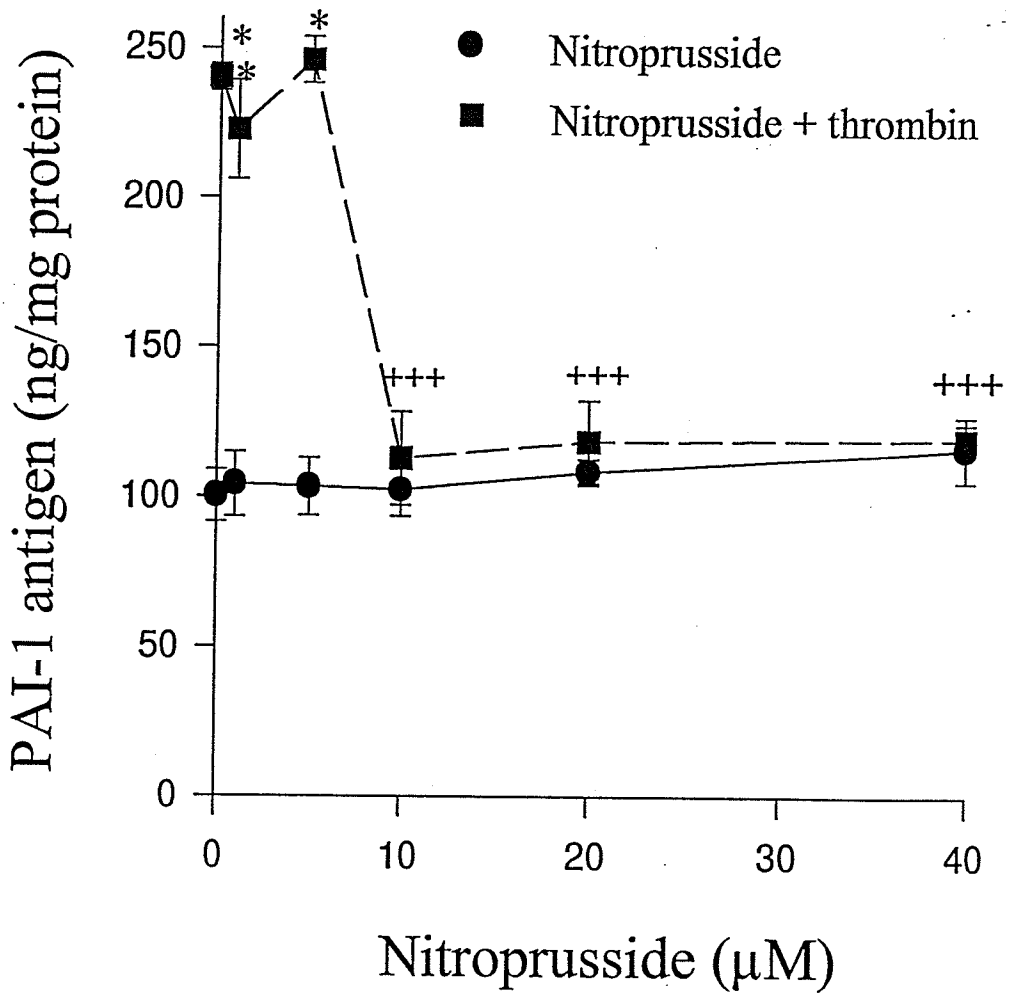


Fig.23. Dose-response of nitroprusside on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with 1-40 μM of nitroprusside ± 10 U/ml of thrombin for 3 h. The procedures for analyses of PAI-1 antigen were the same as described in the legend of Fig.4. Data was presented as mean ± SD (n=4). *: $P<0.05$ versus controls. +++: $p<0.001$ versus thrombin treated cells.

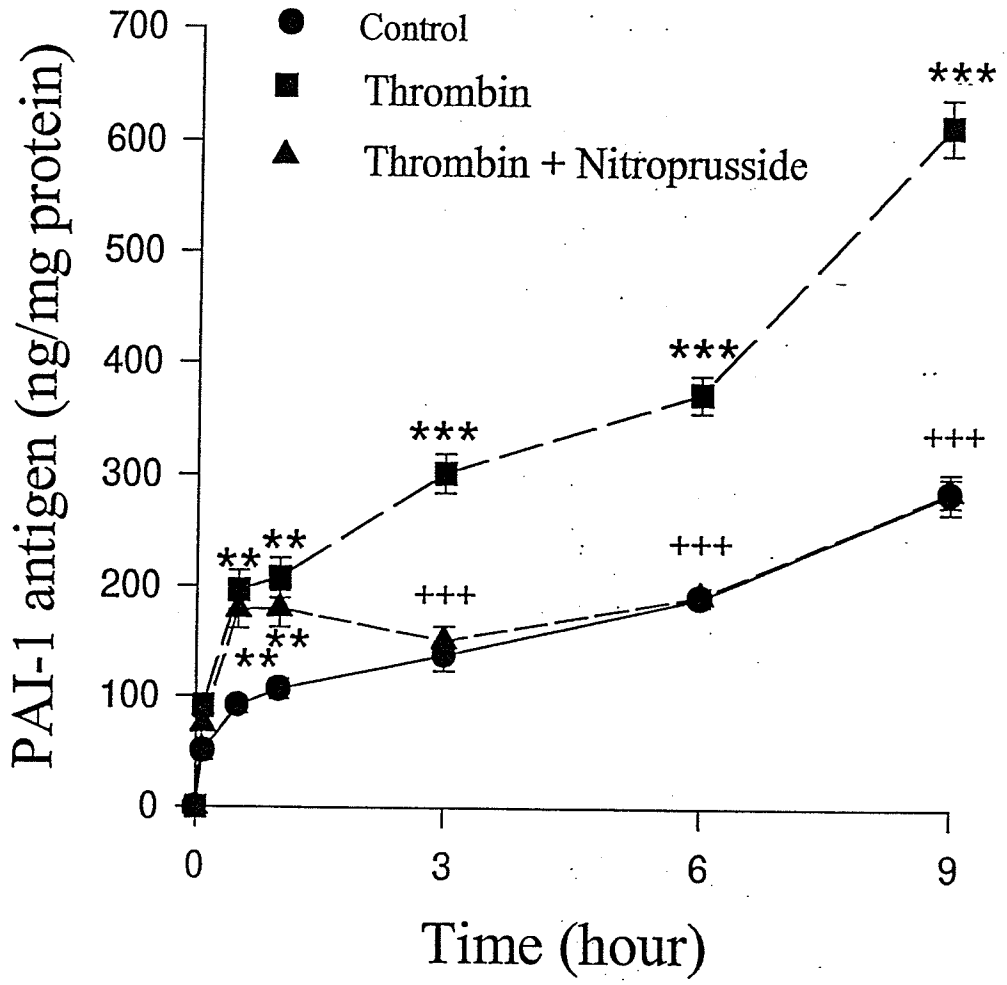


Fig.24. Time course of nitroprusside on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were incubated in the medium containing 1% FBS, or treated with 10 μM nitroprusside ± 10 U/ml of thrombin for 5 minutes to 9 hours. The procedures for analyses of PAI-1 antigen were the same as described in the legend of Fig.4. Data was presented as mean ± SD (n=4). ***: $P<0.001$ versus controls. +++: $p<0.001$ versus thrombin.

4.4 Effects of thrombin inhibitors on PAI-1 production:

1) Hirudin: Pretreatment of thrombin with hirudin, a natural inhibitor of thrombin, completely suppressed the thrombin-induced PAI-1 both at antigen and mRNA level in BASMC ($p<0.001$ versus cells treated with thrombin alone). Treatment with hirudin alone did not affect the basal levels of PAI-1 (Fig.25).

2) Hirulog-1: Hirulog-1 (10 μg/ml, 3 h treatment), a rationally designed thrombin inhibitor based on the structure of hirudin, significantly reduced the thrombin-induced increase of PAI-1 antigen in the conditioned medium of BASMC but not the basal level of PAI-1 release. Treatment with hirulog-1 completely inhibited thrombin-induced increase of PAI-1 mRNA levels in BASMC (Fig.26).

4.5 Dose response to Hirulog-1 on thrombin-induced PAI-1 production:

Hirulog-1 itself has no significant effect on the levels of PAI-1 antigen in the conditioned medium of BASMC. At 5 μg/ml of concentration, Hirulog-1 did not affect thrombin-induced PAI-1 release. With the concentrations equal or above 10 μg/ml, Hirulog-1 significantly suppressed the thrombin-induced increase of PAI-1 antigen ($p<0.05$). In cultures treated with 20 μg/ml of Hirulog-1, thrombin-induced PAI-1 release was inhibited by 91% ($p<0.01$) (Fig.27).

4.6 Time course of Hirulog-1 on thrombin-induced PAI-1 production:

Significant increases in PAI-1 antigen level ($p<0.001$) were detected in the conditioned media of BASMC treated with thrombin from 0.25 to 16 h compared to time-matched control cells. Incubation with 10 μg/ml of Hirulog-1 for 0.25 h did not significantly inhibit thrombin-induced PAI-1 release. Hirulog-1 (10 μg/ml)

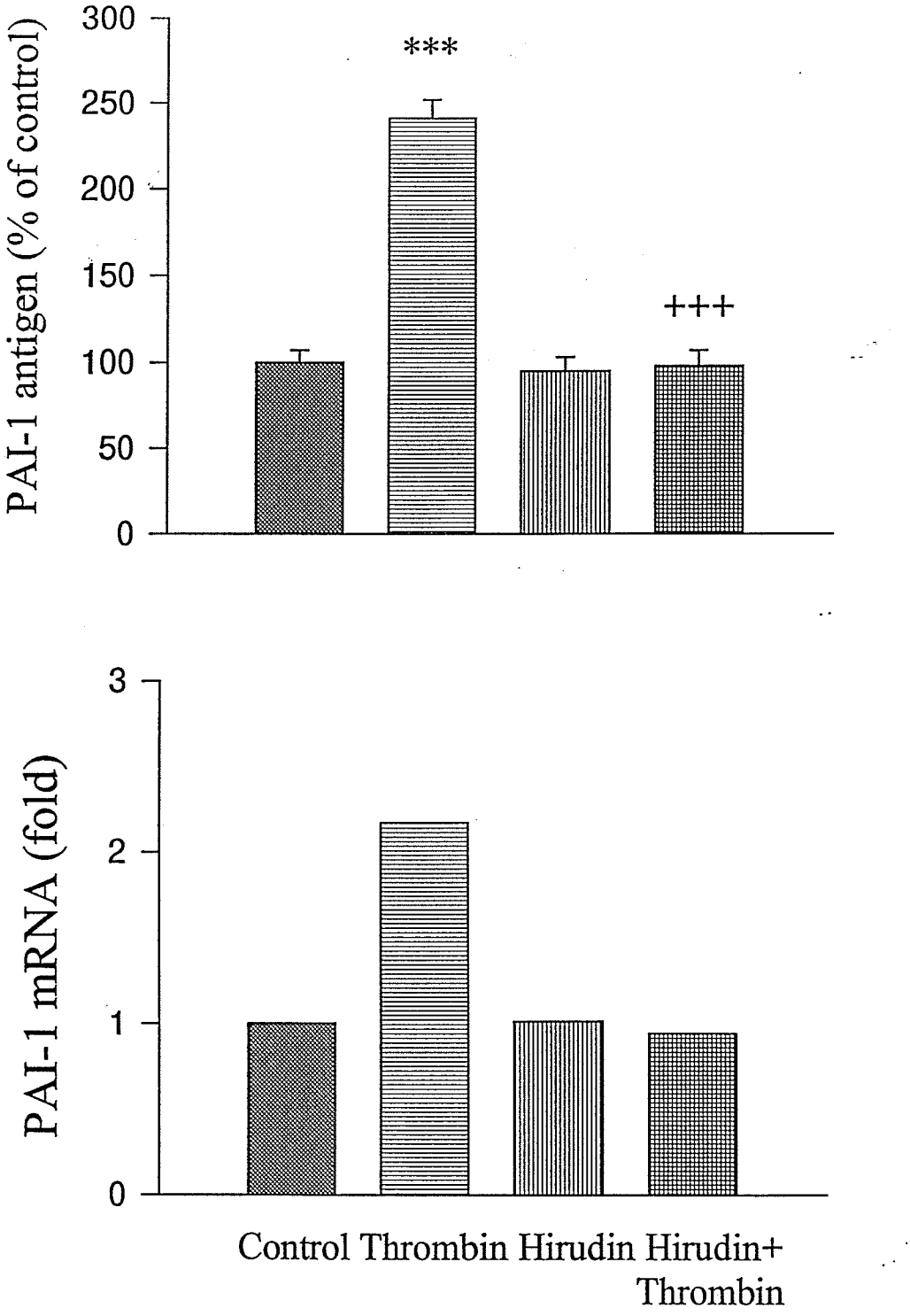


Fig. 25. Effect of hirudin on thrombin-induced PAI-1 production in BASMC. Hirudin was pre-incubated with thrombin in a ratio of 2:1 by units of activity on ice for 30 min. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, hirudin (in the same concentration used for thrombin-treatment) or hirudin-inactivated thrombin 10 U/ml for 3 h. The procedures for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. ***: $p<0.001$ versus controls; +++: $p<0.001$ versus thrombin treated cells.

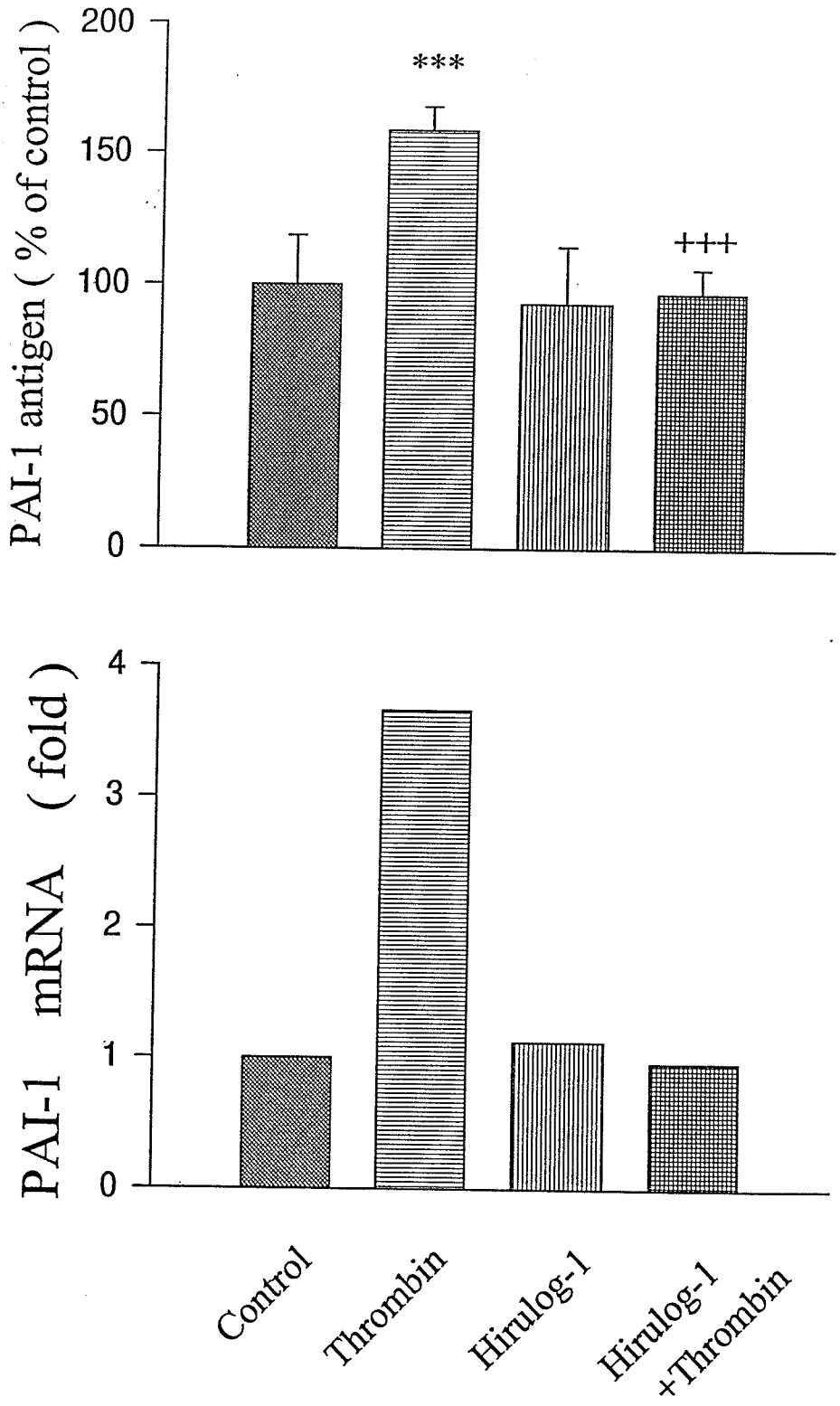


Fig. 26. Effect of Hirulog-1 on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with medium without addition (control), 10 U/ml of thrombin, 10 μg/ml of Hirulog-1 or 10 U/ml of thrombin plus 10 μg/ml of Hirulog-1 for 3 h. The procedures for analyses of PAI-1 antigen (upper, n=4, mean ± SD) and mRNA (bottom, average of two determinations) were the same as described in the legend of Fig.4. ***: $p<0.001$ versus controls; +++: $p<0.001$ versus thrombin treated cells.

significantly inhibited thrombin-induced PAI-1 production following equal or more than 1 h of treatment ($p<0.01$). The maximal inhibitory effect (>99%) of Hirulog-1 reached at 6-8 h. After 16 h of treatment, the inhibitory effect of Hirulog-1 reduced to 59% (Fig.28).

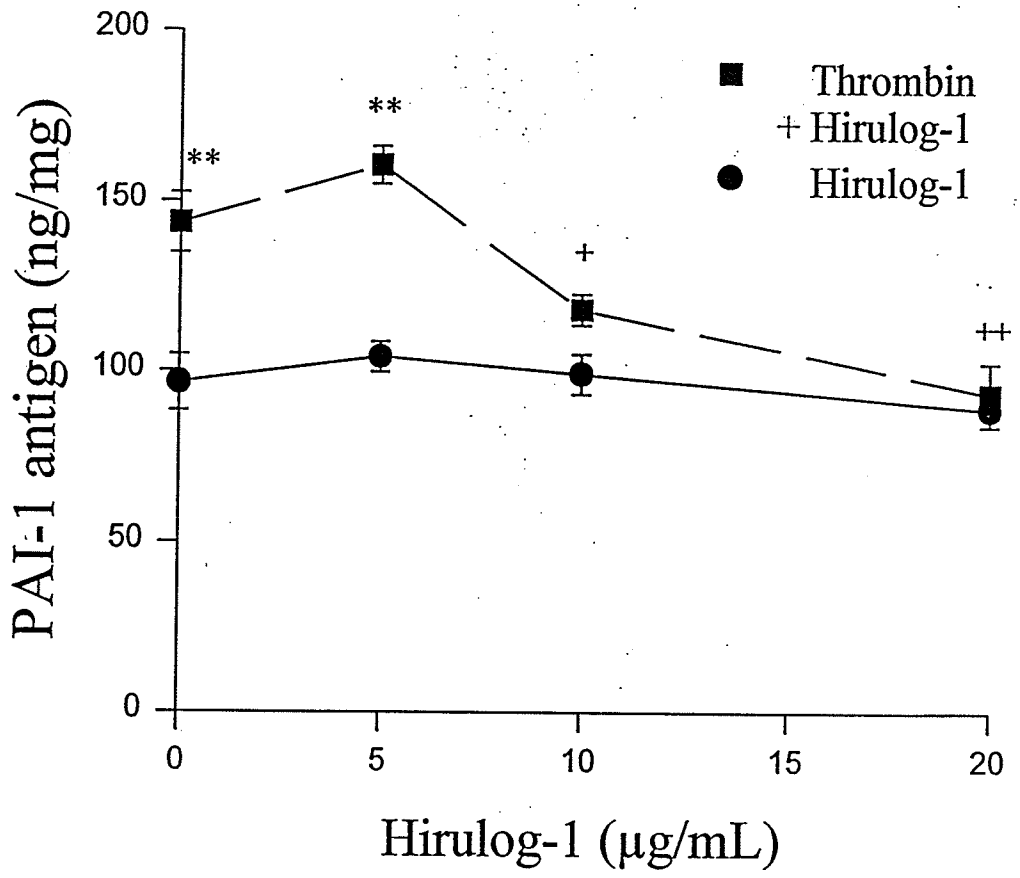


Fig. 27. Dose-response of Hirulog-1 on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were treated with 5-20 μg/ml of Hirulog-1 ± 10 U/ml of thrombin for 3 h. The procedures for analyses of PAI-1 antigen were the same as described in the legend of Fig.1. Data were presented as mean ± SD (n=4). **: $P<0.01$ versus controls. +,++: $P<0.05$ or <0.01 versus thrombin.

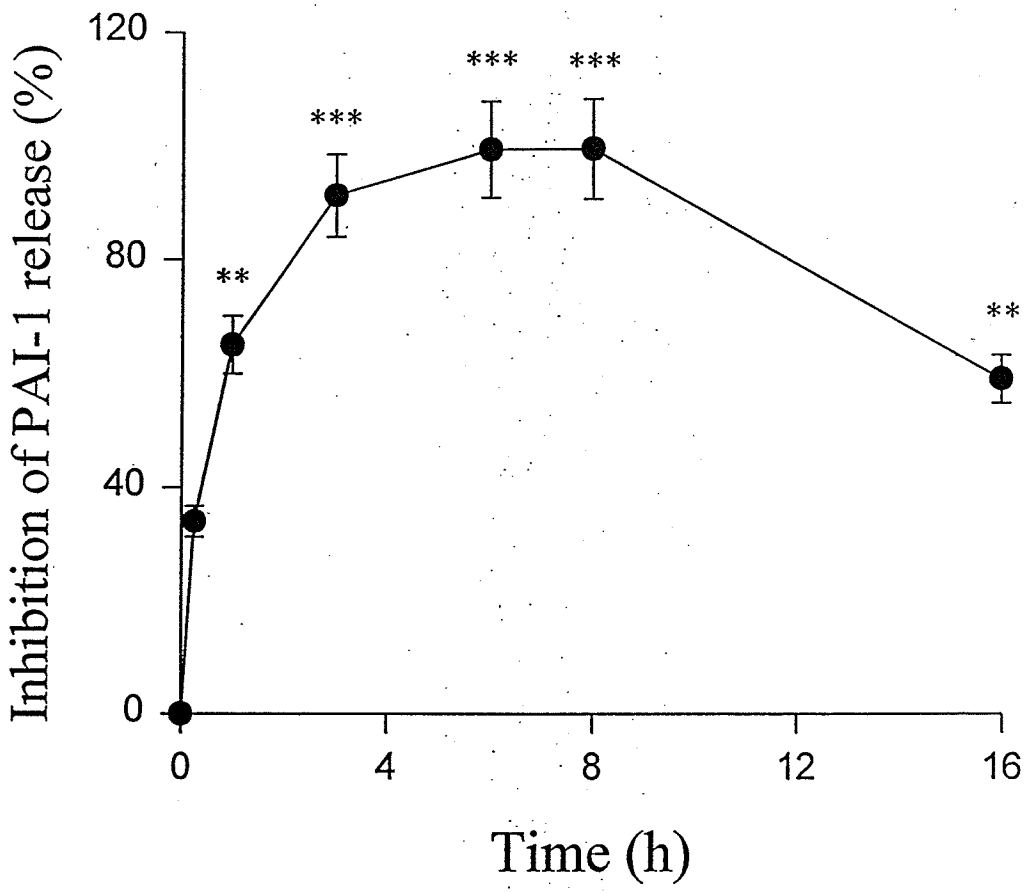


Fig. 28. Time course of Hirulog-1 on thrombin-induced PAI-1 production in BASMC. Confluent BASMC were incubated in the medium without addition (control), 10 U/ml of thrombin or 10 U/ml of thrombin plus 10 μg/ml of Hirulog-1 for 15 min to 16 h. The procedures for analyses of PAI-1 antigen were the same as described in the legend of Fig.1. Data was presented as percentage of inhibition of thrombin-induced PAI-1 release by Hirulog-1. Mean ± SD (n=4). **,***: $P<0.01,0.001$ versus culture treated by thrombin alone.

The results on Hirulog-1 were submitted to Journal of Cardiovascular Pharmacology (paper in revision, Ren et al. 1996c).

DISCUSSION

The results in this thesis indicate that the exposure of primate arterial SMC to physiological levels of thrombin (≤10 U/ml) induces PAI-1 production and the release of biological active PAI-1 from cultured BASMC through multiple pathways. Elevation of PAI-1 antigen in the conditioned medium of the thrombin-treated cultures began within 10 min of thrombin stimulation, which is prior to the appearance of PAI-1 gene overexpression. The thrombin-induced early increase of PAI antigen was associated with reduction of PAI-1 in extracellular matrix and was not suppressed by treatment with cycloheximide, a protein synthesis inhibitor. In the cultures treated with thrombin for more than 1 h, overexpression of PAI-1 gene was noticed, this was followed by further increase of PAI-1 antigen and activity in the medium. These findings suggest that thrombin may increase PAI-1 release from BASMC through at least two pathways: 1) proteolytic cleavage of PAI-1 from extracellular matrix which may generate smaller size of biological inactive PAI-1; 2) increased production at transcriptional level which is associated with the generation of biological active PAI-1.

My studies demonstrate that a transmembrane receptor and multiple signal transduction systems are involved in thrombin-induced PAI-1 generation in vascular SMC. TRAP-6 (SFLLRN) contains minimal requirements in amino acid sequence for activating the thrombin receptor (Beecher et al. 1994). The results of my study suggest that the levels of PAI-1 antigen and mRNA in BASMC were elevated by the treatments of TRAP-

6. γ-Thrombin preserves the structure responsible for the enzymatic activity of thrombin but is disrupted in anion binding exosite. The effect of γ-thrombin on PAI-1 production is much weaker than α-thrombin in BASMC. In addition, enzymatically inactivated thrombin, (ex. PPACK-treated thrombin) is unable to stimulate PAI-1 production in BASMC, which is probably due to its inability to generate a tethered ligand for activating the thrombin receptor by proteolytic cleavage (Fenton et al. 1988b). The combination of these results strongly support the hypothesis that the thrombin receptor mediates the overproduction of PAI-1 in BASMC induced by thrombin.

Hirudin and its analog Hirulog-1 are inhibitors of thrombin, which can block both the binding and activity sites of thrombin. Recent studies indicate that Hirulog-1 is a potential alternative for heparin in prevention of thrombosis in several significant clinical situations associated with increased coagulation and endothelial injury (Lidon et al.1993, Topol et al. 1993). Thrombolysis was enhanced by Hirulog-1 when it was used in combination with tPA in canine coronary artery compared to tPA alone (Yao et al. 1992). Treatment with Hirulog-1 effectively reduced the lethal effect of endotoxin-induced shock in experimental animals (Cicala et al. 1995). My results show that hirudin and Hirulog-1 effectively inhibit thrombin-induced PAI-1 production in BASMC. The investigation on the effect of Hirulog-1 on PAI-1 production may help to understand the mechanism of its effect on prothrombolysis and the prevention of thrombosis. Hirulog-1 is a slow turnover substrate of thrombin compared to hirudin (Yorimuitsu et al. 1993). Its inhibitory effect is decreased along with the exposure to thrombin. This may explain the low incidence of bleeding complications during Hirulog-1 treatment. My observations indicate

that a single dose of Hirulog-1 in BASMC results in hours long of inhibition on thrombin-induced PAI-1 production. Complete inhibition of PAI-1 production was induced by 10-20 μg/ml of Hirulog-1 treatment. These information may be helpful for further studying the effect of Hirulog-1 on PAI-1 production in vivo.

It has been shown that various membrane receptors convey instructions of extracelluar signals by stimulating a receptor-coupled G protein at the inner surface of cell membrane (Gilman 1987). Sodium fluoride (NaF), a G protein agonist, stimulates PAI-1 production in BASMC. Pertussis toxin-sensitive G protein is involved in the activation of the thrombin receptor in Ca^{++} mobilization in rat aortic SMC (Neylon et al. 1992). My study indicates that pertussis toxin inhibits thrombin-induced PAI-1 release and mRNA levels in BASMC. The combination of the results suggest that inhibitory G protein is required for thrombin-induced overproduction of PAI-1 in BASMC. NaF is also a non-specific inhibitor for phosphatase, including those for tyrosine phosphorylation. Therefore, its effect on PAI-1 production also suggests the requirement of tyrosine kinase, in this process.

Previous studies suggest that thrombin-induced PAI-1 production in vascular endothelial cells was inhibited by genistein, a tyrosine kinase inhibitor. Vanadate, a tyrosine protein phosphatase inhibitor, increases PAI-1 production in endothelial cells (van Hinsbergh et al. 1994). Activation of PLC is not necessarily mediated through G protein. Tyrosine phosphorylation is associated with the activation of certain types of receptors, especially those for growth factors or related molecules, and phosphatidylinositol

hydrolysis (Berridge et al. 1984). Some of those receptors possess intrinsic tyrosine phosphorylation activity which phosphorylates on PLC or receptors. The formation of inositol phosphates in platelets through the occupancy of Fc receptor is sensitive to tyrosine kinase inhibitors (Blake et al. 1994). The role of tyrosine kinase involvement in thrombin-induced PAI-1 production was investigated by tyrosine kinase inhibitors, genistein and tyrphostin 25. Genistein may also affect PKC and PKA activity beside tyrosine kinase. Tyrphostin 25 is a selective tyrosine kinase inhibitor. The results of my study demonstrate that both inhibitors effectively suppress thrombin-induced PAI-1 overproduction in SMC. Tyrphostin 1, a structural homologue of tyrphostin 25, did not alter PAI-1 production. The results suggest tyrosine phosphorylation is likely involved in thrombin-induced PAI-1 production in BASMC.

The requirement of PLC in production of PAI-1 induced by thrombin was studied by neomycin and U-73122. Neomycin, an inhibitor for PLC and PLD, inhibits thrombin-induced polyphosphoinositide metabolism in platelets (Siess et al. 1986). U-73122 is a specific inhibitor of PLC. U-73343 is a structural homologue of U-73122 but has a very weak inhibitory effect on PLC (Tatrai et al. 1994). Neomycin and U-73122 inhibited thrombin-induced PAI-1 production in BASMC. While U-73343 has no effect on thrombin-induced PAI-1 production. The results suggest that a PLC posssibly mediates the production of PAI-1 in vascular SMC.

The requirement of a PKC-dependent pathway for PAI-1 production has been shown in several types of cells. The release of PAI-1 antigen from glomerular epithelial

and mesangial cells (Villamediana et al. 1990, He et al. 1992) was blocked by a treatment with H7, a less specific PKC inhibitor (Kawamoto et al. 1984). PMA, a potent PKC agonist, stimulates PAI-1 release from endothelial cells (Grulich-Henn et al. 1990). Thrombin-induced PAI-1 production was partially inhibited by H7 or staurosporine in endothelial cells (Levin et al. 1989). My study demonstrates that PAI-1 mRNA levels in BASMC were greatly elevated following PMA treatment. Treatment with calphostin C, a specific PKC inhibitor (Kobayashi et al. 1989), suppressed basal and thrombin-induced increase of PAI-1 production in BASMC. My observations suggest that a PKC-dependent pathway is essential for PAI-1 generation in BASMC.

It has been shown that IL-1 is a strong inducer for PAI-1 generation in endothelial cells (Bevilaqua et al. 1986, Nachman et al. 1986). Thrombin also stimulates the production of IL-1 in endothelial cells (Stern et al. 1985). Previous studies by Heaton et al. (1992) suggested the overproduction induced by thrombin may be secondary to IL-1α in endothelial cells. Antibody against IL-1α blocked the increase of PAI-1 production by 0.3 U/ml of thrombin in endothelial cells. Addition of 0.3 U/ml of thrombin did not further increase PAI-1 production in endothelial cells in addition to the effect of IL-1α (Heaton et al. 1992). My results indicate that high doses of thrombin induce additional increases of PAI-1 production in IL-1α-treated BASMC. This implies that thrombin may independently regulate PAI-1 production in BASMC.

Elevation of intracellular cAMP level by forskolin inhibited PAI-1 secretion in HUVEC (Santell et al. 1988). cAMP analogues or adenylate cyclase agonists inhibit PAI-

1 secretion from endothelial, epithelial and mesangial cells (Francis et al. 1989, Villamediana et al, 1990, He et al. 1992). The results of my study demonstrate that 8-bromo-cAMP reduces PAI-1 production in SMC. Recent studies by other groups demonstrated that increase in intracellular cAMP levels down-regulates the levels of thrombin receptor in mesangial cells (Zacharias et al. 1995). Increase in intracellular cAMP attenuates tyrosine phosphorylation of PLC gamma 1 in T-cells (Park et al. 1992). It is postulated that increase in cAMP in SMC may modulate the expression of thrombin receptor and the activity of tyrosine kinase, which may be responsible for the effect of 8-bromo-cAMP on thrombin-induced PAI-1 production in BASMC.

Nitric oxide (NO) generated from sodium-nitroprusside (NP) may activate guanylate cyclase system and further increased the cGMP production (Furchgott et al. 1989, Harrison et al. 1993). Lidbury et al. (1990) described an increased fibrinolysis following in vivo NP administration, which is hypothetically due to an inhibitory effect on platelet PAI-1 release. Rogers et al (1988) demonstrated that cGMP inhibits the secretion mediated by PKC in rat pancreatic acini. Nitrovasodilators inhibit platelet function by an increase in cGMP (Mellion et al. 1980). Nitrovasodilators, such as NP, and 8-bromo-cGMP inhibit both the protein kinase C-dependent and calcium-dependent pathways leading to platelet activation (Doni et al. 1991). My results indicate that cGMP structural homologue and NP reduce PAI-1 production in SMC. The results suggest that nitrovasodilators may reduce PAI-1 production in vascular SMC and improve fibrinolytic activity via a cGMP-dependent mechanism.

I also studied the effect of Lp(a) , LDL and their oxidized forms on the generation of PAI-1 from cultured vascular EC. The results of my study indicate that 10 μg/ml of Lp(a) increases PAI-1 generation in HUVEC following ≥ 24 h of treatment. This was consistent with a previous report from Etingin et al (Etingin et al. 1991). PAI-1 antigen levels in the cultured medium was increased by the treatment of 100 μg/ml of LDL (Tremoli et al. 1993). To prevent the possibility of the contamination of Lp(a) in LDL isolated by ultracentrifugation, my study used Lp(a)-free LDL prepared by affinity chromatography to examine the effect of LDL on the expression of the fibrinolytic mediators in HUVEC. Equimolar amounts of Lp(a)-free LDL had no effect on the levels of PAI-1 mRNA. To prevent the influence of of LDL subclasses, same sources of Lp(a) and LDL were used in my study source. My results suggest that Lp(a) specificlly increase PAI-1 production in HUVEC.

Oxidized LDL modified by ultraviolet radiation elevated the levels of PAI-1 antigen in the cultured medium of HUVEC compared to native LDL (Tremoli et al. 1993). Oxidized LDL modified by $CuSO_4$ stimulated the release of PAI-1 and reduced the release of tPA from EC compared to native LDL (Kugiyama et al. 1993). Oxidative modification may induce many kinds of peroxidation products in the protein and lipid components of lipoprotein. Previous studies have detected Lp(a) particles and the peroxidiation products of oxidized LDL in atherosclerotic lesions (Hajjar et al. 1989, Yia-Herttuala et al. 1989), which probably includes the particles of oxidized Lp(a). The results of my study demonstrate that oxidized Lp(a) has stronger effect on PAI-1 generation from vascular EC than native Lp(a). Both native and oxidized Lp(a) increase the levels of cell-

associated PAI-1 in cultured EC. The majority of PAI-1 associated with cultured EC is located in extracelluar matrix (Levin et al. 1994). Measurement of PAI-1 only in conditioned medium possibly underestimates total PAI-1 generated from EC. Native and oxidized Lp(a) may increase the storage of active PAI-1 in cell-associated pool, most likely in extracelluar matrix. PAI-1 associated with EC may be released into extracelluar fluid when tPA is available, with consequent reduction of fibrinolytic activity in blood. Oxidized LDL moderatly increased PAI-1 production compared to native LDL. This suggests that unidentified alteration in LDL and Lp(a) induced by oxidization may contribute to PAI-1 production in vascular EC.

In summary, thrombin may have multiple effects on the generation of PAI-1 from vascular SMC. Thrombin proteolytically cleaves PAI-1 from the extracelluar matrix, which initiates within minutes of thrombin treatment. PAI-1 released from the extracelluar matrix of the SMC by thrombin does not affect PAI activity but may reduce the storage of intact PAI-1 in vessel wall. The transcription of PAI-1 in BASMC increases after more than one hour of thrombin stimulation, which results in an elevation of active PAI-1 in extracelluar fluid. Thrombin regulates PAI-1 production in primate arterial SMC via transmembrane receptor and multiple signal transduction systems. Thrombin activates pertussis toxin-sensitive G protein coupled thrombin receptors in BASMC. Activation of genistein-sensitive tyrosine kinase and phospholipase C are required for thrombin-induced PAI-1 production in BASMC. Increased levels of intracellular cAMP and cGMP may negatively regulate PAI-1 production in vascular SMC. Treatment with thrombin inhibitors and nitroprusside effectively inhibited thrombin-induced PAI-1 synthesis in

cultured vascular SMC. The results of my studies also demonstrate that exposure to Lp(a), an atherogenic lipoprotein, elevates PAI-1 production at mRNA, antigen and activity levels in HUVEC. Oxidative modification enhances the effect of Lp(a) on the levels of PAI-1 generation in HUVEC. The extent of PAI-1 generation induced by native and oxidized Lp(a) or LDL corresponds to increases in PAI-1 mRNA levels in HUVEC. The overproduction of PAI-1 in vascular EC induced by Lp(a), especially its oxidized form, potentially attenuates fibrinolytic activity in blood and favors thrombus formation in vivo. The antifibrinolytic effect of oxidized Lp(a) at the endothelial cell surface suggested by the present study provides additional evidence for the role of the oxidized form of Lp(a) in local thrombogenesis and atherosclerosis.

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